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Supplement 20

3rd International Conference, Zagazig University

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Address: Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov: Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
Tel.: +386 (0)1 47 79 100, 47 79 129, Fax: +386 (0)1 28 32 243
E-mail: slovetres@vf.uni-lj.si

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Original Research Article

ISOLATION AND INITIAL CHARACTERIZATION OF A *Myoviridae* PHAGE FOR CONTROLLING ZOONOTIC *Salmonella* Typhimurium AND *Salmonella* Enteritidis FROM BROILERS IN EGYPT

Abdallah M. A. Merwad[1]*, Mahmoud E. F. Abdel-Haliem[2]

[1]Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, [2]Department of Microbiology and Botany, Faculty of Science, Zagazig University, 44519, Zagazig, Egypt

*Corresponding author, E-mail: merwad.abdallah@yahoo.com

Abstract: This study targeted isolation and characterization of phage against multidrug resistant (MDR) *Salmonella* Typhimurium and *Salmonella* Enteritidis recovered from broilers and to evaluate the lytic effect of the phage on growth of *Salmonella* serovars. *Salmonella* isolates were recovered from caecal contents, liver and breast meat of broiler chickens from retail outlets at Sharkia Governorate, Egypt. *Salmonella* Typhimurium (n=14) and *S.* Enteritidis (n=11) were tested for their antimicrobial susceptibilities against 15 antimicrobials by disc diffusion method. Isolates of *S*. Typhimutium and S. Enteritidis were 100% resistant to seven antimicrobial agents. The phage was isolated from Zagazig sewage water by spot test and double over layer agar assay. The phage designated as phiSalmchick1showed an icosahedral head and contractile tail structure in electron microscopy, indicating a member of the family *Myoviridae*. The phage was a polyvalent infecting a wide host range of all MDR strains of *S*. Typhimurium, *S*. Enteritidis, *S*. Paratyphi, *E. coli* serotypes O26 and O168 and *Klebsiella pneumoniae.* Myovirus phage had burst size of 100 plague forming unit (PFU)/cell with latent period of 60 min. The phage genome had double-stranded DNA by molecular analysis. The lytic effect of phiSalmchick1 phage was *in vitro* assessed on growth of *S*. Typhimurium and *S*. Enteritidis isolates by measuring the optical density (OD) of the liquid media during *Salmonella* growth at 37 °C and the multiplicity of infection (MOI) was equal to 1.0. Significant reductions were observed in OD of *S*. Typhimurium and *S*. Enteritidis treated with the phage after 24 hrs incubation compared to the controls ($P<0.05$). The myovirus has a high potential for phage application to control zoonotic and MDR *Salmonella* serovars isolated from broiler chickens in Egypt.

Key words: multidrug resistant; *Salmonella* serovars; *Myoviridae* phage; broiler chickens; *in vitro* control

Introduction

Salmonellosis is an occupational anthropo-zoonoses and constitutes economic and public health concern (1). *Salmonella* is the main cause of foodborne illness in human as sixteen million human cases had typhoid fever, approximately 1.3 billion cases had gastroenteritis and annually deaths of 3 million were recorded (2). The raw eggs, poultry meat and other chicken products are responsible for the sporadic and epidemic salmonellosis in

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humans (3). In Egypt, the contamination of poultry meat is derived from infected poultry, cross–contamination with bird dropping, instruments and hands of workers during processes of slaughtering, defeathering and scalding (4). The genus *Salmonella* comprises more than 3000 serovars; and the most important serovars are *S.* Pullorum, *S.* Gallinarum, *S.* Enteritidis and *S.* Typhimurium. In Egyptian poultry farms, *S.* Typhimurium and *S.* Enteritidis have been distinguished as the most predominant serovars (5). The antibiotics were inappropriately used in chicken farms in Egypt, which resulted in the spread of multidrug resistant (MDR) *S.* Typhimurium and *S.* Enteritidis (6). The increase of *Salmonella* resistance to most antimicrobials utilized for human therapy creates a zoonotic threat (7). Therefore, bacteriophages are used as potential therapeutic agents for biocontrol of MDR bacteria in poultry (8). Bacteriophages are viruses that have the ability to attack only bacterial cells with rapid killing and self-replication and are safe for eukaryotic cells (9,10). Three phages (Salmacey1, Salmacey2 and Salmacey3) were completely inhibiting the growth of *S.* Kentuckey isolated from broilers in Egypt (11). Thereby, this study was aimed to isolate and characterize phages targeting MDR *S.* Typhimurium and *S.* Enteritidis isolated from retailed broiler chickens in Sharkia Governorate, Egypt, as well as to assess the lytic efficacy of the isolated phage for controlling *Salmonella* serovars in broilers.

Material and methods

Collection and preparation of chicken samples

A total of 150 samples including swabs from caecal contents (n=60), liver (n=50) and breast meat (n=40) were collected from broiler chickens marketed at different retail outlets at Sharkia Governorate, Egypt. The samples were individually collected in sterile plastic bags, then kept in an insulated box with packs of ice and sent immediately to the laboratory.

Isolation and identification of Salmonella

The swabs from caecal contents were pre-enriched in buffered peptone water (BPW, Oxoid, UK); while liver and breast meat (25 g/each) were mixed with 225 ml of BPW then homogenized using a stomacher at 230 rpm for 5 min. The swabs and tissue mixtures were incubated at 37 °C for 18 hrs. The isolation of *Salmonella* from caecal contents, liver and breast meat of retailed broilers was performed according to the method of ISO-6579 (12). In brief, the pre-enriched cultures (0.1 ml) was added to 10 ml of Rappaport-Vassiliadis soy peptone broth (Oxoid,UK) then incubated at 41.5 °C for 18-24 hrs. A Loopful from enriched broth was streaked on the surface of Xylose Lysine Desoxycholate (XLD, Oxoid CM0469, UK) agar plates, and then incubated at 37 °C for 24 hrs**.**

The presumptive *Salmonella* isolates were biochemically identified using catalase, oxidase, indole, triple sugar iron, methyl red, Voges-Proskauer, citrate and urea hydrolysis, H_2S tests according to the described protocols in Bacteriological Analytical Manual (13). The isolates of *Salmonella* were serotyped by using rapid diagnostic *Salmonella* antisera sets (Denka Seiken CO., Japan) at Serology Unit, Animal Health Research Institute, Dokki, Giza, Egypt as previously described (14). Stock cultures of *Salmonella* isolates were stored in 20% glycerol at -80°C.

Antimicrobial susceptibility testing

Twenty-five *Salmonella* isolates including *S.* Typhimurium (n=14) and *S.* Enteritidis (n=11) were tested for their antimicrobial susceptibility against 15 antibiotics by Kirbye-Bauer agar disc diffusion method (15). The antibiotic panels and their concentrations including amoxicillin-clavulanic acid (AMC, 30 µg), ampicillin (AMP, 10 µg), gentamycin (CN, 10 µg), amikacin (AK, 30 µg), tetracycline (TE, 30 µg), imipenem (IPM, 10 µg), sulphamethoxazole (RL, 25 µg), streptomycin (S, 10 µg), rifampin (RD, 5 µg), trimethoprim / sulphamethoxazole (SXT, 25 µg), cefoxitin (FOX, 30 µg), ciprofloxacin (CIP, 5 µg), ceftriaxone (CRO, 30 µg) and

cephazolin (KZ, 30 µg) were obtained from Thermoscientific Oxoid (USA); while trimethoprim (W, 5 µg) were obtained from Bio-Rad (France). The bacterial isolates were enriched in brain heart infusion broth (Oxoid CM0337, UK) then incubated at 37ºC for 24 hrs. A loopful of bacterial growth was mixed with 5 ml of tryptone soya broth (Oxoid, UK), followed by incubation at 37ºC for 24 hrs till reaching the turbidity of 0.5 McFarland standard as previously described (16). The bacterial suspensions were inoculated on Mueller Hinton agar plates, then discs of antibiotics were placed on the plates, and subjected to incubation at 37ºC for 18 hrs. The diameters of inhibition zones and interpretation of *Salmonella* isolates were in accordance to the guidelines of the relevant CLSL document (17).

Bacterial strains

Thirteen bacterial isolates including *S.* Paratyphi from blood of humans (n=3), *Escherichia coli* O168 from dropping of ducks (n=6); *Proteus vulgaris* from urine of patients (n=2) and *Klepsiella pneumoniae* from sputum of patients (n=2) were kindly obtained from Microbiology Department, Faculty of Science, Zagazig University. Also, twelve bacterial strains comprising *E. coli* O26 from milk of dairy cows (n=5), *Staphylococcus aureus* from edible parts of fish (n=4) and *Listeria monocytogenes* from litters of broilers and meat products (n=3) were obtained from Zoonoses Department, Faculty of Veterinary Medicine, Zagazig University.

Phage isolation

The bacteriophage was isolated from sewage water samples from different stations at Zagazig City, Egypt using the enrichment technique (18). Briefly, a sewage sample was centrifuged at 10,000 xg for 15 min, then filtered using a sterile 0.45 µm Millipore filter (Steradisc, Kurabo Industries LTD., Japan). Fifty milliliters of the filtrate was added to an equal volume of Luria-Bertani (LB, Oxoid,UK) broth, which was inoculated with 1 ml of *Salmonella* serovars, then subjected to incubation with shaker at 120 rpm at a temperature of 37ºC/24 hrs. The centrifugation of bacterial cultures was performed at 10,000 xg/10 min. The supernatant was filtered using a sterile 0.45 µm Millipore filter, then used as a phage source to be found on the propagative *Salmonella* isolates. The bacterial lawns of *S.* Typhimurium and *S.* Enteritidis were subjected to propagation on LB plates using the double agar overlay method as previously explained (18). Two hundred µl of each *Salmonella* serovar (OD of 0.4 at 600 nm) were added to semi-solid LB agar (4 ml) then poured over nutrient agar plates. The droplets of formerly prepared bacteriophage source (10 µl) were spotted on the bacterial lawns, and then were left for drying. Incubation of plates was carried out at 37 °C overnight and checked for existence of the lytic zones.

Purification and propagation of phage

The bacteriophage was purified by three single plaque isolation assays using sterile pasture pipette (11,18). In brief, a single plaque was picked up, then placed in 0.5 ml of nutrient broth harboring 100 µl of *Salmonella* Typhimurium, then subjected to incubation at 37ºC with shaking at 1200 rpm. Afterwards, centrifugation of phage-bacteria mixture was done at 10,000 xg for 10 min. The supernatant was filtered via a sterile 0.45 µm Millipore filter to exclude any bacterial cells. Storage of purified phages was done at 4ºC.

Determination of phage host range

The isolated bacteriophage was tested against 50 isolates of pathogenic bacteria including *S.* Typhimurium (n=14), *S.* Enteritidis (n=11), *S.* Paratyphi (n=3), *E. coli* O26 (n=5), *E. coli* O168 (n= 6), *P. vulgaris* (n=2), *K. pneumoniae* (n=2), *S. aureus* (n=4), *L. monocytogenes* (n=3) to detect the host range as previously explained (19). The propagation of bacterial lawns was achieved on LB agar plates, followed by addition of 10 µl of phage droplets (1×10^7 plague forming unit, PFU/ ml). These plates were incubated at 37ºC/ 24 hrs then tested for existence of plaques (lytic zones). Efficient phage was selected on the bases of lysis profile, clarity of plaque and size.

Transmission electron microscopy analysis

A drop of phage suspension (10^7PFU/ml) was located on 200 mesh copper grids with the carbon-coat films, and the excess was removed with a filter paper. The uranyl acetate as a saturated solution was placed on grids, and the excess was removed by filter paper. The purified phage particle was stained with uranyl acetate, and then was examined using an electron microscope (Hitachi H600A, Japan) at Mansoura University, Egypt as previously described (20).

One -step growth experiment

The single-step growth curve of the isolated bacteriophage was determined as previously explained (21). The phage was added at a multiplicity of infection (MOI) = 1.0 to *S.* Typhimurium cells then the phage was allowed to be adsorbed on host cells at 37º C/ 10 min. The phage-host mixture was centrifuged at 10,000 xg for 10 min, and the pellet harboring infected cells was suspended in 10 ml of nutrient broth then incubated at room temperature. The specimens were taken in duplicate at 15 min interval for a period of 150 min. The titer of phage was estimated by the double-layer plague assay. The first sample set was subjected to dilution before titration; while, 1% chloroform (V/V) was used to treat the second sample set for the release of intracellular phages and determination of the eclipse period. Three independent assays were carried out. The means of relative burst size were calculated ± standard deviation (SD).

Nucleic acids characterization of phage particle

The genomic DNA of isolated phage was extracted by the phenol/chloroform method as previously described (22). Three restriction enzymes (*Eco*R1, *Hin*dIII and *Bam*H1) were used to digest phage DNA according to the manufacturer's instructions (Takara Bio Inc., Japan). The restriction required a time ranging from 2-4 hrs. The analysis of DNA digestion mixtures was performed by electrophoresis (at 100 V) in agarose gel (1.0%) stained with the ethidium bromide using a DNA ladder.

Effects of phiSalamchick1 on the growth of Salmonella serovars

The efficacy of isolated phiSalamchick1 were evaluated on the growth of MDR *S.* Typhimurium and *S.* Enteritidis isolated from retailed broilers in a liquid media over a time period from zero time (starting point of host-phage incubation) to 42 hrs by measurement of the optical density (OD 600) by using UV-Spectrophotometer (UNICO 1200, USA). The freshly prepared bacterial culture of each *Salmonella* serovar (0.1 ml) and phage filtrate (0.1 ml) were added to sterile nutrient broth (3 ml) at MOI equals to 1.0 starting from zero time to 42 hrs. The optical density at OD_{600} was measured each 6 hrs for the control (bacterial culture alone) and for the treated bacterial broth (bacteria+phage) as previously described (11). This experiment was performed for independently three times. The means of bacterial OD were calculated ± standard deviation (SD).

Statistical analysis

The data for *Salmonellae* distribution in different samples of broilers was analyzed by IBM SPSS Statistics (vers.22) to compute p-values for Pearson chi-square. The analysis of data for single step growth curve experiment was done by one way analysis of variance (ANOVA) followed by the post hoc Duncan's test (IBM SPSS Statistics, version 22). Also, the data concerning *in vitro* assessment of phiSalamchick1 on the growth of *Salmonella* serovars was analyzed using independent sample T. test (IBM SPSS vers.21).

Results

Isolation and serotyping of Salmonella isolates from broilers

In this study, only 25 *Salmonella* isolates were serotyped into *S.* Typhimurium (n=14) and *S.* Enteritidis (n=11) out of 150 samples from broilers at various retail outlets at Sharkia, Egypt. *Salmonella* Typhimurium showed insignificant distribution (P>0.05): 11.7% (7/60) in caecal contents, 8% (4/50) in liver and 7.5% (3/40) in breast meat. The distribution of

S. Enteritidis was significantly higher (12.5%, 5/40) in breast meat followed by 10 % (6/60) in caecal contents (P<0.05), while it was not detected in liver of retailed broilers.

Antimicrobial susceptibility testing

The antibiotic resistance patterns of recovered *S*. Typhimurium and *S*. Enteritidis to 15 antimicrobials of various classes were determined by the disc diffusion method (Table 1). *Salmonella* Typhimurium strains showed moderate sensitivity (64.2%) to cephazolin followed by ceftriaxone (57.1%), while *S*. Enteritidis isolates illustrated higher sensitivity (81.8%) to cephazolin followed by cefoxitin (72.2%). Moreover, *S*.Typhimurium showed higher resistance (85.7%) to streptomycin followed by ampicillin (71.4%) and then a moderate resistance (64.2%) to amikacin and rifampin.The isolates of *S*. Enteritidis expressed higher resistance (81.8%) to streptomycin then followed by a similar resistance percentage (72.7%) to ampicillin, amikacin and rifampin. Of interest, both isolates of *S*. Typhimurium and *S*. Enteritidis were 100% resistant to seven antimicrobial agents (amoxicillin-clavulanic acid, gentamycin, tetracycline, imipenem, trimethoprim, sulphamethoxazole, trimethoprim / sulphamethoxazole) as shown in Table (1). This result proved that the two isolated *Salmonella* serovars were MDR to several classes of antimicrobials.

Isolation and morphology of phage

The bacteriophage against MDR *Salmonella* serovars was detected from Zagazig sewage water by spot test and double over layer agar technique. We picked up single plaque for propagation and characterization (Figure 1A). Four phage particles were observed during electron micrograph. The isolated phage was designated as phiSalmchick1.Under electron microscopy, the phage had an icosahedral head (130±5 nm in diameter, n=3) and long contractile tail with length of (133±5 nm in diameter, n=3). The phage (phiSalmchick1) appeared to be a member of *Myoviridae* family based on its morphological features (Figure 1B).

Host range of phage

Our results showed that phiSalmchick1 could infect all MDR strains of *S*. Typhimurium, *S*. Enteritidis, *S*. Paratyphi, *E. coli* serotypes O26 and O168 and *Klebsiella pneumoni*ae; and had lytic effects on such strains; while this phage could not infect *Proteus vulgari*s strains or other Gram positive bacteria (*S. aureus* & *L. monocytogenes*) as shown in Table (2). This finding indicated that phiSalmchick1 was a polyvalent phage and had a wide host range.

Characteristics of phage growth

From the curve (Figure 2), the latent period of phiSalamchick1 was 60 min and one cycle took 120 min. The average burst size of this myovirus was 100 PFU per infected cell (Figure 2). There was a significant increase in average burst size at 105 min compared to those burst sizes at time of 90 and 75 min (P<0.001).

Genomic digestion with restriction enzymes

The genomic DNA of phage was sensitive to digestion with *Eco*R1 and *Hin*dIII restriction enzymes. This indicated that the myovirus was a double stranded DNA. However, the phage DNA was resistant to digestion with *Bam*H1 enzyme.

Effects of phiSalamchick1 on the growth of MDR salmonellae

The lytic effects of phiSalmchick1 on the growth of *S*. Typhimurium and *S*. Enteritidis isolates were assessed by measuring OD_{600} of the bacterial culture infected with the phage at MOI of 1.0 and incubated at 37 º C (Figure 3). The values of standard deviation (SD) were small ranging from 0.005 to 0.01, and were not appeared in Figure (3). From zero to 12-18 hrs post infection, the growth of bacterial cells were inhibited by phage infection (OD values were much lower compared with uninfected controls) and after 12-18 hrs post infection, the bacterial lysis was significantly apparent (P<0.05). The net reduction of bacterial density was significantly detected after 24 hrs from the infection point (P<0.05). The growth of MDR salmonellae was inhibited by phiSalmchick1

infection and the bacterial populations were significantly reduced 24 hrs post treatment ($P<0.05$) and reached to complete lysis at 42 hrs incubation (Figure 3).

Table 1: Antimicrobial resistance patterns of *S.* Typhimurium and *S.* Enteritidis isolates from retailed broilers by disc diffusion method

Antimicrobials (disc content/µg)	*S.* Typhimurium (no=14)			*S.* Enteritidis (no=11)		
	R	I	S	R	I	S
AMC(30)	14(100)	-	-	11 (100)	-	-
AMP(10)	10(71.4)	3(21.4)	1(7.1)	8(72.7)	2(18.1)	1(9.09)
CN(10)	14 (100)	-	-	11 (100)	-	-
AK(30)	9(64.2)	2(14.2)	3 (21.4)	8 (72.7)	1 (9.09)	2 (18.1)
TE(30)	14 (100)	-	-	11(100)	-	-
IPM(10)	14 (100)	-	-	11(100)	-	-
W(5)	14(100)	-	-	11(100)	-	-
RL(25)	14(100)	-	-	11(100)	-	-
S(10)	12(85.7)	2 (14.2)	-	9 (81.8)	1 (9.09)	1 (9.09)
RD(5)	9 (64.2)	2 (14.2)	3 (21.7)	8 (72.7)	2 (18.1)	1(9.09)
SXT (25)	14 (100)	-	-	11(100)	-	-
FOX(30)	3 (21.4)	4 (28.5)	7 (50)	1 (9.09)	2 (18.1)	8 (72.7)
CIP(5)	4 (28.5)	4 (28.5)	6(42.8)	2 (18.1)	2 (18.1)	7 (63.6)
CRO(30)	3 (21.4)	3 (21.4)	8 (57.1)	2 (18.1)	3 (27.2)	6(54.5)
KZ(30)	3 (21.4)	2 (14.2)	9 (64.2)	1(9.09)	1(9.09)	9 (81.8)

R: resistant; I: intermediate; S: sensitive.
Data were represented by No (%)**.**
Where AMC: amoxicillin-clavulanic acid; AMP: ampicillin; CN: gentamycin; AK: amikacin; TE: tetracycline; IPM: imipenem; W: trimethoprim; RL: sulphamethoxazole; SXT: trimethoprim/sulphamethoxazole; S:streptomycin; RD: rifampin; FOX: cefoxitin; CIP: ciprofloxacin; CRO: ceftriaxone; KZ: cephazolin.

Table 2: The host range of isolated phage phiSalmchick1

Strains of Bacteria	No. of isolates	PhiSalmchick1	Source of strains
Salmonella Typhimurium	14	+	This study
*S*almonella Enteritidis	11	+	This study
*S*almonella Paratyphi	3	+	MSZU
Escherichia coli O26	5	+	ZVZU
Escherichia coli O168	6	+	MSZU
*Proteus vulgari*s	2	−	MSZU
*Klebsiella pneumoni*ae	2	+	MSZU
Staphylococcus aureus	4	−	ZVZU
Listeria monocytogenes	3	−	ZVZU

+: Bacterial strain was susceptible to the bacteriophage phiSalmchick1 and the plaques were produced.
-: Bacterial strain was resistant to the bacteriophage phiSalmchick1 and the plaques were not produced.
MSZU: Microbiology Department, Faculty of Science, Zagazig University, Egypt.
ZVZU: Zoonoses Department, Faculty of Veterinary Medicine, Zagazig University, Egypt.

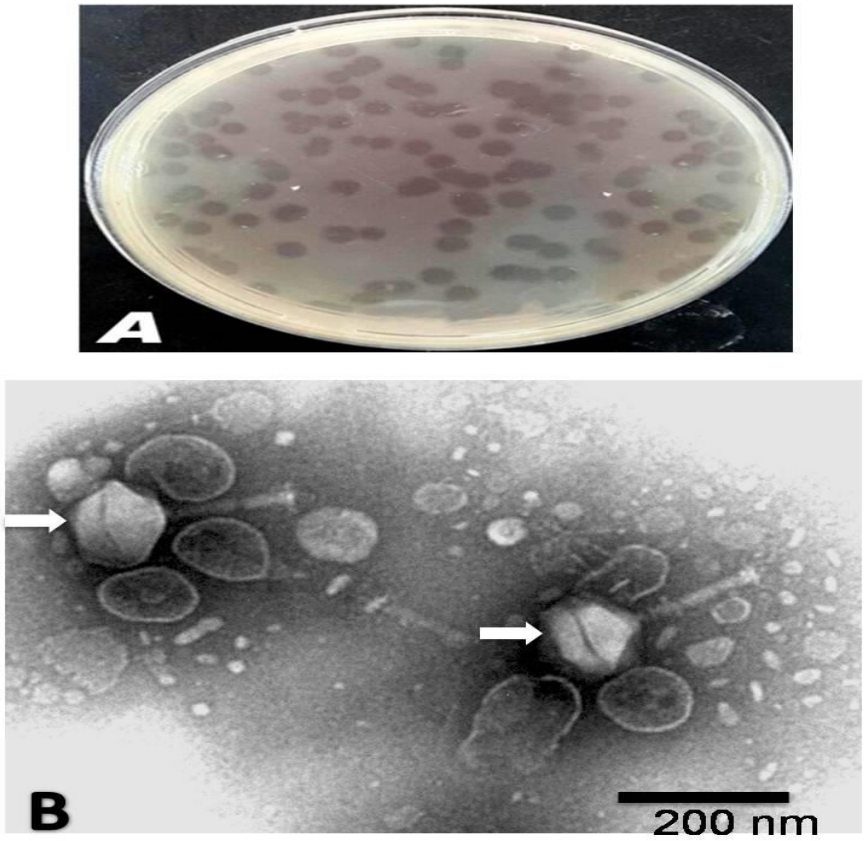

Figure 1: Plaque morphology and electron micrograph of phage phiSalmchick1. 1A: Plaque morphology produced on *S.* Typhimurium as a bacterial host. 1B: Electron micrograph of *Myoviridae* phage (phiSalmchick1) under transmission electron microscope

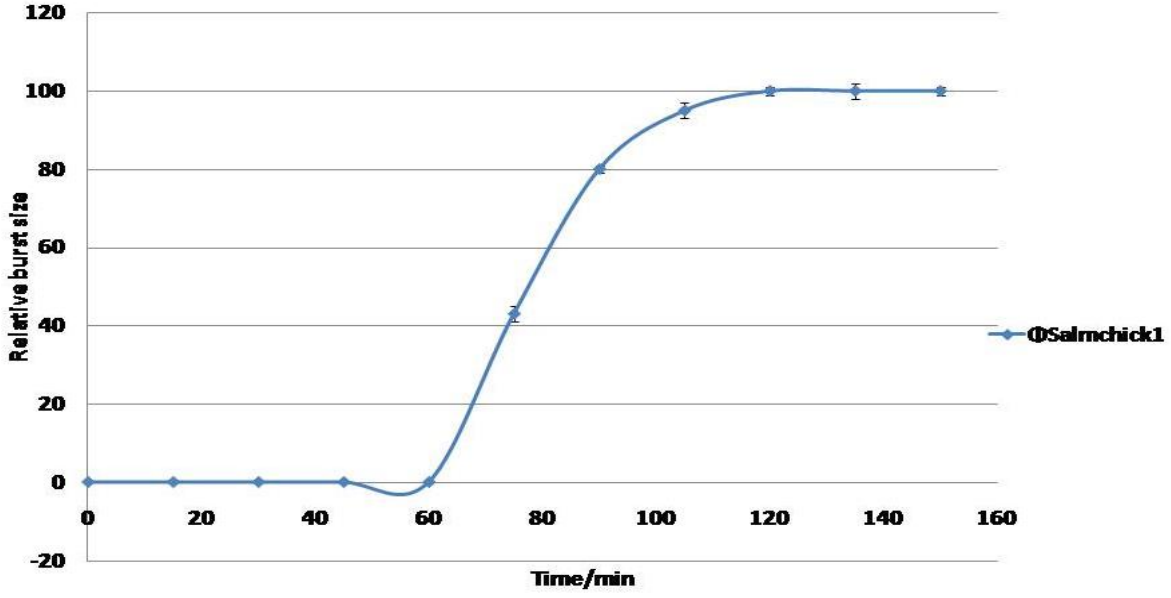

Figure 2: Single-step growth curve of the phage phiSalmchick1. The plaque forming Units (PFUs) per infected cell in *S.* Typhimurium cultures were calculated at different times after treatment. The samples were taken with an interval of 15 min. The means of relative burst size were calculated ± standard deviation (SD)

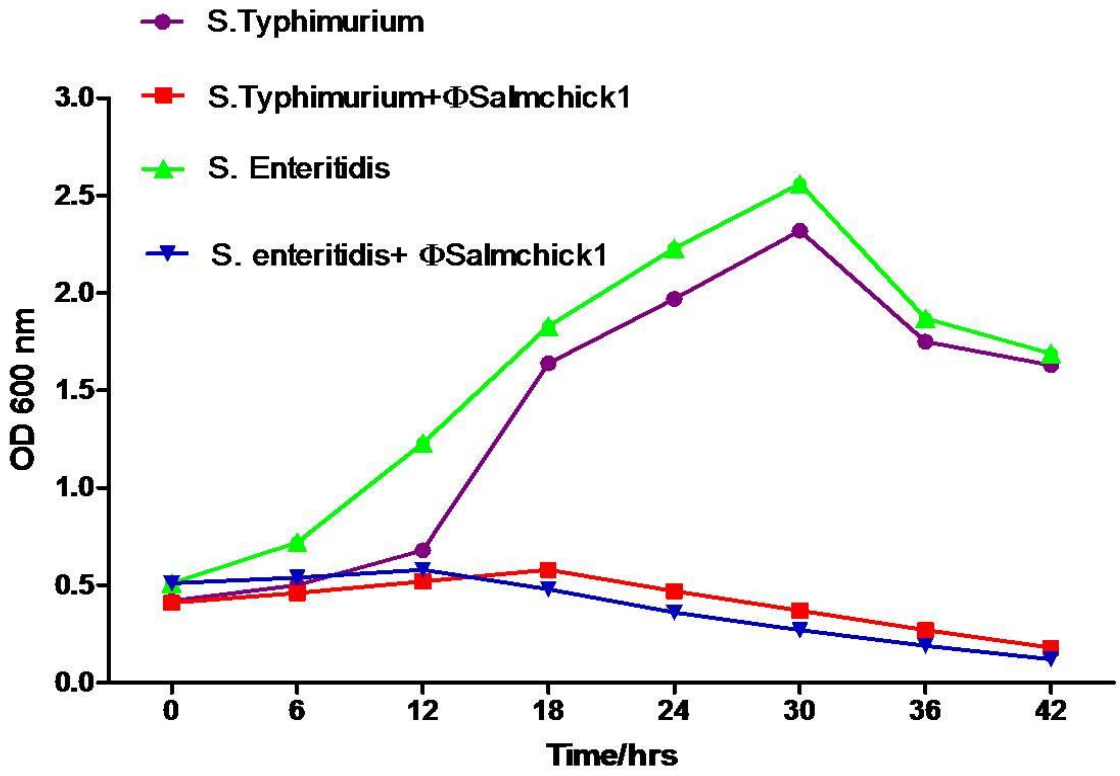

Figure 3: Effects of phage phiSalmchick1 on the growth of *S.* Typhimurium and *S.* Enteritidis at 37ºC at a MOI of 1.0. Each data point was representing means of bacterial OD ± standard deviation (SD). The values of SD were small ranging from 0.005 to 0.01; and were not appeared on the curves

Discussion

Our study showed that all recovered isolates of *S.* Typhimurium and *S.* Enteritidis from retailed broilers showed 100% resistance to seven antimicrobial agents. So, this study was handled with the isolation and characterization of specific bacteriophages targeting those MDR isolates of *S.* Typhimurium and *S.* Enteritidis derived from caecal contents, liver and breast meat of retailed broilers in Sharkia Governorate, Egypt. In this regards, one bacteriophage infecting *Salmonella* serotypes was firstly isolated from sewage water at Zagazig City, Sharkia Governorate, Egypt. It was appeared that the plagues formed by the isolated bacteriophage were clear, indicating that phiSalmchick1 is a lytic phage as previously supported (23). The isolated phage (phiSalmchick1) was belonging to family *Myoviridae* under electron micrograph. Of interest, the recovered myovirus phage was polyvalent and had a broad host range. Similarly, three phages belonging to *Siphoviridae* and *Myoviridae* (Salamacey1, Salamacey2 and Salamacey3) were polyvalent and had lytic effects on *S.* Typhimurium, *S.* Enteritidis and *S.* Kentackey originated from broilers and could infect other *Enterobacter cloacae* and *E. coli* isolates of broiler chicken (11). Moreover, polyvalent phages having ability to infect *E. coli* and *S.* Choleraesuis were reported in previous studies (24,25). However, phage SES8 recovered from chicken skin posed the narrowest host range as it induced lysis for only 2 out of 22 *Salmonella* isolates in Japan (26). The degree of phage specificity or susceptibility relies on phage attachment or adsorption on bacterial cell in the initial stage of phage infection cycle (27). The process of phage adsorption often includes specific binding of receptor-binding proteins (RBPs) in

bacteriophages and the binding of specific carbohydrate receptors located on cell wall of host bacteria (28).

Regarding one-step growth curve of myovirus, phiSalmchick1 phage had a large burst size 100 PFU per infected cell with latent period of 60 min. Similarly, the phages Salmacey3 and STS9 had large burst size of 110 and 209 PFU/ infected cell, respectively (11,26). On the other hand, the *Salmonella*-bacteriophage Felix 01 showed a small burst size of 14 PFU/cell (29). The differences in latent period and burst size of various bacteriophages could be augmented for the variations in bacterial host cell, temperature and pH (30,31). Interestingly, the isolated phage (phiSalmchick1) had a short latent period and a large burst size verifying its applicability for the control and treatment of salmonellosis in poultry and humans as previously supported (32). The genome of phiSalmchick1 was sensitive to digestion with *Eco*R1 and *Hin*dIII enzymes. This was corroborated by a previous study, where the genome of myovirus Salmacey3 was digested by *Eco*R1 and *Hind*III (11).

Phages were used to control food poisoning or zoonotic pathogens (33). Several studies have demonstrated the potential use of lytic phages to treat infections with zoonotic *Salmonella* serovars (25,34). In this study, there were significant reductions in the OD of *S.* Typhimurium and *S.* Enteritidis treated with phage phiSalmchick1 at MOI/1.0 and at 37ºC after 24 hrs incubation when compared to OD values of non-treated *S.* Typhimurium and *S.* Enteritidis ($P<0.05$). Similar studies evaluated *in vitro* the lytic activity of different phages on various *Salmonella* serovars from broiler chickens: Mahmoud et al. (11) cited OD reduction of treated *S.* Kentuckey with three phage cocktails, Salamacey1, Salamacey2 and Salamacey3, after 24 hrs; Duc et al. (25) reported a significant reduction in the count of *S.* Enteritidis co-incubated with phages SEG5, SES8, STG2, STG5, and STS9 to less than 10 CFU/ml after 24 hrs; Atterbury et al. (35) observed significant reductions in counts of *S.* Enteritidis, *S.* Typhimurium and *S.* Hadar co-treated with the phages Φ151, Φ10 and Φ25 by means of 2.2, 5.9 and 2.5 log, respectively after 24 hrs incubation.

Conclusion

This study provided polyvalent, lytic phage (phiSalmchick1) belonging to family *Myoviridae* against MDR isolates of *S.* Typhimurium and *S.* Enteritidis recovered from retailed broilers. Our data confirmed that this phage has a high potential for phage application to control zoonotic and MDR *Salmonella* serovars derived from broilers in Egypt. Further molecular characterization of phiSalmchick1 genome is required for future applications.

Conflict of interest

None of the authors have any conflict of interest to declare

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Original Research Article

GENETIC CHARACTERIZATION OF EGYPTIAN NEWCASTLE DISEASE VIRUS STRAINS ISOLATED FROM FLOCKS VACCINATED AGAINST NEWCASTLE DISEASE VIRUS, 2014-2015

Mohamed M. Megahed[1], Amal A. M. Eid[1], Walaa Mohamed[2], Ola Hassanin[1]*

[1]Department of Avian and Rabbit Medicine, [2]Veterinary Medicine Hospital, Faculty of Veterinary Medicine, Zagazig University, Zagazig, 44511, Egypt

*Corresponding author, E-mail: olahassanin@zu.edu.eg; olafalcon2001@yahoo.com

Abstract: In the present study, forty-five chicken flocks suspected to be infected with Newcastle disease virus (NDV) in Sharkia Governorate were submitted for clinical and postmortem examination as well as virus isolation and sero-molecular identification. Forty samples were positive for hemagglutinating viruses, 15/40 (37.5%) were confirmed positive isolates against NDV antiserum, 12/40 (30%) were positive isolates against both NDV and AIV (H5) antisera and 9/40 (22.5%) were positive against AIV antiserum using serological identification. Twenty-seven NDV isolates were confirmed positive by molecular identification; twelve of them were confirmed as virulent NDV strains using specific primers. A fragment of 766-bp comprising the 3' end of the M gene and the 5' end of the F gene was sequenced from four amplified products, revealing that these isolates carried the 112RRQKRF117 motif, which is characteristic of virulent strains. The investigated strains were clustered with a class II genotype VIId with large genetic distance with the LaSota strain. This is alarming given the potential evolution of different virulent NDVs and may explain the circulation of these virulent strains among Egyptian poultry farms despite the application of intensive vaccination programs.

Key words: Sharkia; NDV; genotype VIId; poultry; PCR

Introduction

Newcastle disease (ND), one of the most important infectious viral diseases in poultry, still causes major economic losses in the poultry industry. The devastating negative effects of the virus, such as high mortality, reduced egg production, a high condemnation rate and the cost of vaccination (1), are considered major limiting factors on industry growth in many countries due to their substantial socio-economic impact (2). ND is classified by the World Organization for Animal Health (OIE) as a list A disease because it is highly contagious, with high morbidity and mortality in susceptible birds (up to 100%) (3).

Newcastle disease virus (NDV) is an enveloped virus containing linear, non-segmented, negative sense, single-stranded RNA (4). NDV is an avian paramyxovirus-1 (APMV-1), one of the most antigenically distinct avian paramyxoviruses 1-11, genus Avulavirus, family Paramyxoviridae and order Mononegavirales (2,5,6). Based on their pathogenicity in chickens, NDV isolates are categorized into three main pathotypes: lentogenic (low virulence), mesogenic (intermediate virulence) and velogenic (high virulence)

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(7-9). The disease is characterized by respiratory involvement, nervous system impairment, and gastrointestinal and reproductive problems (8-10). Since the first confirmed outbreaks of ND in 1926 in Java, Indonesia, and Newcastle-Upon Tyne, UK (10,11), NDV has continued to re-emerge in both epidemic and endemic forms throughout the world and is a potential cause of sporadic outbreaks in formerly disease-free countries around the world (12).

In Egypt, infection with NDV was recorded for the first time in 1948 (13). Thereafter, many ND outbreaks have been reported, and Egypt has been classified as an endemic country for NDV (14,15). Prevention and control of NDV primarily depend on the strict application of biosecurity measures and intensive vaccination programs that have been successfully used throughout the world for many years (8). Nevertheless, in the last few years, NDV has caused several outbreaks in Egyptian domestic poultry flocks, resulting in massive economic losses (14).

The frequent incidence of NDV infection, even in vaccinated birds, is not only related to improper vaccination or immune suppression but may also be due to viral mutation leading to changes in the genomic sequence of the virus, thus altering its biological properties and virulence (16,17). Therefore, the present study was conducted to genetically characterize field isolates of NDV obtained from Sharkia Govronrate in 2014–2015 and analyze their genetic distance from already-existing vaccine strains.

Material and methods

Flock histories and sample collection

A total of 318 birds representing 45 chicken flocks between 10–240 days of age suspected to be infected with NDV which located in different districts of Sharkia Governorate during 2014–2015 were selected based on the presence of respiratory and/or nervous manifestations as well as greenish diarrhea despite previous vaccination against NDV (Table 1). Birds were subjected to both clinical and postmortem examination before samples collection. Forty-five tissue pools from recently dead birds (3 birds/pool) were collected from the digestive and respiratory organs (trachea, lung, proventriculus and cecal tonsils) under completely aseptic conditions. The samples were ground in sterile iced mortars to create a 10% suspension in PBS. The homogenized suspensions were transferred to centrifuge tubes for centrifugation at 1000 g for 10 min to remove extraneous materials. The supernatants were collected, and antibiotics (streptomycin at 2 mg/ml and penicillin at 2000 IU/ml) were added to inhibit bacterial contamination (18).

Virus isolation and propagation

A total of 600 (9–11-day-old) embryonated chicken eggs (ECE) obtained from non-vaccinated and non-infected chicken flocks were used for virus isolation and propagation. Processed samples (0.2 ml) were inoculated into the allantoic cavity of the ECE under completely aseptic conditions. All inoculated embryos were incubated at 37ºC for 4–7 days. Daily candling and observation were carried out. Embryos that died within the first 24 hours post-inoculation were discarded. After 24 hours, dead embryos were chilled overnight in a refrigerator for embryonic examination and a direct hemagglutination assay of the harvested allantoic fluid was performed (18). The negative samples were passaged on ECE for another two passages, and samples that showed negative results (no embryonic lesions and negative hemagglutinating activity) after the third passage were recorded as negative samples.

Micro-plate hemagglutination and hemagglutination inhibition assays

Positive hemagglutinating allantoic fluids were subjected to micro-plate hemagglutination and β-hemagglutination-inhibition tests using either NDV (Veterinary Serum and Vaccine Research Institute®) or H5AIV (Harbinweike Biotechnology Development Company®) antisera according to a previously described method (18).

Viral RNA purification and RT-PCR

Viral RNA was extracted from the harvested amino-allantoic fluid which were screened positive HI against NDV antiserum. All the purification steps were performed according to the manufacturer's instructions using a GenJet viral DNA and RNA purification kit®. Three one-step RT-PCR amplification reactions targeting different fragments of the F gene were performed using a one-step Maxime RT-PCR Premix kit®. Different primers were used to amplify three different fragment of NAD genome to diagnose and characterized the isolated strains; A (F:-5'-TTGATGGCAGGCCTCTTGC-3'), B (R:-5'GGAGGATGTTGGCAGCATT-3'); C (R:-5'-AGCGT (C/T) T CTGTCTCCT3'); NDV-M2 (F:-5`TGG AGC CAA ACC CGC ACC TGCGG3`) and NDV-F2(R:-5`GGA GGA TGT TGG CAG CATT3`) (19-20). A and B were used for amplification of 362 bp fragment of F gene for all NDV pathotypes and were tested for all NDV positive HI isolates examined in this study. A and C were used for amplification of 254 bp fragment of F gene of virulent ND strains and were tested for selected 12 NDV isolates according to their geographical distribution and breeding purposes. NDV-M2 and NDV-F2 primers were used for amplification of 766 bp from matrix and F genes and were tested for the same virulent 12 NDV isolates for sequencing purpose. The amplifications were carried out in a BOER thermocycler under the following conditions: 45ºC/30 m (minute) followed by an inactivation step and denaturation at 94ºC/5 m and 40 cycles of 94ºC/30 s (second), 50–54ºC/30 s and 72ºC/1 m with a final extension step at 72ºC/5 m. The amplified fragments were separated on agarose gels (1%) and visualized under ultraviolet light after staining with ethidium bromide.

PCR purification and sequencing

PCR purification was performed using a QIAquick Gel Extraction Kit® Protocol according to the manufacturer's instructions. Sequencing was performed commercially at the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Animal Health Research Institute, Dokki, Giza, Egypt.

Four choosen 766-bp purified RT-PCR products containing a region comprising both the M and F genes were sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA).

Genomic analysis and phylogenetic tree

Alignment and phylogenic analyses were performed using several software programs such as MEGA (Molecular Evaluation Genetic Analysis, www.megasoftwere. net), BioEdit and DNA Star.

Results

Clinical findings

The examined birds showed one or more of the following signs: respiratory signs in the form of coughing, sneezing, nasal discharge and eye inflammation; nervous signs in the form of paralysis, tremors and torticollis; or digestive disorders such as greenish diarrhea. These signs were accompanied by general signs of illness, such as depression, ruffled feathers and decreased food consumption. Cyanosis of the combs and wattles were also observed. Decreases in egg production among layer flocks sometimes reached 50%. The mortality rate ranged from 8–70%. Necropsies revealed congestion of the brain blood vessels and signs of septicemia in the form of congested subcutaneous blood vessels, congestion of the liver, spleen and lungs, and gallbladder enlargement. Tracheitis and airsacculitis were also observed in the respiratory tract. Hemorrhage on the tips of the proventriculus gland (Figure 1a), greenish mucous content in the gastrointestinal tract, elliptical raised ulcers in the intestine (Figure 1b) and enlarged hemorrhagic cecal tonsils were also observed.

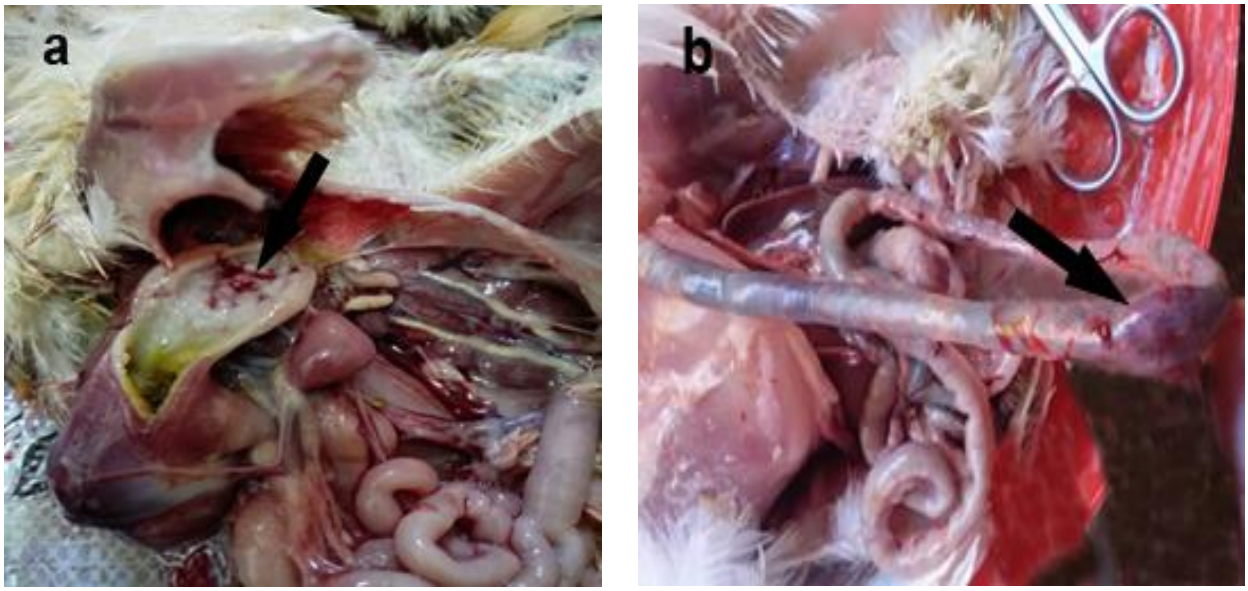

Figure 1: Pathological features of the naturally infected NDV flocks. A: Petechial hemorrhage on the proventriculus glands of 42-day Cobb broiler chick. B: Elliptical ulcer in the intestinal tract in 25 day Hubbard broiler chick

Virus isolation

As shown in Table (1), 40 out of 45 (88.8%) inoculated samples demonstrated positive hemagglutination activity in the harvested allantoic fluid from the first passage. The inoculated embryos showed extensive hemorrhage, congestion and death within 48–96 hours after the first passage. The remains of the negative samples were passaged on ECE for another two passages, and samples that showed negative results (no embryonic lesions and negative hemagglutinating activity) after the third passage were recorded as negative samples.

Micro-hemagglutination inhibition results

Positive HA isolates that were subjected to HI tests against both NDV and AIV (H5) antisera revealed 15/40 (37.5%) positive isolates against NDV antiserum and 12/40 (30%) positive isolates against both NDV and AIV (H5) antisera. Nine isolates (22.5%) were positive against AIV antiserum, and four isolates (10%) did not demonstrate positive activity against either NDV or AIV (H5) antisera (Table 1).

Table 1: Descriptive data and NDV detection steps of the examined chicken flocks

Flock No.	Locality	Breed	Age	Virus Isolation	HI	HI	PCR common type	PCR pathogenic type	PCR primers
					NDV	AIVH5	Common type	Pathotype	M-F primer
1	Zagazig	SASSO	18	+	+	+	+	+	+
2	Menia Kamh	Cobb	41	+	+	-	+	Nd	Nd
3	Zagazig	Native	20	+	+	-	+	Nd	Nd
4	Abo Hamad	SASSO	21	+	+	-	+	Nd	Nd
5	Zagazig	Hubbard	25	+	+	-	+	Nd	Nd
6	Faquos	Hubbard	27	+	+	-	+	Nd	Nd
7	Zagazig	Native	45	-	Nd	Nd	Nd	Nd	Nd
8	Faquos	Cobb	33	+	+	+	+	+	+
9	Elsalhia	Hubbard	25	+	+	-	+	Nd	Nd
10	Elsalhia	Cobb	32	+	-	+	Nd	Nd	Nd
11	Faquos	Lohman (Brown)	180	+	-	+	Nd	Nd	Nd
12	Faquos	Hubbard	24	+	+	+	+	+	+
13	Faquos	Hubbard	31	+	+	+	+	Nd	Nd
14	El-Ibrahemia	Cobb	34	+	+	+	+	+	+
15	Hehia	Cobb	34	+	-	-	Nd	Nd	Nd
16	Faquos	SASSO	22	+	-	-	Nd	Nd	Nd
17	Faquos	Hy-line (Brown)	10	-	Nd	Nd	Nd	Nd	Nd
18	Elsalhia	Lohman (white)	77	+	-	+	Nd	Nd	Nd
19	Elsalhia	Lohman (Brown)	70	+	-	+	Nd	Nd	Nd
20	Faquos	Hy-sex (Brown)	124	+	+	+	+	+	+
21	Abo Kabeir	SASSO	50	+	+	+	+	+	+
22	Abo Kabeir	Cobb	23	+	-	-	Nd	Nd	Nd
23	Abo Kabeir	Cobb	35	+	-	+	Nd	Nd	Nd
24	Abo Kabeir	Saso	30	+	+	+	+	+	+
25	Abo Kabeir	Saso	28	+	+	+	+	Nd	Nd
26	Faquos	Hy-line (Brown)	240	+	-	+	Nd	Nd	Nd
27	Faquos	Hubbard	33	-	Nd	Nd	Nd	Nd	Nd
28	Faquos	Hy-sex (Brown)	86	+	-	+	Nd	Nd	Nd
29	Abo Kabeir	Hy-line (red)	200	+	-	+	Nd	Nd	Nd
30	Hehia	Balady	34	+	+	+	+	Nd	Nd
31	Abo Hamad	Hy-line (Brown)	46	-	Nd	Nd	Nd	Nd	Nd
32	Faquos	Cobb	28	-	Nd	Nd	Nd	Nd	Nd
33	Abo Kabeir	Lohman (Brown)	200	+	-	+	Nd	Nd	Nd
34	Hehia	Cobb	35	+	+	-	+	+	+
35	Faquos	Hubbard	35	+	+	-	+	+	+
36	Abo Kabeir	Baladi	40	-	Nd	Nd	Nd	Nd	Nd
37	Zagazig	Cobb	25	+	+	+	+	+	+
38	Faquos	Hubbard	24	+	+	-	+	Nd	Nd
39	Zagazig	Hubberd	28	+	+	-	+	Nd	Nd
40	Elknayat	Hy-line (Brown)	90	+	+	-	+	Nd	Nd
41	Belbais	Lohaman (white)	28	+	+	+	+	+	+
42	Faquos	Cobb	28	+	+	-	+	Nd	Nd
43	Zagazig	Cobb	35	+	+	-	+	Nd	Nd
44	Menia Kamh	Hubbard	28	+	+	-	+	+	+
45	Zagazig	Cobb	28	+	+	-	+	Nd	Nd

Flock 08: NDV/chicken/Egypt/1/2015, Flock 14: NDV/ chicken/Egypt/2/2015, Flock 37: NDV/chicken/Egypt/3/2015
Flock 44: NDV/ chicken/Egypt/4/2015, Nd: note done

Molecular identification

i. RT-PCR (common type)

RT-PCR using primers A and B resulted in a 362-bp fragment amplified from all 27 NDV tested samples.

ii. RT-PCR (pathotype)

Twelve selected positive RT-PCR isolates were subjected to RT-PCR using primers A and C. A 254-bp fragment was detected in all 12 tested isolates, indicating they were virulent strains.

iii. RT-PCR flanking the M and F genes

The third RT-PCR test was carried on the 12 NDV virulent isolates using the M2 and F2 primer set, which revealed the presence of a 766-bp fragment in the tested samples.

Partial sequencing of the M and F genes

Sequencing of the 766-bp fragment comprising the 3' end of the M gene and the 5' end of the F gene in the four amplified PCR products revealed that they carried the 112 RRQKRF 117 motif, which is characteristic of virulent strains. The four obtained sequences were designated
NDV/chicken/Egypt/1/2015,
NDV/chicken/Egypt/2/2015,
NDV/chicken/Egypt/3/2015 and
NDV/chicken/Egypt/4/2015
and were published in GenBank under the accession numbers KX231852, KX231853, KX231851 and KX231854, respectively.

Alignment and sequence analysis

Alignment and sequence analysis of the four sequenced viral fragments was carried out on a selected region including the cleavage site. The truncated 413-bp fragment, spanning 5' (1–413) 3' within the F gene, was analyzed using MEGA 6.06 software. When the four sequences were aligned together, they showed a high similarity with nucleotide identity ranging from 98.3% to 99.5%. When the four sequenced strains were compared with 37 other NDV reference strains (Table 2), they were shown to be closely related to EG/ CK/ NDV/ 19/Luxor-Esna/2011, NDV/ chicken/ VRLCU138/ Egypt/2012 and NDV/ Chicken/ China/ SDYT03/2011 with average identities of 99.2%, 98.3% and 98.3%, respectively. Nucleotide identity with the LaSota reference strain ranged from 79.4–80.5%, identity with B1 ranged from 79.2–80.4% and identity with Komarov ranged from 79.2–80.4%. As shown in Figure 2, the above-mentioned variations in nucleotide identity led to several amino acid substitutions, either when the four sequences were compared together or were compared with other reference strains. NDV/ chicken/ Egypt/ 1/2015 and NDV/ chicken/ Egypt/ 3/2015 shared two amino acid substitutions at positions (4k→4R) and (13L→13P) with NDV/ Chicken/ China/ SDYT03/2011. Other substitutions were present at position 28 (L→S) in NDV/ chicken/ Egypt/1/2015 and at position 43 (V → I) in NDV/ chicken/ Egypt /3/2015. The other two studied strains were 100% identical with each other and with NDV/ Chicken/China/ SDYT03/2011. When the four studied strains were compared with the LaSota strain, 27 amino acid substitutions at different positions were found. Regarding the cleavage site motif, the four studied strains contained the same cleavage site specific to NDV virulent strains such as NDV/ Chicken/ China/ SDYT03/ 2011.

Table 2: Newcastle disease reference strains used in the phylogenetic analysis

Strain	Accession number	Genotype
NDV/99-0655	AY935494	I
NDV/99-1997PR-32	AY935497	I
NDV/Queensland-V4	AF217084	I
NDV/lreland/Ulster/67	AY562991	I
NDV/LaSota	AY845400	II
NDV/clone30	Y18898	II
NDV/Ostrich/lsmailia/2010	JN 193503	II
N DV/B1	NC-002617	II
NDV/ turkey/USA/VGGA/89	AY289002	II
NDV/chicken/Cameroon/CAE08-061/2009	HF969123	II
NDV/chicken/Egypt/4/2006	FJ969395	II
APMV-1/chicken/U.S.(TX)/GB/1948	GU978777	II
NDV/chicken/Egypt/2/2006	FJ969393	II
NDV/ Beadutte C	X04719	II
NDV/Chicken/Egypt/1/2005	FJ9393I3	II
NDV/Komarov	KT44590l	I
NDV/JS/9/05/Go	FJ430160	III
APMVI-australia-victoria	M21881	III
NDV/Herts/33	AY741404	IV
NDV/ltalien	EU293914	IV
NDV/rAnhinga	EF065682	V
NDV/ mixed species/U.S./Largo/71	AY562990	V
NDV/ASTR/74	Y190I2	VI
NDV/chicken/U.S.(CA)/1083(Fontana)/72	AY562988	VI
NDV/chicken/Egypt/2/2015	KX231853	VII
NDV/chickenEgypt/4/2015	KX23I854	VII
NDV/chicken/Egypt/1/2015	KX231852	VII
NDV/chicken/Egypt/3/2015	KX231851	VII
NDV-F388-RLQP-CH-EG-I4	KP316016	VII
EG/CK/NDV/19/Luxor-Esna/2011	KP 119144	VII
NDV-B161-RLQP-CH-EG-I2	KM288620	VII
NDV/B131/RLQP/CH-EG- 12	KM288610	VII
NDV/Chicken/China/SDYT03/2011	JQ015297	VII
NDV/turkey/lsrael/111/2011	JN979564	VII
NDV/chicken/VRLCU138/Egypt/2012	JX885868	VII
NDV/QH1	FJ751918	VIII
NDV/Mali-ML029_07/2007	JF966386	XIV
NDV/chicken/Togo AK018'2009	JX390609	XVII
NDV/Chicken/lvoryCoast/CIV08-069/2007	HF969127	XVIII
NDV/Chicken/CentralAfrican Republic/CAF09-014/2008	HF969125	XVII
NDV/chicken/Nigeria/N IE10-124/2011	HF969168	XVII

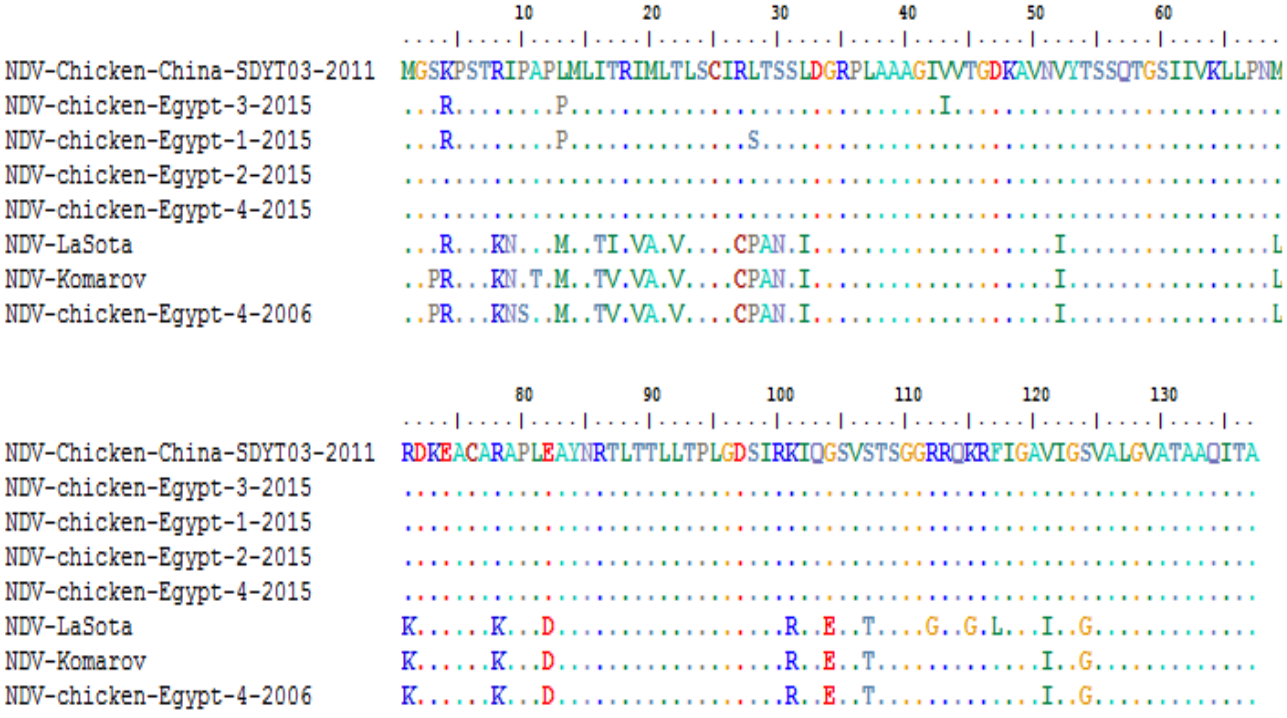

Figure 2: F protein amino acids alignment between the 4 studied strains and other vaccine and reference strains. The alignment was performed using bioedit software

According to the phylogenetic analysis of the studied strains, the nucleotide sequences of 41 strains isolated from different areas of the world, including vaccine strains, were aligned. As shown in Figure 3, the four studied strains clustered with genotype VII (d) of previously published velogenic NDV strains. The nearest neighbors for the four studied strains were EG/CK/NDV/19/Luxor-Esna/2011, NDV/F388/RLQP/CH-EG/14, NDV/chicken/VRLCU138/Egypt/2012, NDV/turkey/Israel/111/2011 and NDV/Chicken/China/SDYT03/2011, with average distances of 0.9%, 1.4%, 1.6%, 1.6% and 1.9%, respectively. The average distance between the four studied strains and the lentogenic LaSota vaccine was 24%, while the average distance was 22% in the case of the mesogenic Komarov vaccine.

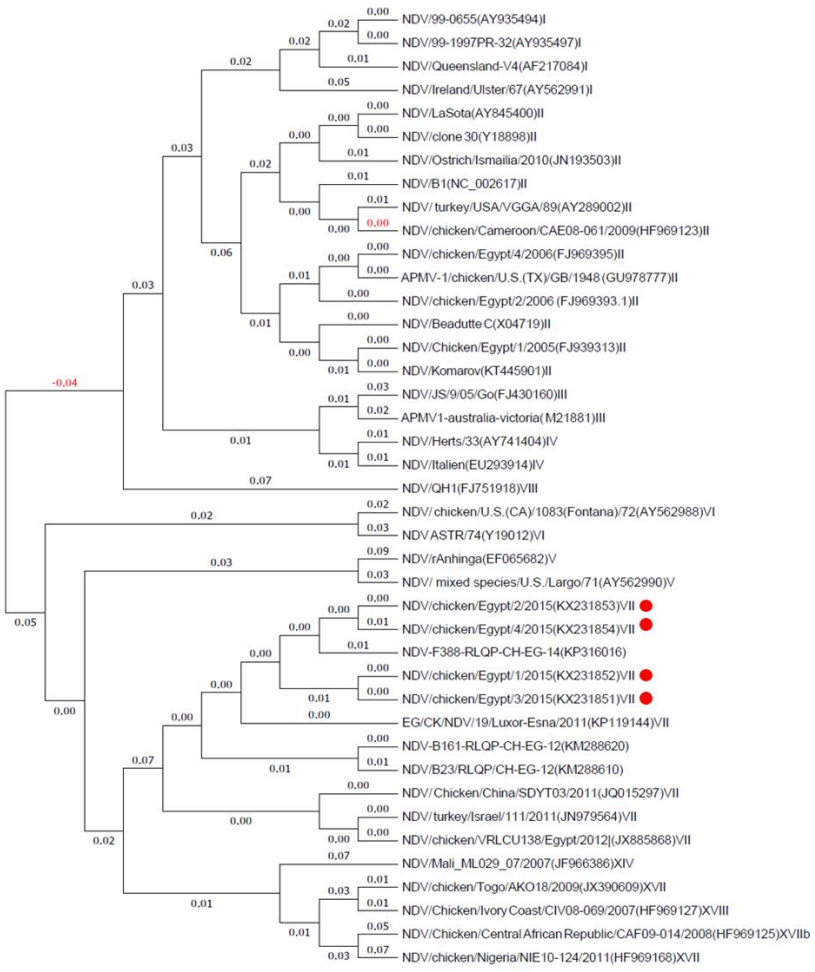

Figure 3: Phylogenetic analysis of the nucleotide sequence of NDV strain based on partial F-gene. The four sequences in this study are labelled. Phylogenic relationship through a bootstraps trail of 1000 was determined with the MEGA Version 6 using Cluster W alignment algorism and neighbour-joining method for tree construction.

Discussion

Undoubtedly, ND has been considered as one of the highly destructive disease (12). Not only the highly contagious nature of disease but also the high morbidity and mortality rate play a vital role to classify ND as list A disease by OIE (21). Since 1948, ND has been recognized as an endemic disease after it was discovered for the first time in Egypt (13). The virus still causes severe outbreaks on poultry farms a cross Egypt, although intensive vaccination programs against NDV has been continuously applied either on large scale or small scale poultry farming.

The genetic composition of the NDV genome isolated from chicken flocks in Sharkia Governorate was studied previously in 2005-2006 (15). However, it is of interest to identify the changes characterizing NDV in the past 10 years. Clinically, the examined birds presented respiratory signs including sneezing, coughing, and nasal discharge; nervous signs manifesting as torticollis, opisthotonus, and leg paralysis; and/or digestive troubles such as greenish diarrhea surrounding the cloaca. Cyanosis of the comb and wattles was also observed. Postmortem examination revealed congestion (brain, trachea, lungs and liver) and hemorrhages (proventriculus, intestine and cecal tonsils). Thickening of the air sacs and greenish mucous content in the intestinal tract were observed. The above-mentioned clinical findings were previously shown to be associated with

NDV and AIV virus infection by many authors (7,22,23). The presence of elliptical ulcers in the intestinal tract was reported in 20 poultry flocks as a characteristic pathogenomic lesion of vvNDV (23).

Viral inoculation in ECE led to congestion and hemorrhages in chicken embryos with deaths between 48–96 hours post-inoculation as well as positive HA activity in the allantoic fluids of forty investigated samples. Similarly, Alexander (8) stated that NDV induces hemorrhage and mortality in chicken embryos as well as hemagglutinating activity in chicken RBCS. However, these embryonic lesions and viral HA activity are potentially attributable to any of the avian paramyxoviruses or avian influenza viruses (21). Therefore, it is necessary to confirm the presence of NDV using an HI test due to its low cost, availability and simplicity (24,25). In the present study, the HI results revealed 15 flocks were infected by NDV alone, 12 flocks were infected by both NDV and AIV (H5), and only 9 flocks were infected by AIV (H5) alone. In Nigeria, a serological survey using an HI test proposed that a concurrent infection between pathogenic avian influenza and NDV was possible (26).

In the current study, the RT-PCR technique was utilized to schematically identify, characterize and pathotype the isolated NDVs. First, the A+B primer set was used for the preliminary identification of NDVs via amplification of the 362-bp fragment of the F gene, which is conserved in all NDV pathotypes (20). Twenty-seven isolates were confirmed as positive for NDV via PCR amplification. NDV isolates are divided into three groups based on their virulence in poultry: velogenic (high virulence), mesogenic (moderate virulence) and lentogenic (low virulence) (3,18). Second, another set of primer pairs (A+C), which included conserved forward primer and reverse primers representing the cleavage site of the F gene for virulent strains, was used to amplify a 256-bp fragment only in cases of virulent NDV (20). Our selected 12 samples were based on their geographical distribution and breeding purpose were tested and confirmed as positive virulent strains. These data confirmed that RT-PCR was a successful and rapid tool for NDV strain characterization and pathotyping during outbreaks (20,27).

Because all conserved NDV-positive samples originated from flocks that were subjected to intensive NDV vaccination programs, including live and inactivated vaccine, pathotyping and genotyping of the circulating virus among these flocks was a critical issue. The OIE defines an outbreak of ND as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that has multiple basic amino acids at the C-terminus of the F2 protein and a phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term multiple basic amino acids refers to three or more basic amino acids, specifically lysine (K) or arginine (R), present in residues 113–116 and phenylalanine (F) at position 117 (21,28). Failure to demonstrate the characteristic amino acid residue pattern described above requires characterization of the isolated virus with an ICPI test (21).

On the other hand, avirulent viruses contain the 112 G/E-K/R-Q-G/E-R-L 117 motif at the F protein cleavage site, which fails to meet the above-mentioned criteria for virulent strains (29). Therefore, sequencing of the 766-bp fragment from four PCR products obtained from virulent NDV isolates (spanning position 980 of the M gene to position 485 of the F gene) revealed that they contained the F-gene cleavage site characteristic of virulent NDV strains (112RRQKRF117). Based on genome length and the F gene sequence, NDV strains have been classified into two classes within a single serotype: class I (nine genotypes, 1–9) and II (eighteen genotypes, I–XVIII) (30,31). Currently, the circulating strains associated with disease outbreaks worldwide predominantly derive from genotypes V, VI and VII of class II (6).

Interestingly, phylogenetic analysis of the nucleotide sequence of the F gene clustered the four sequenced strains with class II genotype VII strains (EG/CK/NDV/19/Luxor-Esna/2011, NDV/F388/RLQP/CH/EG/14 and NDV/chicken/VRLCU138/Egypt/2012), which are responsible for recent NDV outbreaks at other areas in Egypt (32,33). The four sequenced strains demonstrate low distance and high

identity with EG/ CK/ NDV/ 19/Luxor-Esna/2011 and Chicken /China / SDYT03/2011 (98.8–99.5% and 97.5–98.5%, respectively). Analysis of the obtained results clearly indicates a considerable rate of mutation among circulating NDVs between 2005 and now. Saad and colleagues recorded the presence of class II genotype II among chicken flocks in Sharkia Governorate (34). Later, a similar study performed on samples obtained from 2005–2006 in the same Governorate concluded that the NDV circulating in the Sharkia Governorate clustered with class II genotype II and demonstrated high identity with the LaSota strain (93.4–98.2%) (15).

In the current study, the four studied strains clustered with class II genotype VII and demonstrated a lower range of identity (79.2–80.6%) and an overall amino acid distance of 24.4% with the vaccine strains, including the LaSota strain. This finding is very similar to a previous study in which a distance matrix analysis of the F gene from NDV genotype VII strains isolated from Malaysia indicated an overall amino acid distance of 21% with the genotype II LaSota vaccine and 12.8% with genotype VIII isolates (35). The large genetic distance with the LaSota strain may be alarming regarding the potential evolution of different virulent NDV and may explain the circulation of these virulent strains among Egyptian poultry farms despite the application of intensive vaccination programs. On the other hand, other studies have suggested that the circulation of virulent NDV among vaccinated flocks may be attributed to poor flock immunity due to inadequate vaccination practices and concurrent infection with immuno-suppressive agents rather than antigenic variation (36-38).

Moreover, the studied strains had several amino acid substitutions with the previously isolated strain from the same locality (NDV/Chicken/Egypt/4/2006). A previous study described NDV genotype VII strains isolated from China as immune response-escaping antigenic variants (39). Furthermore, previous studies concluded that superior protection against genotype VII is achieved using the closest vaccine to the circulating field isolates considering the induction of a higher and earlier immune response, protection against morbidity and mortality, and reduction of challenge virus shedding (33,40). The presence of specific amino acid residues may help in subtyping NDV genotypes. Hence, genotype VII is sub-classified into 5 subtypes (genotypes VIIa, b, c, d and e), each with a specific molecular characterization. One example of a genotype VIId isolate involves a unique pattern consisting of V52-for-I, K101-for-R, S176-for-A and Y314-for-F (39). In our study, the four sequences were genotype VIId. Interestingly, this subtyping method provides researchers with highly valuable information about the origin and epidemiology of the studied strains. For example, genotypes VIIa and b represent viruses that emerged in 1970 in the Far East and spread to Europe and Asia or South Africa, respectively. Another example is genotypes VIIc and d, which originated from China, Kazakhstan and South Africa (40,41). Therefore, there is a strong probability that the studied strains are from China or Middle Eastern countries such as Israel and enter Egypt through infected poultry products or wild birds.

Conclusion

In conclusion, genotype VIId class II strains are the currently circulating virus strains responsible for NDV outbreaks among poultry farms in Sharkia Governorate. Despite current vaccination regimens against NDV, this virus continues to impact the Egyptian poultry industry. Further investigation to evaluate the efficacy of the available vaccines against challenge with recently isolated viruses is recommended, as is the improvement of vaccine application to ensure proper vaccination.

Conflict of interest

The authors have no conflict to declare.

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Original Research Article

EFFECT OF GINGER DIETARY SUPPLEMENTATION ON GROWTH PERFORMANCE, IMMUNE RESPONSE AND VACCINE EFFICACY IN *Oreochromis niloticus* CHALLENGED WITH *Aeromonas hydrophila*

Ahmed El-Sebai[1], Abd El-Hakim El-Murr[2], Azza A. A. Galal[1]*, Sabry M. A. Abd El-Motaal[1]

[1]Department of Pharmacology, [2]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Zagazig University, 44511 Zagazig, Egypt

*Corresponding author, E-mail: azzapharma@yahoo.com; azzagalal@zu.edu.eg

Abstract: Ginger powder was used in the current study for the improvement of the growth performance and the efficacy of *Aeromonas hydrophila* (*A. hydrophila*) vaccination in *Oreochromis niloticus*. A total of 120 apparently healthy fish were classified into 2 equal groups. The control fish received a basal diet and ginger group received a basal diet enriched with 1% ginger powder. The feeding period lasted for 2 months and the growth performance indicators were measured. Next, each group was allocated into 2 equal groups to be 4 groups. G1: received basal diet, G2: received basal diet then vaccinated with *A. hydrophila* vaccine, G3: received ginger supplemented diet, and G4: received ginger supplemented diet then vaccinated with *A. hydrophila* vaccine. One-week post vaccination, fish were challenged with *A. hydrophila* bacteria. Fish fed 1% ginger supplemented diet had a marked increase in growth performance parameters and utilized their feed more efficiently than those received the basal diet. Nitric oxide, IgM and lysozyme activity were significantly differed among fish groups in order of G4 >G2 > G3 > G1. Ginger and/or *A. hydrophila* vaccine reduced the clinical signs, mortality rate as well as the elevation of alanine aminotransferase and creatinine which induced by *A. hydrophila* infection. It could be inferred that ginger has been suggested as growth promoter and immune-stimulant in *O. niloticus* and increase efficacy of *A. hydrophila* vaccine, which increase protection against *A. hydrophila* infection.

Key words: ginger; *O. niloticus*; growth performance; immunity; vaccine

Introduction

Fish is an important source of protein for human in most countries all over the world and aquaculture represents an important source for fresh water and marine water fish (1). *Oreochromis niloticus* (*O. niloticus*), Nile tilapia, is the most popular cultured species among tilapia in many countries around the world (2). Tilapia are the species of choice in the field of aquaculture due to their high growth rate, high marketable value, reproduce easily and withstand the environmental conditions and rough handling (3). Motile Aeromonas Septicemia (MAS) is a standout amongst the most well-known bacterial diseases of tilapia which is caused by *Aeromonas hydrophila* (*A. hydrophila*) and causing high mortalities in tilapia (4). Millions of dollars were lost per year due to the diseases caused by this bacterium (5). *A. hydrophila* can affect a variety of fish species including tilapia

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(6), common carp (7), eel (8), cat fish (9), and goldfish (10).

Vaccination has a fundamental role in vast scale commercial aquaculture. The use of vaccines in the aquaculture industry has been important in reducing economic losses caused by the disease (11,12) and in the reduction of antibiotic use (13). Excessive use of antibiotics in fish farms results in the emergence of antibiotic-resistant bacteria and creation of toxicants, which may lead to environmental risk (14).

The vaccine is a preparation of killed micro-organisms which are attenuated, fully virulent or non-virulent. Once enhanced by a vaccine, the antibody-producing B-cells still sensitized to the infectious micro-organisms and respond to reinfection by producing more antibodies, so re-stimulating the immune response (15).

Ginger (*Zingiber officinalis*, Roscoe), is as a safe medicinal plant (16); contains flavonoids, alkaloids, polyphenols, steroids, saponin, fibre, tannin, carbohydrate, carotenoids, vitamins, and minerals (17); natural antioxidants as gingerols, shogaols and zingerone and essential oils that has strong anti-inflammatory effect (18). Supplementation of fish diets with ginger may improve the disease resistance by strengthen the host growth and innate immune response that are important for safeguard against infectious diseases (19). Herbs such as ginger have been reported for their biological effects such as growth promotion and immune-stimulation (20). The current experiment delineated to investigate the improvement of the growth performance and the efficacy of *A. hydrophila* vaccination in *O. niloticus* by ginger supplementation.

Material and methods

Experimental fish

A total number of 120 apparently healthy *O. niloticus* with an average body weight 31±0.5 g purchased from Central Laboratory for Aquaculture, Abassa Fish Farm at Sharkia Governorate. Fish kept in glass aquaria filled with 90 L de-chlorinated fresh water. The water temperature, dissolved oxygen, pH, ammonium and nitrite were measured and found to be 27 ± 2 ºC, 5.4 mg/l, 7.2, 0.20 mg/l and 0.02 mg/l, respectively. They received normal diet, which was prepared according to NRC (21) to fulfil the nutrient needs of Nile tilapia. Fish supplemented with the basal diet daily at 3% of their body weight throughout the adaptation period (2 weeks).

Fish diets and feeding

The ginger bulbs purchased from local farms in Sharkia Governorate, Egypt. They washed, sun dried, and powdered. The ginger supplemented diet was prepared at Fish Research Center, Faculty of Veterinary medicine, Zagazig University, Egypt. It was prepared by mechanical mixing as 1% ginger powder with the basal diet ingredient, and then finally pelleted. The pellets were dried at room temperature (26ºC for 48 h) and stored in a refrigerator at 4ºC until use. It contained (2940 kcal/kg ME and 30.80% CP) in the form of dry pellets and prepared to fulfil the nutrient needs of Nile tilapia (21). Feedstuffs used in diets preparation examined according to A.O.A.C. (22). All fish provided with their diets at a level of 3% of body weight three times daily for 60 days.

Preparation of vaccine

Formalin-killed *A. hydrophila* bacterin prepared by the addition of formalin (0.3%) to the bacterial culture, previously incubated at 35°C for 48 hrs according to Baba et al. (23). The formalized bacterial culture kept at room temperature overnight, and then subjected to sterility and safety tests as mentioned by Cardella et al. (24). The sterility test performed by culturing washed bacterin on Tryptic Soy agar and plates incubated at 37°C for 24 hrs and then examined for bacterial growth. The safety test performed by the intraperitoneal injection of the prepared bacterin cells (0.1 ml) to twenty susceptible tilapia fish. The fish kept under observation for 2 weeks post-inoculation and then dead fish subjected to necropsy for re-isolation of *A. hydrophila* using Aeromonas selective base media. Vaccine placed at 4°C. Immediately before use, the prepared vaccine washed twice with sterile saline solution and was prepared at a concentration of 3 mg wet-weight/ml saline.

Experimental design

Fish were classified into two equal groups (each group has three replicates; 20 fish/ replicate). The control group received a basal diet and ginger group received a basal diet enriched with 1% ginger powder. The feeding period lasted for 2 months and growth performance indicators measured.

Next, each group was allocated into two equal groups so become four groups each group contain 30 fish. Group 1: supplemented with normal fish diet, group 2: supplemented with normal fish diet then vaccinated with *A. hydrophila* vaccine, group 3: received ginger supplemented diet, and group 4: received ginger supplemented diet then vaccinated with *A. hydrophila* vaccine.

Fish in-group 2 and 4 were vaccinated two times with one week interval. Fish vaccinated with 0.1 ml formalin killed *A. hydrophila* diluted in 0.1 ml sterile saline according to Badran et al., (25)**.**

Challenge test

One week post vaccination, all group were challenged intraperitoneally with 0.1 ml of pathogenic *A. hydrophila* (10^8 CFU mL^{-1}) that had previously isolated from moribund fish and confirmed to be pathogenic (26). The groups are, G1 (infected- non-vaccinated group), G2 (infected- vaccinated group), G3 (infected-ginger supplemented group) and G4 (infected-vaccinated- ginger supplemented group). Challenged fish observed for clinical signs and mortality for 14 days. Any dead fish subjected immediately to post-mortem examination and routine bacteriological examination.

Blood samples

Blood samples (n=9/group) were collected 3 days after the 2nd vaccination (for evaluation of some immunological parameters) and 3 days post-challenge (for assessment of some biochemical parameters) according to Stoskopf (27). Blood samples were taken without EDTA and were centrifuged at 3000 rpm for 15 minutes for serum separation.

Growth performance parameters

The fish weighed at the beginning and after 2 month of feeding. Average body weight (BW), body gain (g), body gain percent (%), feed conversion ratio (FCR) and specific growth rate (SGR) were determined according to Merrifield et al., (28).

Determination of some immunological and biochemical parameters

Nitric oxide (NO) level was detected as the method described by Rajaraman et al., (29). Lysozyme activity detected according to Parry et al., (30). Immunoglobulin (IgM) measured by an ELISA method according to Fuda et al., (31). Alanine aminotransferase (ALT) assessed as described by Reitman and Frankel (32). Serum creatinine assessed by using the method of Henry (33).

Data analysis

The statistical significance of the immunological and biochemical data was assessed by one way ANOVA using SPSS statistical software package. Data of growth performance parameters were analysed by Student's t test. The level of significance was taken as $p < 0.05$.

Results

Effect of ginger supplementation on growth performance parameters:

The results revealed that the fish fed 1% ginger supplemented diet for 60 days had a highly significant ($p<0.05$) elevation in total final BW (53.45 g), BWG (21.8 g), BG % (68.88%) and SGR (0.87%), and a significant ($p<0.05$) improvement in the FCR (1.95) than those fed the control diet (48.5 g, 16.88 g, 53.38%, 0.71 and 2.48, respectively). There was non-significant ($P>0.05$) change in average daily feed intake between control and ginger supplemented group (Table 1).

Effect of ginger supplementation and/or A. hydrophila vaccine on some immunological parameters:

Nitric oxide, IgM and lysozyme levels were significantly (p<0.05) differ among fish groups. Regarding to the above-mentioned parameters the positive effect was recorded in group 4 that fed on diet contained 1% ginger and vaccinated with *A. hydrophila* vaccine followed by group 2 and 3 then control group (Table 2).

Table 1: Effect of feeding of 1% ginger supplemented diet for 60 days on growth performance parameters of *O. niloticus* (means ±SE)

Parameters	Experimental groups		*Sig. (2-Tailed)*
	Control	Ginger	
Initial BW (g)	31.62±0.38	31.65±0.51	0.415
Final BW (g)	48.50±0.58	53.45±0.48*	0.000
Body gain (g)	16.88±0.20	21.80±0.25*	0.000
Body gain (%)	53.38±0.17	68.88±1.45*	0.000
Specific growth rate	0.71±0.002	0.87±0.010*	0.001
Feed intake (g)	41.88±0.68	42.66±1.11	0.130
Feed conversion ratio	2.48±0.01	1.95±0.05*	0.000

Values are represented as the mean±SE. * denote significant difference (p<0.05). Control group, fed a basal diet. Ginger group fed a basal diet supplemented with 1% ginger powder.

Table 2: Effect of feeding of 1% ginger supplemented diet and/ or *A. hydrophila* vaccine on some immunological parameters of *O. niloticus* (means ±SE)

Parameters	Experimental groups				*p*-value
	G1	G2	G3	G4	
Nitric oxide (µg /ml)	34.35±1.95^{b}	40.37±0.90^{a}	36.93±1.92^{b}	40.25±0.58^{a}	0.006
IgM (µg /ml)	0.20±0.003^{b}	0.29±0.004^{a}	0.28±0.005^{a}	0.30±0.0005^{a}	0.000
Lyzozyme (µg /ml)	0.22±0.003^{c}	0.34±0.003^{ab}	0.31±0.0.004^{b}	0.44±0.039^{a}	0.000

Values are represented as the mean±SE. ^{abc} Within-row different superscript letters denote significant difference (P<0.05). G1: fed on basal diet, G 2: fed on basal diet then vaccinated with *A. hydrophila*. G3: fed on ginger supplemented diet. G4: fed on ginger supplemented diet then vaccinated with *A. hydrophila* and infected with *A. hydrophila* bacteria.

Effect of ginger supplementation and/or A. hydrophila vaccine on clinical signs post-mortem lesions, mortality rate and some biochemical parameters of A. hydrophila infected fish:

Fish in control infected group showed loss of equilibrium, ascites, skin darkness, exophthalmia and ulcers. Enlargement and congestion of internal organs were appeared in post-mortem examination. The severity of clinical signs and post-mortem lesions differed following the order of G1 (infected- non-vaccinated group) > G2 (infected- vaccinated group) and G3 (infected-ginger supplemented group)>G4 (infected-vaccinated- ginger supplemented group). The survival rate was 76.7% in G1, 90% in G2, 83.4 in G3 while 96.7 % in G4 (Table 3).

The vaccinated fish group (2nd) or groups fed on 1% ginger with or without vaccination revealed a marked (p<0.05) lowering of renal injury marker (creatinine) and hepatic injury biomarker (ALT) concentrations compared to infected non vaccinated group (Table 3).

Table 3: Effect of feeding of 1% ginger supplemented diet and/ or *A. hydrophila* vaccine on mortality rate and serum levels of ALT and creatinine of *A. hydrophila* infected *O. niloticus* (means ±SE)

Parameters	Experimental groups				*p*-value
	G1	G2	G3	G4	
ALT (IU/L)	40.67±2.02[a]	34.33±1.20[b]	33.67±1.20[b]	28.67±0.88[c]	0.000
Creatinine (mg/ dl)	0.48±0.02[a]	0.47±0.02[a]	0.41±0.01[b]	0.38±0.01[b]	0.014

Values are represented as the mean±SE. [abc] Within-row different superscript letters denote significant difference (P<0.05). G1: fed on basal diet and infected with *A. hydrophila* bacteria, G 2: fed on basal diet then vaccinated with *A. hydrophila* and infected with *A. hydrophila* bacteria. G3: fed on ginger supplemented diet and infected with *A. hydrophila* bacteria. G4: fed on ginger supplemented diet then vaccinated with *A. hydrophila* and infected with *A. hydrophila* bacteria.

Discussion

Our results revealed that the fish fed 1% ginger supplemented diet for 60 days had a significant (p<0.05) elevation in total final BW, BWG, BG % and SGR %, as well as a significant (p<0.05) improvement in the total FCR than those fed the control diet. These results clearly showed that the ginger stimulated fish growth. Our data are supported with those of Talpur et al., (34) who suggested that supplementation of Asian sea bass diet with 5 and 10 g ginger /kg feed was most effective for the growth and FCR. In addition, Sukumaran et al., (19) showed that dietary enrichment with 0.8% ginger enhanced the growth performance of *L. rohita*. The positive growth promoting effect of ginger might be due to their chemical and physical properties; their positive immune-stimulating effect or acted as an appetizer which led to stimulates digestion and improving protein and fat metabolism (34) or contains bioactive compounds which improving anti-oxidant status of the fish (35), anti-microbial (36)**,** various pharmacological effects (37) and favourable effects on gut function, which is the primary mode of action for growth promoting feed additives (38)**.** Additionally, growth performance was affected by supplementation of *Onchorhynchus mykiss* (39) or *Lates calcarifer* (34) diets with the ginger.

Fish vaccination is one of the strategies that can be applied to prevent the infectious diseases by means of inducing the immune response (40). *A. hydrophila* Vaccine significantly increased the immunity of vaccinated groups compared to the parallel control groups as shown in our results. These results supported by those of Sumiati et al., (41) who detected improvement of the specific immune response in *O. niloticus* vaccinated against *A. hydrophila* and the increase of antibody in fish involved in the elevation of the survival rate fry fish. Moreover, Sukenda et al., (42) stated that vaccination in tilapia brood stock succeeded in improving the specific and non-specific immune responses, and protecting fry tilapia from *A. hydrophila* infection. The mechanism of its action, the antigen slowly releases into the blood or tissue, stimulating and prolonging the humeral response (42)**.** The success of vaccine is often regulated by the individual antigens concentration, cross reactivity and competition among different antigens (43)**.**

Nitric oxide has serum potent bactericidal activities and showed a variety of biological functions as microbicidal and tumoricidal activity, and a range of immunopathologies (44)**.** In our results, a significant increase NO level was recorded group 2 and 4, which indicate the stimulation of the fish immune system. This may be due to ginger has a special phytochemical properties which increase efficacy of vaccine to activate fish macrophage–inducible NO synthase and stimulate NO generation in response to infection. NO can inactivate several respiratory terminal oxidases and aconitase or react with reactive oxygen intermediates (such as superoxide) to form several powerful oxidizing reactive nitrogen intermediates (such as peroxinitrite) that can effectively kill

microorganisms (45). Further, the serum NO is elevated in the *Renibacterium salmoninarum*-infected rainbow trout with (46) or in the *Edwardsiella ictaluri* infected channel catfish (45).

The soluble IgM is present in the blood and other fluids and act as an immune stimulator molecule (47). Current work clarified an elevation of serum IgM level in ginger supplemented groups (3rd and 4th). These results coincide with those of Hassanin et al., (48) who demonstrated that IgM were significantly improved in Nile tilapia due to supplementation of the diets with ginger.

Lysozyme is considered a serum bactericidal activity thus resulting in the reduction of disease (49). Our findings revealed a significant increase of lysozyme activity in 3rd and 4th groups which indicates that the immune system was enhanced in the fish (34). This may be due to lysozymes are catalysing the peptidoglycans hydrolysis of bacterial cell walls and acts as non-specific innate immunity molecules against the incursion of detrimental bacteria (50). Also, Hassanin et al., (48) demonstrated that lysozyme activity in Nile tilapia was significantly improved due to supplementation of the diets with ginger. Moreover, Sukumaran et al., (19) showed that 0.8% ginger dietary supplementation could strengthen immunity of *L. rohita*. Similarly, Clara et al., (20) recorded a significant advancement of fish resistance against *A. hydrophila* infection by dietary *Zingiber officinale* supplementation. Positive immunological results of ginger could be due to the bioactive compounds polyphenols, flavonoids, tannins and saponins that present in ginger may protect fish from bacterial infection by activating immune system (34) or have a better coordination of their stimulatory and anti-oxidant scavenging properties (51). However, unlike this study, lysozyme activity wasn't influenced in juvenile beluga fed with ginger (52).

Increased serum ALT level may indicate hepatic cellular damage (53) and elevated creatinine level signifies impaired kidney function or kidney disease. The vaccinated fish group (2nd) or groups fed on 1% ginger with or without vaccination revealed a marked reduction of ALT and creatinine concentrations compared to control infected group. The active ingredients such as flavonoids, polyphenols, saponins and tannins that present in ginger may safeguard fish from bacterial infection by stimulating immune system and its supplementation might prevent lipid peroxidation of cell membranes and inhibit the hepatic and renal damage (34). Similarly, feeding of Indian catfish with *Zingiber officinale* incorporated diet at dose of 0.5g resulted in lowering the level ALT in ginger treated group than control-infected groups (54). Furthermore, the immune-stimulating effect of 1% ginger and /or *A. hydrophila* vaccine could increase the resistance of *O. niloticus* to *A. hydrophila* infection which manifested by reduction of the mortality rate and the severity of the clinical symptoms and post-mortem lesions in G2, G3 and G4 when compared to infected–non vaccinated group.

Conclusion

The findings of the current study revealed that addition of 1% ginger to diet improved growth performance and immune status in *O. niloticus*. Ginger increases the efficacy of *A. hydrophila* vaccine, which increase protection against *A. hydrophila* infection and could represent a suitable way against this infection in *O. niloticus* aquaculture.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

NEUROCHEMICAL, HEMATOLOGICAL AND BEHAVIORAL ALTERATIONS RELATED TO ESZOPICLONE ADMINISTRATION IN RATS

Mohamed A. Kamel[1], Hesham H. Mohammed[2]*, Nora E. Abdel-Hamid[3]

[1]Pharmacology Department, [2]Veterinary Public Health Department, [3]Physiology Department, Faculty of Veterinary Medicine, Zagazig University, Egypt

*Corresponding author, E-mail: heshamvet_hosny@yahoo.com

Abstract: This study aimed to shed light on the effect of eszopiclone (ESZ) administration once daily for 30 consecutive days at night time on some neurochemical, behavioral and hematological criteria. A total of 27 male Wister albino rats were assigned to one of three drug treatment groups, vehicle, Eszopiclone (3mg/kg) and (6mg/kg). After 30 days of Eszopiclone administration, the neurochemical analysis revealed a significant reduction in serotonin and glutamate (306.44 ng/ml, 4.33 nmol /µl, respectively) in 6mg/kg treated animals, furthermore dopamine levels were significantly higher in rats treated with Eszopiclone (3mg/kg or 6mg/kg) in compare to control group. Reduced glutathione, superoxide dismutase and catalase levels revealed a significant decrease (0.15 µmol /gm tissue, 25.24 µmol /gm tissue, 1.93 Unit/gmtissue, respectively), while the malondialdehyde levels (15.79 nmol /gm tissue) demonstrated a significant increase in animals treated with 6 mg of ezopiclone. Behavioral assessment was carried out 3 times throughout the study (once/2 weeks) by video recording. It was recorded in 5 tests, including open field, the hole-board, inclined plain, grip and tail suspension tests. There were no changes between the rats in 3 mg/kg of ezopiclone and those in the control group. The rats in 6 mg/kg of ezopiclone showed less response in all behavioral observations, with significant decreases in inclined plain angle (23.35), exploratory time (17 second) and exploratory frequency (2 frequencies) in compare to other groups. The results support the concept that the administration of eszpiclone more than 3 mg/kg may lead to the behavioral changes. There were no serious adverse events regarding hematological indices. It is concluded that eszopiclone administration causes an imbalance between different neurotransmitters in the cerebrum. A marked decrease in antioxidant scavenging capacity with a behavioral alteration in 6mg/kg treated animals. The maximum safe dose of eszopiclone was 3mg/kg and more than this dose could lead to a deleterious reactions as evidenced in this study.

Key words: eszopiclone; neurotransmitters; behavior; hematology

Introduction

Eszopiclone is a soporific, mesmerizing agent that is used for treatment of difficulty maintaining sleep during the night and early morning. It is the S (+) enantiomer of the dextrorotatory form of zopiclone, a cyclo-pyrrolone with no structural likeness to the

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mesmeric drugs zolpidem and zaleplon, or to the benzodiazepines (BZs) and barbiturates (1). Eszopiclone, along with zaleplon and zolpidem, are member of grade of therapies notorious as nonbenzodiazepine benzo-diazepine receptor agonists. These drugs tie up to sites on the gamma aminobutyric (GABAA) receptor agonists that are familiar to or overlap with the sites at which BZs take action (2).

Attic BZs bind equally to GABAA receptors involving all the α-subunit isoforms with exclusion of α4 and α6 (3-5). Recently, the use of BZs has been signified to have a number of damaging undesirable effects, for example, anterograde amnesia, and discontinuation pathologies (6-9). Recognition of these side effects of BZs led to the development of a brand new cohort of non-benzodiazepine hypnotics, incorporating zolpidem and eszopiclone. Zolpidem has an elevated affinity for GABAA receptors containing the α1 subunit, low resemblance for α2 and α3 containing receptors, and no remarkable affinity for α5 encompassing receptors (2,4,5,10). Conversely, eszopiclone evinces significant activity at GABAA receptors containing α1, α2, α3 and α5 subunits (5, 10-13).

Thalamus has a mandatory responsibility in sleep act and rhythmicity, where two well defined subtypes of synaptic GABAA receptors; α1β2γ2 and α3β3γ2 are articulate in thalamocortical relay neurons and in interneurons of the reticular thalamic nucleus, so impairment to the thalamus stand in the way of normal sleep (14). Behavioural studies are crucial not only for increasing the knowledge on the status of animal but also for assessing the animal's response to its environment (15). There were some of behavioral tests that used to assess fear and anxiety in animals, and therefore attained the status of one of the most widely used instruments in animal psychology (16). These tests primarily involve placing the subject animal in a novel “open” space from which escape is prevented by a surrounding wall and then measure the elicited behaviors of the subject in this test situation. In these tests the most commonly used parameters to assess an animal’s state of anxiety are behaviour (body movements; mainly general level of activity and exploration such as movement, ambulation, rearing and freezing) and defecation (17), although other forms of behaviour such as grooming and vocalization, and other parameters such as physiological parameters (heart and respiration rate, electromyogram recording and plasma glucocorticoids levels) have also been included (16). It is generally thought, in the open field tests, that animals which show a high level of activity (all forms of locomotion) and have low defecation scores are considered to be less emotional (18).

Due to the adverse effects of benzodia-zepines, the uses of non-benzodiazepines were more popular in last few years. However, non-benzodiazepines receptor agonists had adverse effects, including dementia, delirium, sleepwalking, serious injuries and fractures (19-23), furthermore, it is associated with an increase in hospitalizations and motor vehicle accidents. Insomnia may be predisposing factor to develop anxiety disorders (24,25).

Therefore, the objective of this study was to quantify changes in some neurotransmitters and behavioral profile following the administration of Eszopiclone (3mg and 6mg). Another objective was to determine oxidative stress related to Eszopiclone administration in addition to the assessment of alterations in hematological profile.

Material and methods

The ethical approval was taken from the Animal Welfare and Research Ethics Committee, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Drug

Eszopiclone (Sepracor Inc., Marlberough, MA) was dissolved in 50 ml of acetate buffer and the dose of the drug was 3 mg/kg and 6 mg/kg of body weight, according to Huang et al. (26).

Animals

A total of 27 male Wister rats aged 7-8 weeks and weighed 180-200 gm. The experimental procedures were carried out in research unit, Faculty of Veterinary Medicine, Zagazig University, Egypt. The animals were

assigned randomly into 3 groups (9 rats in each one) and each group was subdivided into 3 replicates (3 rats in each/ cage). The rats were housed in stainless-steel cages (40 cm L*25 cm W*20 cm H), maintained in a climate-controlled environment at 23±2ºC; 60±10% of humidity with a 12/12 dark/light cycle (6 am: 6 pm).

Experimental strategy

Rats were randomized to 3 groups, each group holding 9 rats. Group 1 received 1 ml of saline (control animals). Group 2 was given 3mg/kg eszopiclone. Group 3 received 6mg/kg eszopiclone. All treatments were given orally once daily for 30 days at night time (10 pm).

Blood samples

Samples were taken from retro-orbital sinus (27) and Sodium salt of EDTA was used as an anticoagulant for estimation of some hematological frameworks.

Analysis of tissue samples

The brain of each rat was pulled out and cleaned from the stuck tissues and weighted. It was homogenized in 0.05 ml potassium phosphate buffer (pH 7.4) using Electronic Homogenizer and was kept at -80 °C until estimation of dopamine, serotonin, glutamate, glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels.

Dopamine estimation

Whole brain was dissected out, wet tissue was homogenized in HCL-butanol for about 1 minute (1:10 ratio), centrifuged for 10 minutes at 3000 rpm, and one ml of supernatant was taken after centrifugation for dopamine estimation as described by Shyamjith et al. (28).

Serotonin and Glutamate estimation

The content of brain from serotonin was estimated by the method mentioned by Schlumfjf et al. (29), while the glutamate levels were measured by multiple development paper chromatography (30).

GSH and SOD estimation

The levels of GSH and SOD in the brain were measured as mentioned by Davies et al. (31) and Ohkawa et al. (32) respectively.

Estimation of CAT and MDA

CAT was estimated by the decomposition of H2O2 after adding 1 ml of H2O2 to 2 ml of the prepared sample (0.1 ml of supernatant +1.9 ml of phosphate buffer). While, malondialdehyde levels were estimated by thio-barbituric acid reaction (32).

Hematological studies

Total RBCs count and WBCs were scrutinized according to the method illustrated by Dacie and Lewis (33) while hemoglobin concentration was estimated colorimetrically following method of Wintrobe (34). The packed cell volume was determined using the microhematocrite method according to Darcourt et al. (35).

Behavioural observation

The behavioural assessment was carried out in an experimental room under the same environmental conditions. The experimenter was blind to the treatments of the animals. It was carried out 3 times (once/2 weeks) during the light phase of the light–dark cycle. The behaviour of the rat was recorded using a video camera fixed over the experimental cage and a focal sampling technique was used to record the behavior from the electronic compact disc.

Open field test

The rats were held from the tails and placed in one corner of the open field arena (70 L*70 W*40 H cm), which was divided into 16 equal squares. Behaviours were recorded by video recording for 5 minutes after 2 minutes setting the rat in the apparatus (for acclimation) and the arena was cleaned with 5% ethanol before the next rat. The behavioural parameters recorded were ambulation or locomotion frequency (the number of floor sections entered with two feet), rearing frequency (the number of times the animal stood on its hind legs), stereotype counts (the number of grooming movements), and immobility (freezing) duration (total time in

seconds without spontaneous movements). The movement of rats in squares reflected the level of anxiety-like behavior (36).

Hole-board test

This test included a board (80 cm L* 80 cm W), which had 4 equal holes distributed in four quarters of board. The exploratory behavior (time and frequency) was recorded (37) through head entrance of rat in holes of this apparatus.

Inclined plain test

According to the procedures followed by Abou-Donia et al. (38), rats were placed in the horizontal position on a flat plane and the angle of falling rat from flat plane was calculated.

Grip strength test

The strength of rat's forepaw was recorded by hanging in wood dowel (5mm diameter) (39). Time of grip in wood dowel was recorded in seconds to assess forepaw strength.

Tail suspension test (TST)

TST has been adopted as a behavior model to predict antidepressant effect of drugs and chemicals in rodents (40). In this test, the rats were suspended 50 cm above the floor and throughout a 5-minute, the total duration of mobility and struggling was recorded.

Statistical analysis

Data were statistically analyzed using SAS statistical system Package (41), where it was analyzed by one-way ANOVA using the SPSS 16.0 computer program. Duncan's Multiple Range test was performed to compare means value between experimental groups. The results were presented (mean ± SE) with normal distribution and significantly differed at $P<0.05$.

Results

The neurochemical analysis presented a marked reduction in serotonin and glutamate with a noticeable elevation in dopamine levels in brain homogenate, alterations that were more observable in 6mg/kg treated animals (Table 1). Reduced glutathione, superoxide dismutase and catalase levels revealed a significant decrease (0.15 µmol /gm tissue, 25.24 µmol /gm tissue, 1.93 Unit/gmtissue, respectively), while the malondialdehyde levels (15.79 nmol /gm tissue) evoked a significant increase in the same group (Table 2). There were no serious adverse events regarding hematological indices (Table 3). There were no clear changes between the first and second group, while the rats in group 3 had less response to all behavioral observations, with significant differences in inclined plain (23.35) and exploratory test (17 second and 2 frequencies) (Table 4,5).

Table 1: Effect of oral administration of Eszopiclone (3mg/kg and 6mg/kg) for 30 consecutive days on serum levels of serotonin, glutamate and dopamine in male Wistar rats

Groups	Serotenin (ng / ml)	Glutamin (nmol /µl)	Dopamin (µmol /gm tissue)
Control	488.29±5.40^{a}	8.03±0.59^{a}	50.94±2.39^{b}
3 mg/kg Ezopiclone	428.49±14.96^{b}	5.64±0.35^{b}	60.66±1.68^{a}
6 mg/kg Ezopiclone	306.44±6.88^{c}	4.33±0.38^{b}	59.28±2.18^{a}

^{abc} Means within the same column having different superscripts are significantly different at $P \leq 0.05$).
ng= Nanogram; ml=Milligram; nmol=Nanomole; µl=Micromilly; µmol=Micromole; gm=Gram and kg=Kilogram

Table 2: Effect of oral administration of Eszopiclone (3mg/kg and 6mg/kg) for 30 consecutive days on antioxidant parameters (MDA, SOD, CAT and GSH) in male Wister rats

Groups	MDA (nmol /gm tissue)	SOD (µmol /gm tissue)	CAT (Unit/gm tissue)	GSH (µmol /gm tissue)
Control	6.28±0.28[c]	54.16±1.93[a]	5.79±0.17[a]	0.344±0.016[a]
3 mg/kg Ezopiclone	13.05±0.45[b]	41.69±1.38[b]	3.03±0.18[b]	0.257±0.010[b]
6 mg/kg Ezopiclone	15.79±0.62[a]	25.24±1.30[c]	1.93±0.10[c]	0.15±0.007[c]

[abc] Means within the same column having different superscripts are significantly different at P ≤ 0.05).
nmol=Nanomole gm=Gram µmol=Micromole mg=Milgram kg=Kilogram

Table 3: Effect of oral administration of Eszopiclone (3mg/kg and 6mg/kg) for 30 consecutive days on some hematological parameters in male Wister rats

Groups	RBCS (10^6/ µl)	HB (gm/dl)	PCV (%)
Control	7.24±0.10	10.7±0.43	40.29±0.15
3 mg/kg Ezopiclone	6.98±0.13	10.72±0.32	40.28±0.67
6 mg/kg Ezopiclone	7±0.19	11.06±0.08	40.56±0.69

µl=Micromilly; gm=Gram; dl=Deciliter; mg=Milgram and kg=Kilogram

Table 4: Effect of oral administration of Eszopiclone (3mg/kg and 6mg/kg) for 30 consecutive days on the open field test in male Wister rats

Groups	Latency time (S.)	Grooming frequency	Rearing frequency	Ambulation frequency
Control	2.33±0.15 [b]	2±0.20 [a]	6±0.29 [a]	34.67±0.49 [a]
3 mg/kg Ezopiclone	3±0.16 [a]	1.33±0.07 [b]	6.33±0.27 [a]	33±0.64 [a]
6 mg/kg Ezopiclone	0.33±0.02 [c]	0.33±0.03 [c]	3.33±0.14 [b]	23±0.56 [b]

[abc] Means within the same column having different superscripts are significantly different at P ≤ 0.05).
mg=Milgram; S.=Second and kg=Kilogram.

Table 5: Effect of oral administration of Eszopiclone (3mg/kg and 6mg/kg) for 30 consecutive days on sensorimotors tests (grip, tail suspension and inclined plain) and exploratory behaviour in male Wister rats

Groups	Grip time (S.)	Tail suspension time (S.)	Inclined plain angle	Exploratory test	
				Time (S.)	Frequency
Control	3.67±0.11[a]	8.67±0.14[a]	43.33±3.04[a]	35.67±95[a]	5.33±0.12[a]
3 mg/kg Ezopiclone	2.5±0.09[b]	7.67±0.12[b]	43.12±3.05[a]	29±0.62[b]	4.33±0.12[b]
6 mg/kg Ezopiclone	1.5±0.1[c]	4.66±0.13[c]	23.35±1.82[b]	17±0.63[c]	2±0.08[c]

[abc] Means within the same column having different superscripts are significantly different at P ≤ 0.05).
S=Second; mg=Milgram and kg=Kilogram.

Discussion

Eszopiclone among others like zolpidem, zopiclone and zaleplone are medications which commonly referred to as non-benzodiazepine agonists. In 1980, the sedative molecules were developed to overcome the deleterious aspects of benzodiazepine therapy (1). This cyclopyrro-

lone derivative was classified as a potentiator of GABAA receptors, binds to all subtypes inducing sedation and hypnosis for removal of the sleep disorders (35,42). However, Buxton et al. (43) had recently found that eszopiclone in a dose of 3mg for two months treatment did not significantly influence sleep.

GABA is the major inhibitory neurotransmitter in the brain, and activation of GABAA receptors causes neuronal inhibition by increasing chloride ion conductance. GABAA receptors exist as multiple subtypes and these subtypes are differently located throughout the brain. Winsky (44) mentioned that the different GABAA receptor subtypes may cause differences in clinical effects caused by various benzodiazepine and non-benzodiazepine. Still, it is important to evaluate other neurotransmitters if there are any alteration in their levels could be attributed to eszopiclone nightly treatment for 30 consecutive days.

Neurochemically, it is inevitable that eszopiclone acts through modulation of GABAA receptor sites, despite a research by Kumar et al. (45) demonstrated that ESZ sleep induction doesn't require activation of GABAergic sleep-regulatory neurons in the preoptic hypothalamus. Our findings revealed that eszopiclone led to a significant decrease in serotonin levels in brain tissue homogenate, which was more pronounced in the 6mg/kg treated rats (306.44 ng / ml). Dopamine levels showed a marked elevation in eszopiclone 3 mg (60.66 μmol /gm tissue) and 6mg (59.28 μmol /gm tissue) treated rats, respectively, while glutamate values displayed a marked reduction in both treated groups (5.64 nmol /μl and 4.33 nmol /μl, respectively). The identified correlations between eszopiclone and other neurotransmitters than GABAA are unknown. Literature is small since inadequate work had been done on this matter, however, ESZ was found to suppress serotonergic and non-serotonergic neurons (45). Interestingly, Russell et al. (46) found that ESZ 3mg/kg for 18 successive days besides alleviated social defeat stress in mice, its neurochemical findings were elusive.

The decrease in serotonin level may be due to anxiety, obesity, insomnia, and fibromyalgia (47). This might explain the behavioral changes in rats treated with ESZ for 30 consecutive days, especially the group received 6mg/kg body weight. There were certain neurons in brain had the ability to synthesize, store, and released the serotonin. Serotonin in human being plays the important role in the regulation of a variety of processes within the brain, including, depression, mood, emotions, aggression and sleep (48,49).

To our knowledge, non-benzodiazepines have been scarcely investigated from the point of view of dopamine, glutamate, and other neurotransmitters estimation. Apart from potential confounding variables, what might be the reason for the association between eszopiclone treatment and neurotransmitters, neurotransmitter activity is delicately balanced, a shift in an inhibitory neurotransmitter alters other excitatory ones and vice versa. The findings of this paper declared a marked elevation in dopamine levels and a decrease in the glutamate levels in groups 2 and 3 in comparison to control group.

Despite the fact that activation of dopamenirgic receptors in the DRN increases the activity of serotoninergic neurons (50), the increase in dopamine levels in our findings did not necessarily elevate the serotonin levels as we presented a significant decrease in serotonin values after eszopiclone nightly treatment for 30 days suggesting the direct effect of this non-benzodiazepine on serotoninergic receptors, necessitating the need for more research in this area.

Glutamate is a group of endogenous amino acids which act as excitatory neurotransmitters for learning and memory. As neurotoxins, they are believed to be involved in the pathogenesis of a variety of neurodegenerative disorders in which cognition is impaired. McEntee and Crook (51) found that cerebral structures may be particularly vulnerable to the neurotoxic actions of these excitatory amino acids, especially in the elderly who are most susceptible to impairments of mnemonic function. The affinities of (S)-DMZ and N-oxide zopiclone (two major metabolites) to the central BZD receptor site were > 20-fold and >250-fold lower than (S)-zopiclone. (S)-DMZ

was shown to enhance GABAA current by increasing the affinity of receptors for GABA but dose dependently inhibited agonist evoked currents at both N-methyl-D-aspartate receptor NMDA (glutamate) and nACh (acetylcholine) receptors, an effect that was mimicked by zopiclone. Inhibitory actions at NMDA and nACh receptors are also evident at a 20 μM (52).

Rats in group 2 (3mg of Eszopiclone/kg) showed lower GSH, SOD, CAT activities compared to rats in group 3 (6 mg). While, MDA level was significantly higher in group 2 than group 3. These values were more pronounced in animals treated with ESZ (6mg/kg), indicating that the oxidative stress could be gained on brain tissue after administration of ESZ.

Brain tissue is uniquely susceptible to oxidative stress as cellular residents of brain demonstrating markers of oxidative damage in major depressive disorders, insomnia and bipolar disorders (53). Eszopiclone showed deviations from reasonable antidepressants outcome treating oxidative stress accompanying brain disorders, administration of ESZ increased lipid peroxidation and suppressed antioxidant enzymatic activity. In contrast to our findings, Szebeni et al. (53) found that cellular residents of brain white matter revealed markers of oxidative damage in major depressive disorder (MDD), medications that interfere with oxidative damage or pathways activated by oxidative damage have potential to improve treatment for MDD.

One of the most possible explanations of the presented oxidative stress is the reduction of serotonin level in the brain caused by eszopiclone treatment. Serotonin acts as a scavenger of hypochlorous acid (HOCl) in the cerebrum. Serotonin was shown to inhibit the oxidation of 2-thio-5-nitrobenzoate by HOCl in a biphasic fashion. Serotonin might act to decrease chlorinative tension in the brain (54).

There were no serious adverse events during the study regarding hematological parameters. On the other hand, Lovett et al. (55) suggested that over dose of ESZ (30 times normal dose) evoked a marked hemolytic anemia and methemoglobinemia in adult women.

The data of behavior as shown in Table 4 and 5 revealed that rats in group 2 and 3 showed behavioural alterations as compared to control group. The changes in behavior are considered as a marker of emotional condition of the living organism (56), representing in grooming frequency and freezing time. Furthermore, frequencies of rearing and ambulation in this test help in the measurement of hyperactivity (57), locomotory activity (58) and anxiety-like behavior (59). In the current study, animals receiving 6 mg/kg of Eszopiclone had an increase in rearing frequency and freezing time, while frequencies of ambulation and grooming were the highest in control and rats treated with 3 mg/kg of eszopiclone.

In the current finding, the exploratory time and frequency were significantly higher in control and rats treated with 3 mg/kg, while it was the lowest in rats treated with 6 mg/kg. These results were consistent with locomotory behavior in open field test. These results may be due to the positive effect of Eszopiclone in treatment of insomnia (60). In this regard, the durations of immobility in tail suspension were higher in rats treated with 6 mg/kg. Moreover, grip strength delineated the motor neurotoxicology (61).

There was difference in grip strength among experimental groups, where it was higher in control and rats treated with 3 mg/kg, respectively than 6 mg/kg. Also, the marked depletion of the neurotransmitter here may be responsible for this neurobehavioral alterations especially lack of exploration and depression (62). The most of behavioural changes may be due to histopathological alteration of brain, as mentioned before by Khalil et al. (61), who cited that neuronal degeneration and cerebral oedema led to behavioural alteration.

Conclusion

In the present study, Eszopiclone administration caused an imbalance between different neurotransmitters in cerebrum presented by an elevation in dopamine and a marked reduction in serotonin and glutamate levels. Therefore, there are potentials suggesting cross-talking between GABAergic, serotoninergic, dopaminergic and NMDA

systems when eszopiclone is used. Behavioral activity highlighted side effects of 6mg/kg ESZ and oxidative stress was more conspicuous in 6mg/kg ESZ than in 3mg/kg treatment as substantiated by the decrease in antioxidant enzymes and the increase in lipid peroxidation.

Conflict of interest

None of the authors have any conflict of interest to declare

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Original Research Article

TOXIC METAL RESIDUES IN NON-EDIBLE ANIMAL BYPRODUCTS

Wageh S. Darwish*, Abd-El Salam E. Hafez, Aya A. Khairy

Food Control Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt

*Corresponding author, E-mail: wagehdarwish@zu.edu.eg

Abstract: Environmental pollution by heavy metals is a major problem worldwide. Domesticated animals such as cattle and camel share the same environmental conditions like human and they are exposed to heavy metals via different sources. Therefore, these animals are considered as ideal bio-indicators for human exposure to heavy metals. Heavy metals accumulate in the different tissues of the animals. Estimation of toxic metal residues such as arsenic (As), mercury (Hg), lead (Pb) and cadmium (Cd) in the animal edible tissues had been extensively studied. However, estimation of such toxicants in the non-edible animal byproducts had received little attention. Additionally, non-edible animal byproducts are frequently used in many industries such as animal feed additives and leather fabrication. Therefore, this study was undertaken to estimate the residual concentrations of As, Hg, Pb and Cd in the hair, hides and bones of cattle and camel slaughtered at Zagazig, Abo-Hammad and Belbies cities, Sharkia Governorate, Egypt. Metal-metal correlations were additionally calculated. The achieved results indicated exposure of cattle and camel to high levels of heavy metals, particularly lead and arsenic. Camel had higher concentrations (mg/kg ww) of arsenic compared with cattle particularly in hair (38.57 ± 8.77 and 22.48 ± 1.91 in camel and cattle, respectively). Bone had the highest load of the measured metals among examined samples. For instances, in camel, elemental concentrations (mg/kg ww) in bone were 34.53 ± 6.16 (As), 3.41 ± 0.56 (Hg), 2.76 ± 0.36 (Pb) and 0.11 ± 0.01 (Cd). Samples collected from Zagazig city were highly contaminated compared with other locations. Significant positive correlations were observed between lead - mercury, lead - cadmium and arsenic- mercury (r <0.0001 in each). Contaminated non-edible animal byproducts should be hygienically disposed and avoid its introduction to downstream industries. It is highly recommended to control environmental pollution with heavy metals in Egypt.

Key words: bone; domesticated animals; hair; heavy metals; hide

Introduction

Heavy metals are highly accumulative environmental pollutants that can get entry into the ecosystem via anthropogenic activities and subsequently find their way into human and animal bodies via consumption of contaminated food and water leading to several adverse outcomes (1).

Heavy metals such as cadmium (Cd), lead (Pb), arsenic (As) and mercury (Hg) are classified as toxic heavy metals. For instances, Cd exposure is linked to kidney damage and skeletal muscle deformities (2). Metals like Pb

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may cross blood brain barrier leading to nervous manifestations in both children and adults (3). Arsenic is reported to cause hyperpigmentation and hyperkeratosis of the skin (4). Mercury can lead to multiple organ damage as it had pulmonary, nervous and cardiac symptoms (5).

Domesticated animals such as cattle and camel are reared for their meat and milk and other non-edible animal byproducts, such as hair, hides, bones, that can be used in many industries such as leather fabrication and as ingredients in the animal feed additives (6). These animals are sharing humans in the same environment and therefore are considered as ideal bio-indicators for human exposure to environmental pollutants like heavy metals (7).

Estimation of heavy metals in the edible tissues of domesticated animals had been extensively studied. However, limited information is available about the residual concentrations of the toxic metals in the non-edible animal byproducts such as hair, hides and bone in Egypt. Therefore, this study was undertaken to estimate the residue levels of four toxic metals including As, Hg, Pb and Cd in three non-edible animal products, namely hair, hides and bone of cattle and camel slaughtered at Zagazig, Abo-Hammad and Belbies cities in Sharkia Governorate, Egypt. Additionally, the inter-metal correlations in the examined samples were calculated. Finally, the public health significance of the examined metals was also discussed.

Material and methods

Guidelines of Zagazig University Animal Ethics Committee were followed during conducting this research. All chemicals were the highest quality available and purchased from Merck, Darmstadt, Germany.

Collection of samples

One hundred and eighty samples were collected randomly and equally from non-edible animal byproducts (hair, hides and bone) of both cattle and camel slaughtered at Zagazig, Abo-Hammad and Belbies cities, Sharkia Governorate, Egypt. The sampling sites are characterized by being rural and the animal farms are usually located far from highways. Samples were collected in the period of March to October 2017. Samples were kept frozen in plastic falcon tubes at -20˚C until the time of heavy metal extraction and measurements.

Preparation of samples

Hair samples were washed using distilled water several times for complete removal of any dirt, dried at room temperature, and then cut into small pieces to be easily digested by the acid mixture in the following step.

Hide samples were brushed under running water to remove any dirt followed by washing using distilled water, then cut into small pieces and kept dried.

Bone samples were washed with distilled water to remove any dirt. After washing, bones were cut into small pieces and then treated with a 30% H_2O_2 solution to remove any traces of flesh, fat and blood followed by another wash with distilled water (7). Then one gram from each sample was heated in a hot air oven at 100°C for 2 h followed by another heating cycle for 2 h after mixing with 5 ml of perchloric acid 70% (8).

Digestion of samples

One gram of each sample was mixed with digestion mixture consisted of 5 ml HNO_3 (65%) and 2 ml perchloric acid 70% (9). The mixture was heated at 50°C for 3 h in the water bath, followed by filtration, and dilution. Samples were kept at room temperature until metal analysis.

Analytical procedures

All analytical procedures were done at the Central Laboratory, Faculty of Veterinary Medicine, Zagazig University, Egypt. Residues of arsenic and mercury were estimated using hydride generation/cold vapor atomic absorption spectroscopy, however, graphite furnace was used in case of lead and cadmium (Perkin Elmer® PinAAcle™ 900T atomic absorption spectrophotometer) (Shelton, CT, USA).

Quality assurance and quality control

Reference material (fish protein) named DORM-3 purchased from the National Research Council, Canada was used to validating the analytical procedures of heavy metals. The reference material was exposed to the same analytical method as in the field samples, and the tested metals were estimated. Then the obtained concentrations were compared with the metal concentrations loaded on the reference material to obtain the recovery rates. Recovery rates ranged from 80% to 115% for all metals examined. The detection limits (mg/kg) for the analyzed metals were 0.02 for arsenic, 0.01 for mercury, 0.1 for lead and 0.001 for cadmium.

Statistical analysis

Statistical significance was evaluated using one-way analysis of variance (ANOVA) and Tukey-Kramer test with P-value < 0.05 considered to be significant. Pearson Correlation coefficient (*r*) was obtained using SPSS program version (23.0).

Results and discussion

Levels of toxic metals in non-edible animal byproducts

The achieved results in Table (1) showed that arsenic was detected in all examined samples. The residual concentrations of arsenic (mg/kg ww) in hair, hides and bone of cattle were 22.48 ± 1.91, 7.59 ± 0.65 and 16.53 ± 1.47, respectively. These concentrations were 38.57 ± 8.77, 14.56 ± 3.67 and 34.53 ± 6.16 in the examined hair, hides and bone of camel (Table 1). It is clear that, camel had higher arsenic residual concentrations compared with cattle in all examined samples. This may be attributed to the ability of camel to live under low water levels and subsequently concentrates the metals into its different tissues (10). Hair samples had the highest residue levels of arsenic followed by bone and hides in both of cattle and camel. This may be due to the frequent exposure of hair to contaminated dusts and water with arsenic. Presence of high concentrations of arsenic in the hair samples despite being washed during sample preparation indicates accumulation of arsenic in hair due to past exposure and it is reported that hair is the target site for arsenic (11). The recorded concentrations of arsenic in the present study is extremely high when compared with the washed cattle hair pastured in the vicinity of the Glogow copper smelter; West Bengal, India and in antlers of red deer in Poland (12-14). This may reflect the high load of as in the Egyptian environment, probably due to the uncontrolled agricultural and industrial activities.

Mercury was detected in all examined samples, where, its levels in the examined samples of cattle were comparable to those of camel. The load of mercury (mg/kg ww) in hair, hides and bone of cattle were 0.77 ± 0.19, 0.51 ± 0.17 and 3.75 ± 0.52, respectively. While in camel, these concentrations were 0.83 ± 0.19, 0.10 ± 0.04 and 3.41 ± 0.56 in the examined hair, hides and bone, respectively (Table 1). It is obvious that, bone had significantly higher mercury residues compared with hair and the hide. The high mercury concentrations in the bone tissue agreed with that reported in Chinese rhesus monkeys (15). Hair had significantly higher concentrations of mercury compared with that of the hide, particularly in the camel. It is reported that hair is rich in sulfur containing amino acid cysteine, which is a major mercury-binding protein (16).

Table 1: Arsenic and mercury residues (mg/kg ww) in the non-edible animal byproducts of cattle and camel

	Arsenic				Mercury			
	Cattle		Camel		Cattle		Camel	
	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE
Hair	12.28 –32.88	22.48 ± 1.91[a]	4.41 –73.91	38.57 ± 8.77[a*]	0.04 – 1.71	0.77 ± 0.19[b]	0.03 – 1.72	0.83 ± 0.19[b]
Hide	5.47 –12.21	7.59 ± 0.65[c]	2.13 –36.24	14.56 ± 3.67[b*]	0.01 – 1.79	0.51 ± 0.17[b*]	0.01 – 0.33	0.10 ± 0.04[c]
Bone	10.11 –23.06	16.53 ± 1.47[b]	9.33 –62.03	34.53 ± 6.16[a*]	0.84 – 6.77	3.75 ± 0.52[a]	0.72 – 6.03	3.41 ± 0.56[a]

a-b-c: Means in the same column carrying different superscript letter are significantly different with each other (Comparisons among different tissues of the same species). Means carrying star mark indicate significant difference between same tissue in cattle and camel (p <0.05).

The lead was detected in all examined samples in the present study. Levels of lead residues were comparable among both cattle and camel. Interestingly, bone had significantly higher lead residues followed by hair and hides, respectively (Table 2). Lead has the tendency to accumulate in the bone tissue (17). The residual concentrations of lead in the bone (mg/kg ww) were 2.46±0.28 and 2.76 ± 0.36 in cattle and camel, respectively, while that in the hair were 0.99±0.25 and 0.86±0.24 in cattle and camel, respectively. Hide had the lowest residues of Pb (0.44±0.11 mg/kg ww) in cattle and (0.58±0.09 mg/kg ww) in camel (Table 2). The results of the current investigation agreed with that reported in washed cattle hair pastured in the vicinity of the Glogow copper smelter and in the hair samples collected from camels in Aswan (12, 18). Lower concentrations were recorded in red deer antler in Poland (14). Lead was detected previously in the edible offal of cattle and sheep collected from the same locations (7). Erythrocytes are considered the target site for lead binding in 99% of the absorbed lead. Therefore, lead found its way to soft tissues (liver and kidney) and to bones with the highest precipitation percentages in the bones (18).

Cadmium was detected in all examined tissues in the present study (Table 2). The residual concentrations of cadmium (mg/kg ww) in the examined samples of cattle were 0.13±0.01 in hair, 0.07±0.01 in hides and 0.21±0.02 in bone. These concentrations were 0.09±0.06, 0.01±0.003 and 0.11±0.01 in the examined hair, hides and bone of camel (Table 2). It is clear that, bones had the highest residual contents of cadmium followed by hair and finally the hide. Cattle bone had the highest cadmium residues among the examined samples, which may be explained by the interactions between cadmium and calcium in the skeletal system (19). However, cadmium has no ability to pass the skin barrier (20). The recorded concentrations of cadmium in the current study were slightly higher than that reported in washed cattle hair pastured in the vicinity of the Glogow copper smelter and in red deer antler bone in Poland (12,14), but lower than that recorded in Kalabsha and Halaiub (18). Similarly, high cadmium residues were recorded in the liver and kidneys of cattle and sheep slaughtered in Zagazig city (7).

Arsenic, mercury, lead and cadmium residues were extremely high in samples collected from Zagazig city compared with Abo-hammad and Belbies (Figure 1A, B, C and D). This may be explained by the increase in the anthropogenic activities and traffic deposition in Zagazig city compared with the rural nature of the other locations. Heavy metal load is positively correlated with the traffic deposition (21). Moreover, the Egyptian population in Sharkia Governorate depends mainly on agricultural and small industrial activities, so, many regions of the Governorate have been contaminated with wastewater and solid wastes (7).

In general, presence of such toxic metal residues in animal byproducts reflects the exposure of the living animals to a vast array of xenobiotics including, heavy metals. Subsequently, this indicates contamination of the environment surrounding animals such as air, food and water with such toxicants. Additionally, contamination of such animal

byproducts with toxic metals may lead to loading of the downstream products such as animal feeds and leather products with such toxic metals. Then these metals can find their way to other animals or humans using such contaminated products.

Table 2: Lead and cadmium residues (mg/kg ww) in the non-edible animal byproducts of cattle and camel

	Lead				Cadmium			
	Cattle		Camel		Cattle		Camel	
	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE
Hair	0.06 –1.94	0.99 ± 0.25^{b}	0.05 –2.08	0.86 ± 0.24^{b}	0.04 – 0.18	0.13 ± 0.01^{b}	0.01 – 0.64	0.09 ± 0.06^{a}
Hide	0.05 –0.94	0.44 ± 0.11^{c}	0.01 –1.16	0.58 ± 0.09^{b}	0.04 – 0.15	$0.07 \pm 0.01^{c*}$	0.001 – 0.04	0.01 ± 0.003^{b}
Bone	0.72 –3.66	2.46 ± 0.28^{a}	1.27 –4.26	2.76 ± 0.36^{a}	0.13 – 0.32	$0.21 \pm 0.02^{a*}$	0.05 – 0.17	0.11 ± 0.01^{a}

a-b-c: Means in the same column carrying different superscript letter are significantly different with each other (Comparisons among different tissues of the same species). Means carrying star mark indicate significant difference between same tissue in cattle and camel (p <0.05).

Table 3: Significant Pearson correlation's coefficient values between toxic metals in the examined non-edible byproducts of cattle and camel

Cattle			Camel		
Metal	***r*** ***p value***	**Tissue**	**Metal**	***r*** ***p value***	**tissue**
Pb-Cd	0.949 <0.0001	bone	Pb-Hg	0.947 <0.0001	bone
Pb-Cd	0.914 <0.0001	hide	Pb-Hg	0.847 <0.0001	hide
Pb-Hg	0.901 <0.0001	hair	Pb-Hg	0.965 <0.0001	hair
As-Hg	0.906 <0.0001	bone	As-Cd	0.887 <0.0001	bone
As-Hg	0.988 <0.0001	hide	As-Cd	0.800 <0.0001	hide
As-Hg	0.944 <0.0001	hair	As-Cd	0.539 0.0043	hair

Values in the table are the significant correlations at p <0.05. r: Pearson correlation coefficient; p: probability; Pb: lead; Cd: cadmium; As: arsenic; Hg: mercury

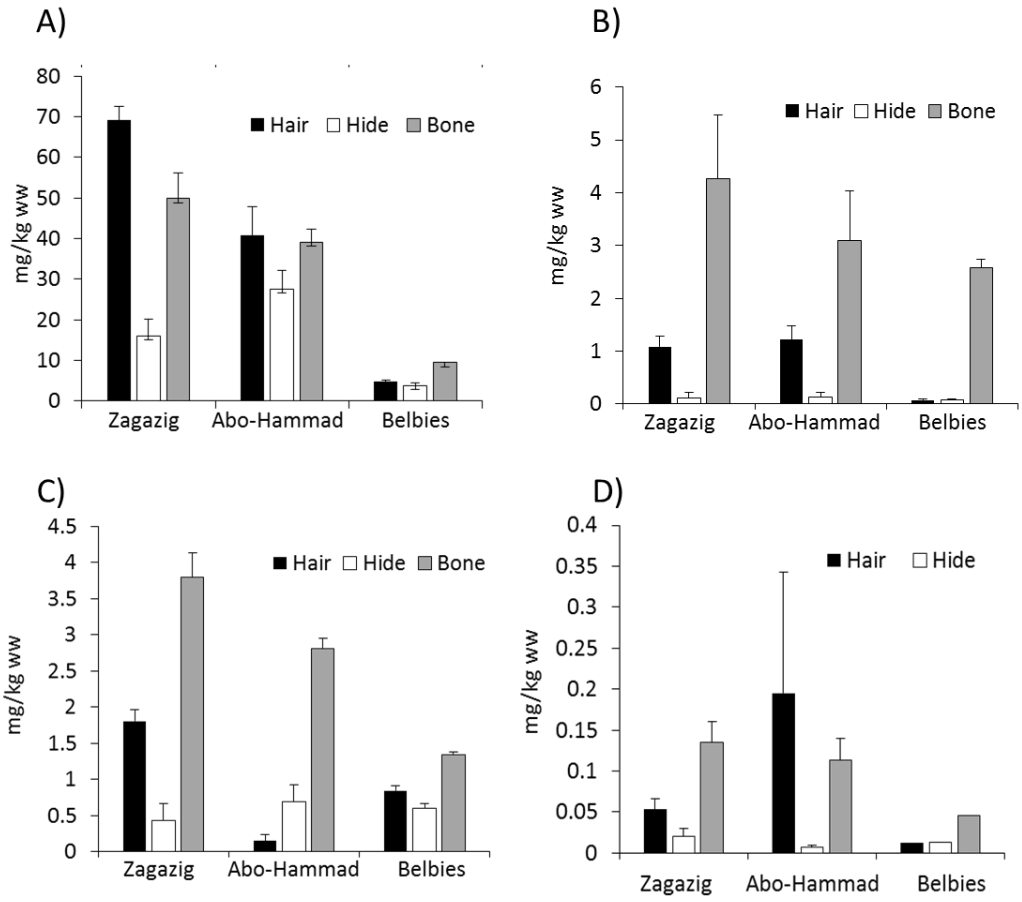

Figure 1: Toxic metal residues in non-edible animal byproducts of cattle and camel in different localities at Sharkia Governorate, Egypt (2017). A) Arsenic, B) mercury, C) lead and D) cadmium residues (mg/kg ww) in hair, hide and bone of cattle and camel from Zagazig, Abo-Hammad and Belbies. Values represent mean ± SE (n = 90 from each animal species divided as 10 samples from each byproduct/ each animal species/each location)

Correlations among metals in the non-edible animal byproducts

As a method of metal bio-detoxification, interactions between toxic and essential metals take place in the animal body. For instances, cadmium and lead absorption increases by iron deficiency. Selenium can protect against mercury-induced cytotoxicity. Lead replaces zinc on haeme enzymes (21). The results described in Table (3) showed the significant positive correlations among the examined metals in the non-edible byproducts of cattle and camel. It is clear that there were positive correlations between lead-cadmium (p <0.0001) in bone and hides of cattle; lead-mercury (p <0.0001) in all examined tissues of camel; arsenic-mercury in cattle tissues and arsenic-cadmium (p <0.0001) in tissues of camel. Nearly similar correlations were detected in the edible tissues of cattle and sheep (7) and in the non-edible tissues of camels (18). Future studies are still needed to estimate the essential trace elements such as iron and selenium to have comprehensive image about correlations among metals in such animal byproducts. Furthermore, levels of toxic metals in the blood should be measured in future studies.

Conclusion

The attained results of the present study declared that non-edible animal byproducts of cattle and camel collected from Sharkia Governorate, Egypt were contaminated with toxic metals including arsenic, mercury, lead and cadmium. Therefore, such contaminated byproducts should be hygienically disposed and

not introduced into manufacturing purposes. In addition, strict control measures should be adopted to reduce the exposure of animal and human to such toxicants.

Conflict of interest

The authors declare no conflict of interest.

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Original Research Article

CLINICOPATHOLOGICAL AND IMMUNOLOGICAL EFFECTS OF USING FORMALIZED KILLED VACCINE ALONE OR IN COMBINATION WITH PROPOLIS AGAINST *Pasteurella multocida* CHALLENGE IN RABBITS

Mohamed A. Hashem, Essam A. Mahmoud, Mohamed F.M. Farag*

Department of Clinical Pathology, Faculty of Veterinary Medicine, Zagazig University, Egypt

*Corresponding author, E-mail: farag_cell@yahoo.com

Abstract: Pasteurellosis is a common and troublesome issue of rabbits causing serious disorders. The immunization procedures are constantly the greatest preventive measures. In the present study, 40 New Zealand rabbits were used to investigate the protective efficacy of formalized *Pasteurella multocida* vaccine alone or in combination with propolis. The animals were divided into four equal groups (I-IV); negative control group, challenged non-vaccinated group, vaccinated challenged group and vaccinated propolis administered challenged group respectively. At the end of the 2nd and 6th weeks of the experiment, blood samples were collected from ear vein of rabbits for hematological, plasma, and serum examinations. The rabbits were then anaesthetized and sacrificed to collect tissue specimens from liver, kidneys, spleen and lungs for histopathological study. The results showed that using of propolis in combination with killed vaccine of *Pasteurella multocida* improved the immune response by increasing the leukocyte phagocytic activity against *Pasteurella multocida* (from 23.80% to 60.80%). Moreover, the clinicopathological findings including hemogram (RBCs count, Hb content, PCV, RBCs indices, platelets, total and differential leukocytes count), and hepato-renal function tests (ALT, AST, ALP, bilirubin, urea and creatinine), as well as, histopathological findings were better in infected rabbits treated with propolis- killed vaccine than using killed vaccine alone.

Key words: propolis; pasteurellosis; phagocytosis; nitric oxide; *P.* formalized killed vaccine

Introduction

One of the common diseases that affect rabbits' generation is pasteurellosis (1). Pasteurellosis affects rabbits of 4– 8 weeks old causing manifestations ranging from lethal septicemia, serious pleuritis, and pneumonia to less serious condition as multiple abscesses, rhinitis, and otitis media. The utilization of antibiotics has been in part effective in controlling pasteurellosis in rabbits, since they don't totally dispose of the bacterium (2). Control of pasteurellosis in rabbits is achieved by vaccination against *Pasteurella multocida* infection. Vaccination of rabbits with killed vaccine (bacterin) frequently brings about insufficient protection in the field when used in a single dose (3).

Immunization studies on rabbits' pasteurellosis have been accounted for the utilization

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of inactivated forms as formalized (4), joined with oil adjuvant (5) or heat treated (6). Propolis (honey bee stick) is a resinous hive item, created by bumble bees from different plant sources. It has a few natural properties as antimicrobial, anti-inflammatory and immuno-modulatory (7-9). Propolis has a several clinicopathological impacts on rebuilding of renal and hepatic functions (10). The propolis extract may have gone about as a right hand adjuvant substance, potentiating the humoral response initiated by the antigen (11). Thus, the aim of the present work was to study the effect of formalized killed *P. multocida* vaccine either injected alone or in combination with propolis as an adjuvant against experimental challenge of rabbits with *P. multocida* strain based on selective hematological (RBCs, Hb, PCV, MCV, MCHC, TLC, neutrophils, lymphocytes, monocytes, eosinophils, basophils and platelets), biochemical (ALT, AST, ALP, bilirubin, urea, creatinine and nitric oxide), immunological (phagocytic percent) and histopathological (liver, kidneys, spleen and lungs) investigations.

Material and methods

Animals

A total of 40 apparently healthy white New Zealand rabbits of 1 kg average body weight and 6–8 weeks old were obtained from laboratory animal house, Faculty of Veterinary Medicine, Zagazig University. All rabbits were not previously vaccinated against pasteurellosis and with no history of pasteurellosis. They were kept under hygienic conditions, housed in metal cages at Experimental Animal Unit, Faculty of veterinary Medicine, Zagazig University and fed on balanced ration and water *ad–libitum* and maintained in a 12 h light-dark cycle at a controlled temperature (21–24°C) and humidity (50–60%). They were kept for 15 days without medication for acclimatization before beginning the study. The care and welfare of animals conformed to the guidelines of the Animal Welfare and Research Ethics Committee, Faculty of Veterinary Medicine, Zagazig University, Egypt.

The used vaccine, propolis, challenging bacteria and chemicals

The formalized killed vaccine for *P. multocida* (9-10^9 bacterial cell/ml- batch number: 56) that was obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.

Propolis that was obtained from bee hives located in Sharkia Governorate, Egypt. Propolis bulk was cut into small pieces, mixed with deionized water and then shook at 95°C for 2 h to prepare the therapeutic dose (50 mg prpolis in each 1 ml). Then it was cooled to room temperature and centrifuged at 1500 rpm for 5 min to obtain the supernatant (12).

Challenging bacteria

The strain of *P. multocida* was obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt, as lyophilized ampoules(1 ml containing 5×10^9 bacterial cell of *P. multocida* serotype A). It was activated by culturing in nutrient broth followed by intraperitoneal inoculation (I/P) in Swiss mice, and then re-isolated from heart blood of mice on nutrient agar plates (Difco). Bacterial colonies were suspended in sterile saline, and the density was adjusted to contain 5×10^9 bacterial cell/ ml. The suspension was used for S/C injection of rabbits in the challenge test (13). All chemicals and stains were of analytical grade and obtained from Sigma Co., and El Gomhoria Co., Egypt.

Experiment design

Forty New Zealand rabbits were divided into 4 groups (I-IV), each group contained 10 rabbits as following; group (I): non vaccinated and non challenged control group, group (II): non-vaccinated challenged group and group (III): the vaccinated challenged group in which rabbits were injected S/C with single dose of (1 ml/kg BW) formalized killed *P. multocida* vaccine according to Okerman and Spanoghe (3) and Osama (14), group (IV): the vaccinated propolis administered group in which rabbits were injected S/C with *P. multocida* vaccine (a single S/C dose of 1 ml/kg BW) mixed with propolis (50 mg /kg BW) as an adjuvant according to Nassar et al. (15). At the end of the

5th week from starting the experiment, rabbits in groups II, III and IV were challenged S/C with broth culture of virulent *P. multocida* strain (0.2 ml/ kg BW) (15).

Sampling

Blood samples were collected from the marginal ear vein of rabbits in all groups at the end of 2nd and 6th weeks of the experiment. They were divided into three parts. The 1st part (1ml rabbit blood) was placed in clean Wasserman tubes containing disodium salts of EDTA for hematological examination. The 2nd part (5ml rabbit blood) was placed in a chemical free test tube without anticoagulant to separate a clear serum for biochemical analysis (16). The 3rd part (3ml) was collected in heparinized tubes for phagocytic activity. Tissue specimens from liver, kidneys, spleen and lungs from all experimental groups were collected at the end of the 6th week of the experiment for histopathological examination.

Hematological studies

Complete blood picture was carried out using an automated hematology analyzer, Sysmex IV2000 (hematology analyzer), UK. This was done at Veterinary Animal Reseasch, Sharkia.

Clinicobiochemical analysis

All biochemical tests were performed using colorimetric kits (BioMerieux, ABC Diagnostics, Vitro Scient, Quimica Clinica Aplicada S.A. (QCA), Spinreact, ELITech and Diamond-Diagnostics) according to the manufacturer's instruction. The applied biochemical tests were measured the serum levels of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) according to the method of Reitman and Frankel (17), Alkaline Phosphatase (ALP) according to modified method of Tietz (18), Bilirubin levels (total and direct) according to Fuehr (19), serum creatinine and urea according to the method of Henry (20) and Fawcett and Scott (21), respectively. Nitric oxide based on Griess reaction as cited in Montgomery and Dymock (22). Indirect bilirubin was calculated by subtracting the direct bilirubin level from the total bilirubin level obtained.

Phagocytic activity

Heat-inactivated *Candida glabrata* (*C. glabrata*) was used as a microbial model to evaluate the phagocytic activity of the rabbit monocytes. In brief, leukocytes were separated from the heparinized blood samples (23,24). One ml of the adjusted viable leukocytes suspension (leukocytes in RPMI 1640 with 5% of pooled rabbit serum) was placed in a sterile plastic tube, to which 1 ml of the prepared heat inactivated *Candida glabrata* was added. The tubes were then incubated at 27ºC for 30 min in a humidified 5% CO2 incubator. Then the tubes were centrifuged at 2500 rpm for 5 min, and the supernatant was removed with Pasteur pipette leaving a small part into which the sediment was re-suspended. Slide smears were prepared from the deposit, air dried and then stained with Leishman's stain. Under a light microscope using oil immersion lens, phagocytic cells were counted randomly in about ten microscopic fields. The number of phagocytic cells with engulfed yeast was recorded. The phagocytic activity was evaluated according to the following equation (25):

Phagocytic percentage **(P %)** =

$$\frac{\text{Number of phagocytes with engulfed yeast cells}}{\text{Total number of phagocytes}} \times 100$$

Histopathological examination

Specimens from the liver, kidneys, spleen and lungs from all groups were collected at the end of 6th week post-treatment, and then fixed in 10% neutral buffered formalin for 48h, then washed overnight under running water. The washed specimens were dehydrated by using up graded concentrations of ethyl alcohol starting with 75% and ending with absolute alcohol, cleaned in xylene two times, each for 2 hrs. There after the specimens were placed in crucible containing soft paraffin and kept in an oven at 60ºC for 12h. Paraffin sections of 5 microns thickness were prepared and stained

with H & E stains for histopathological examination (26).

Statistical analysis

The obtained data was analyzed using one way ANOVA (SPSS Inc. Released 2007. SPSS for windows, version 16.0. Chicago, SPSS Inc.) (27). Means at the same column followed by different letters were significantly different and the highest value was represented with the letter a ($p = 0.5$).

Results

Hematological findings

Regarding the erythrogram, as shown in Table (1) comparing with the control non-infected group (I), the non vaccinated challenged group (II) showed non-significant changes at the values of RBCs (5.68±0.07), Hb (11.38±0.10), PCV (35.71±0.90), MCV (62.89±1.44), MCHC (31.94±0.67), and platelets (456.8±13.58) at the end of the 2nd week from starting the experiment. Meanwhile, at the end of the 6th week, it showed macrocytic hypochromic anemia with thrombocytopenia. On the other hand, the vaccinated challenged rabbits of group (III) showed normocytic normochromic anemia at the end of the 2nd week with non-significant changes at the counts of platelets (448.8±36.12) but at the end of the 6th week, they showed non-significant changes at the erythrogram indices with non-significant changes at platelets count also. On the other hand, vaccinated propolis administered challenged group (IV) did not show any significant changes in the erythrogram indices and platelets count during all the experimental periods.

Leukogram and Immunological results

At the end of the 2nd week of the experiment compared to the control group as illustrated in Table (2), the infected non-vaccinated group (II) showed a non significant change in the counts of total leukocytes (8.84±0.42) including neutrophils (3.84±0.28), lymphocytes (4.21±0.28), monocytes (0.77±0.09), eosinophils (0.08±0.02) and basophils (0.24±0.09) counts. Meanwhile, at the end of the 6th week, group (II) showed a highly significant increase in total leukocytic (13.35±0.20) and neutrophilic (8.01±0.25) count with non-significant changes at the other leukocytes. On the other side, group (III) revealed leukocytosis (11.63±0.39) and lymphocytosis (5.99±0.26) at the end of the 2nd week with non significant change in leukogram variables at the end of 6th week of the experiment.

Group (IV) showed leukocytosis, neutrophilia, lymphocytosis and monocytosis with non significant change in eosinophils and basophils along the experimental periods. The phagocytic percent of group (II) (Figure 1b) was non-significantly changed (25.6±1.5 and 23.8±1.24) than the control group (I) along experimental periods (Figure 1a), elevated in group (III) (35.8±1.24) (Figure 1c,d) and highly increased in group (IV) (60.20±1.59 and 60.8±1.74) (Figure 1e,f).

Biochemical results

At the 2nd weeks of the experiment as shown in Table (3), group (II) showed non significant changes in serum ALT (16.5±0.39), AST (15.2±0.41), ALP (40.12±0.94), total (0.94±0.06), direct (0.24±0.02), and indirect bilirubin (0.71±0.07), urea (34.74±1.05), creatinine (0.96±0.05) and nitric oxide (0.7±0.12) but these parameters were increased at the 6th week. However, group (III) at the 2nd week showed a significant increase in serum ALT (18.32±0.85), AST (19.58±0.38), total (1.36±0.06), indirect bilirubin (1.1±0.08) and nitric oxide (0.78±0.07) with non significant changes in the serum ALP (44.82±1.59), direct bilirubin (0.25±0.02), urea (33.79±1.33) and creatinine (0.98±0.03). However, at the 6th week, it showed marked decrease in the serum activity of ALT (24.49±0.53), AST (22.37±1.04), ALP (44.31±1.21), total (1.61±0.06), indirect bilirubin (1.32±0.05), urea (34.83±1.05), creatinine (0.98±0.05) and nitric oxide (2.06±0.05) with non-significant changes at serum level of direct bilirubin (0.29±0.01).

On the other hand, group (IV) at the 2nd week did not show any significant changes in all previously mentioned parameters comparing

with control group (I), but at the 6th week, it showed a decrease in urea (22.0±0.95), creatinine (0.6±0.03) and direct bilirubin (0.22±0.01) levels with non significant changes in other parameters. However, group (IV) showed good improvement of these parameters when compared with group (II).

Histopathological alterations

In group (II), Liver of rabbit showed multiple focal areas of coagulative necrosis surrounded by line of demarcation (Figure 2A). Furthermore, vascular congestion and mononuclear cell infiltration with widening in the glomerular space of kidney were recorded (Figure 2D). Meanwhile, spleen showed marked polymorphonuclear cell infiltration (Figure 2G). Lung also showed extensive hemorrhage (presence of large numbers of extravasated RBCs in the alveolar lumens) (Figure 2J). The vaccinated challenged group (III) showed mononuclear cell infiltration in the portal areas of liver (Figure 2B), vascular congestion, with perivascular edema and mononuclear cell infiltration (Figure 2E) of kidney, marked thickening of the splenic capsule of spleen (Figur 2H), pneumonic area (represented by marked vascular congestion, and leukocytic infiltration) of lung (Figure 2K). The vaccinated propolis administered challenged group (IV) showed normal hepatocytes with mild portal congestion of liver (Figure 2C), normal renal epithelium except for very mild vacuolations (Figure 2F) of kidney, normal red and white pulps except for mild hemosiderosis of spleen (Figure 2I), normal pulmonary tissue except for a focus having mild congestion and few mononuclear cell infiltration of lung (Fig. 2L).

Table 1: Erythrogram and platelets count (mean values±SE) in rabbits' groups vaccinated and challenged with *Pasteurella mulocida* at the end of the 2nd and 6th weeks of the experiment

	RBCs ($\times10^6$/µl)	Hb (g/dl)	PCV %	MCV (fl)	MCHC%	Platelets ($\times10^3$/µl)
			2nd week			
Control (GI)	5.63±0.14^a	11.52±0.31^a	37.46±1.00^a	66.58±1.82^{abc}	30.77±0.55	483.60±57.53
Non-vaccinated Challenged (GII)	5.68±0.07^a	11.38±0.10^a	35.71±0.9^{ab}	62.89±1.44^c	31.94±0.67	456.80±13.58
Vaccinated Challenged (GIII)	5.08±0.11^b	9.94±0.10^b	33.24±1.51^b	65.29±1.77^{bc}	30.11±1.15	448.80±36.12
Vaccinated Propolis administered (Challenged GIV)	5.69±0.03^a	11.86±0.20^a	36.64±0.74^{ab}	64.4±1.20^{bc}	32.39±0.37	437.80±30.05
			6th week			
Control (GI)	5.60±0.09^a	11.34±0.20^a	34.30±0.65^a	61.33±1.78^b	33.11±0.90^a	469.00±48.10^a
Non-vaccinated Challenged (GII)	3.34±0.14^b	8.46±0.16^b	31.10±0.44^b	102.43±6.87^a	25.11±0.96^b	213.20±46.43^b
Vaccinated Challenged (GIII)	5.16±0.26^a	10.90±0.18^a	33.86±1.19^{ab}	60.76±2.51^b	32.54±0.32^a	428.20±22.19^a
Vaccinated Propolis administered (Challenged GIV)	5.19±0.16^a	.1084±0.19^a	33.13±1.04^{ab}	63.82±1.16^b	31.29±0.73^a	441.40±12.15^a

Means at the same column followed by different letters were significantly different and the highest value was represented with the letter a.

Table 2: Leukogram (x 10^3/µl) and phagocytic percentage (mean±SE) in rabbits' groups vaccinated and challenged with *Pasteurella mulocida* at the end of the 2nd and 6th weeks of the experiment

	TLC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Phagocytic %
			2nd week				
Control (GI)	9.16±0.74^{c}	4.11±0.15^{bc}	3.98±0.47^{b}	0.78±0.28^{b}	0.06±.01	0.23±0.06	26.20±1.39^{c}
Non-vaccinated Challenged (GII)	8.84±0.42^{c}	3.84±0.28^{c}	4.21±0.28^{b}	.077±0.09^{b}	0.08±0.02	0.24±0.09	25.60±1.50^{c}
Vaccinated Challenged (GIII)	11.63±0.39^{b}	4.53±0.18^{b}	5.99±0.26^{a}	0.81±0.07^{b}	0.09±0.01	0.20±0.08	35.80±1.24^{b}
Vaccinated Propolis administered (Challenged GIV)	14.41±0.38^{a}	6.44±0.17^{a}	6.03±0.26^{a}	1.63±0.18^{a}	0.08±0.02	0.24±0.06	60.20±1.59^{a}
			6th week				
Control (GI)	9.29±0.41^{b}	4.40±0.19^{c}	4.03±0.60^{b}	0.59±0.04^{bc}	0.06±0.01	0.21±0.08	25.60±1.50^{c}
Non-vaccinated Challenged (GII)	13.35±0.20^{a}	8.01±0.25^{a}	4.50±0.15^{b}	0.63±0.04^{b}	0.05±0.00	.020±0.05	23.80±1.24^{c}
Vaccinated Challenged (GIII)	10.21±0.71^{b}	4.24±0.29^{c}	5.01±0.41^{ab}	0.68±0.05^{b}	0.06±0.01	0.22±0.07	35.80±1.24^{b}
Vaccinated Propolis administered (Challenged GIV)	13.98±0.09^{a}	6.32±0.14a	6.38±0.13^{a}	0.98±0.09^{a}	0.08±0.01	0.21±0.06	60.80±1.74^{a}

Means at the same column followed by different letters were significantly different and the highest value was represented with the letter a

Table 3: Clinicobiochemical results (mean values ± SE) in rabbits' groups vaccinated and challenged with *Pasteurella mulocida* at the end of the 2nd and 6th weeks of the experiment

	ALT U/l	AST U/l	ALP U/l	T. bilirubin mg%	D. bilirubin mg%	In. bilirubin mg%	Urea mg⁄dl	Creatinine mg/dl	NO µmol/l
				2nd week					
Control (GI)	16.18±0.37^{b}	15.56±0.41^{b}	41.07±2.08^{ab}	1.00±0.04^{b}	0.22±0.03^{b}	0.77±0.04^{b}	33.49±1.19	0.94±0.05^{a}	0.66±0.11^{b}
Non-vaccinated Challenged (GII)	16.50±0.39^{b}	15.20±0.41^{b}	40.12±1.68^{ab}	0.94±0.06^{b}	0.24±0.02^{b}	0.71±0.07^{b}	34.74±1.05	0.96±0.05^{a}	0.70±0.12^{ab}
Vaccinated Challenged (GIII)	18.32±0.85^{a}	19.58±0.38^{a}	44.82±1.59^{a}	1.36±0.06^{a}	0.25±0.02^{ab}	1.10±0.08^{a}	33.79±1.33	0.98±0.03^{a}	0.78±0.07^{a}
Vaccinated Propolis administered (Challenged GIV)	16.30±0.24^{b}	15.72±0.73^{b}	37.67±1.33^{b}	0.99±0.03^{b}	0.21±0.01^{b}	0.78±0.03^{b}	32.83±0.84	0.65±0.04^{b}	0.63±0.08^{b}
				6th week					
Control (GI)	14.86±0.44^{c}	13.64±0.49^{c}	39.70±2.65^{c}	0.95±0.04^{d}	0.30±0.02^{b}	0.65±0.05^{c}	34.14±1.54^{b}	0.95±0.07^{b}	0.58±0.08^{c}
Non-vaccinated Challenged (GII)	32.03±1.08^{a}	27.57±1.19^{a}	55.45±2.03^{a}	2.05±0.09^{a}	0.43±0.03^{a}	1.62±0.09^{a}	48.65±1.21^{a}	1.57±0.05^{a}	2.98±0.15^{a}
Vaccinated Challenged (GIII)	24.49±0.53^{b}	22.37±1.04^{b}	44.31±1.21^{b}	1.61±0.06^{b}	0.29±0.01^{b}	1.32±0.05^{b}	34.83±1.05^{b}	0.98±0.05^{b}	2.06±0.05^{b}
Vaccinated Propolis administered (Challenged GIV)	13.78±0.45^{c}	13.56±0.33^{c}	41.57±0.85^{bc}	1.03±0.01^{cd}	0.22±0.01^{c}	0.81±0.01^{c}	22.00±0.95^{c}	0.60±0.03^{c}	0.58±0.09^{c}

Means at the same column followed by different letters were significantly different and the highest value was represented with the letter a

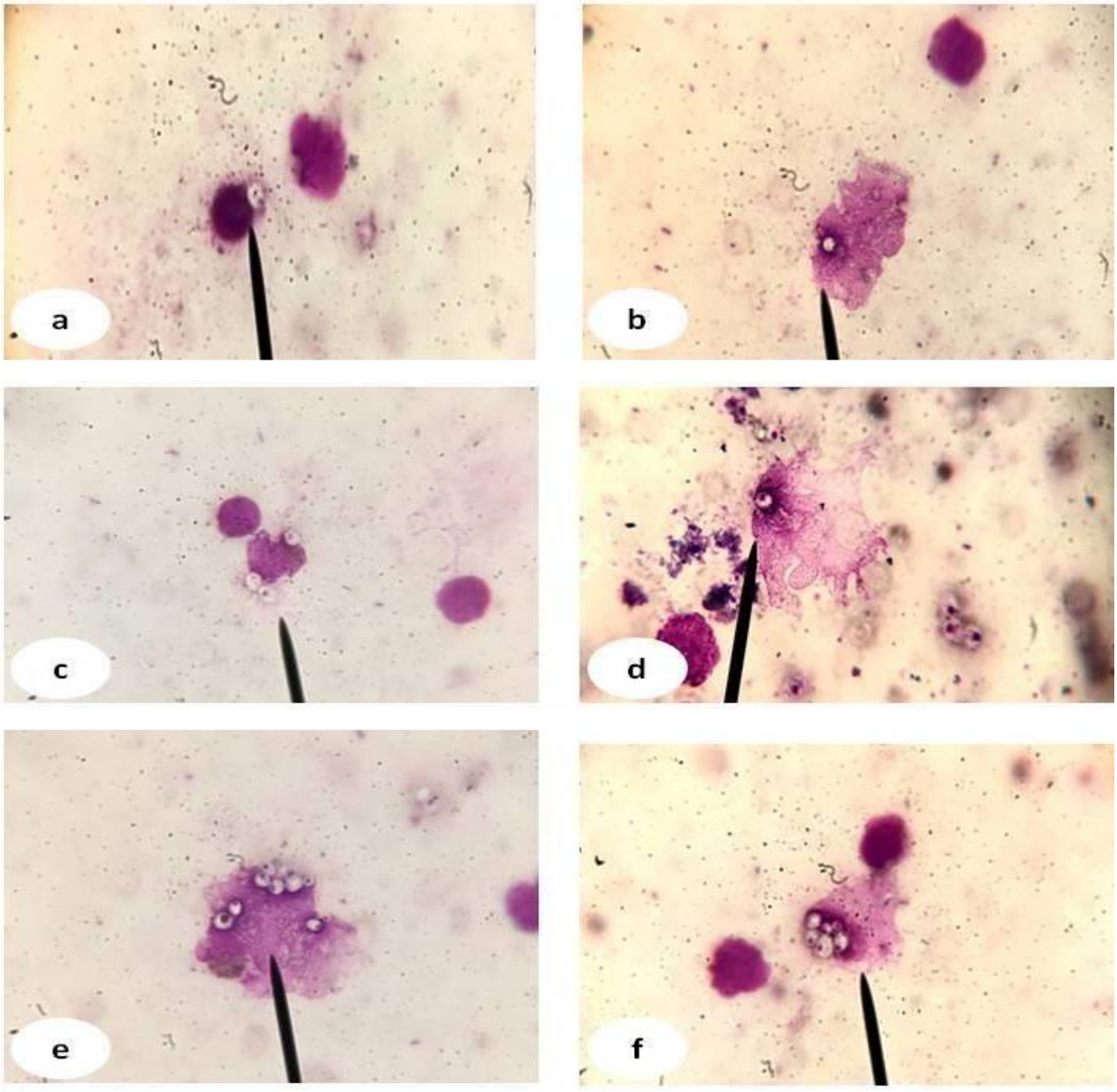

Figure 1: Representative photos showing; mild phagocytic activity in non vaccinated and non challenged control group (I) (a), non-vaccinated challenged group (II) (b), moderate in vaccinated challenged group (III) (c,d) and high in vaccinated propolis administered group (IV) (e,f), arrows indicate phagocytes with engulfed yeast cells

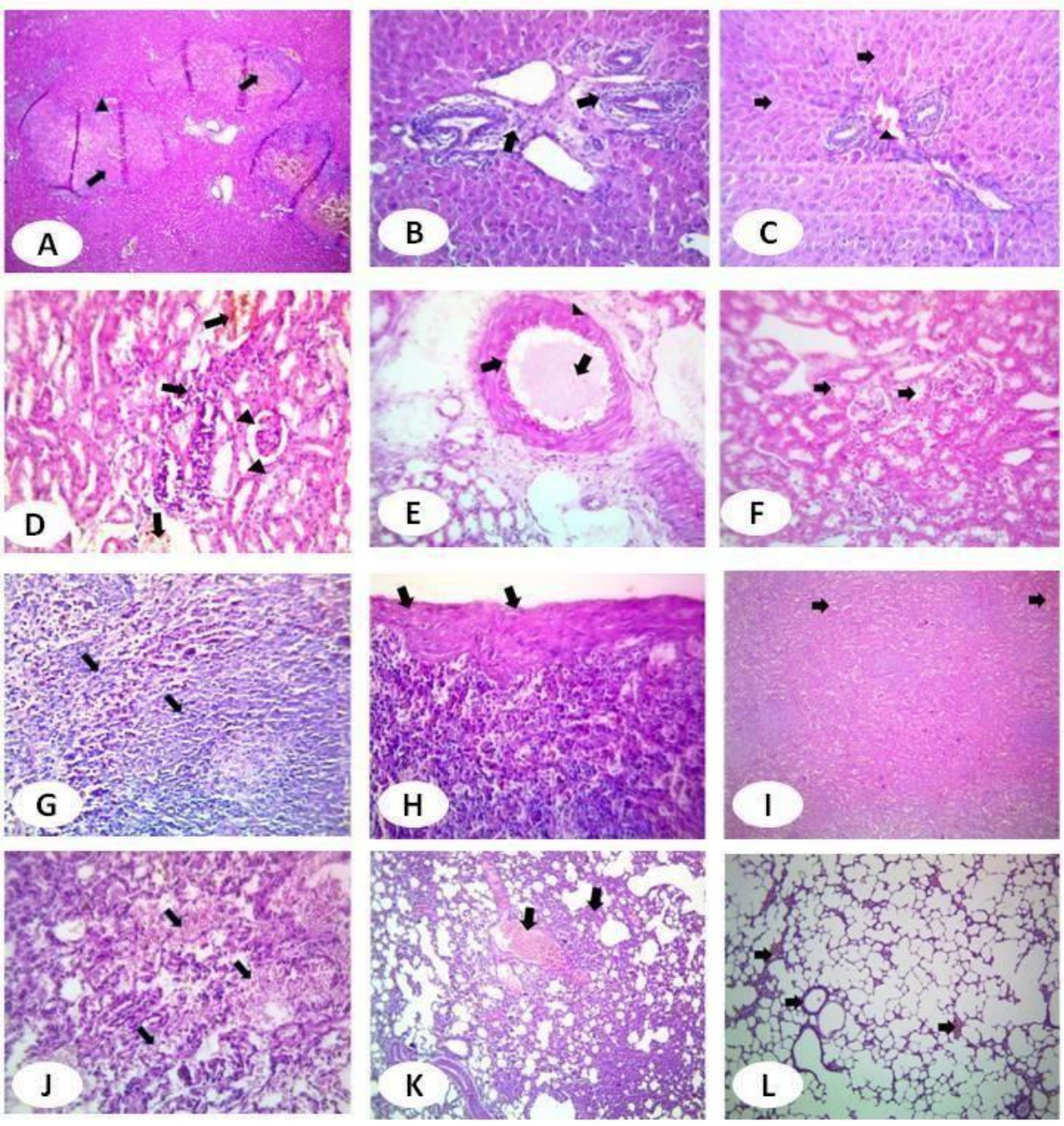

Figure 2: Photomicrograph of H&E-stained tissue sections: Liver (A,B&C) at X100; (A): Multiple focal areas of coagulative necrosis (arrows) surrounded by line of demarcation (arrowhead) (non-vaccinated challenged group II), (B): Mononuclear cell infiltration in the portal areas (arrows) (vaccinated challenged group III), (C): Normal hepatocytes (arrows) with mild portal congestion (arrowhead) (vaccinated propolis administered group IV). Kidneys (D,E&F) at X400; (D): Vascular congestion, mononuclear cell infiltration (arrows) with widening of the glomerular space (arrowheads) (non-vaccinated challenged group II), (E): Vascular congestion, with perivascular edema (arrows) and mononuclear cell infiltration (arrowhead) (vaccinated challenged group III), (F): Almost normal renal epithelium except for very mild vacuolations (arrows) (vaccinated propolis administered group IV). Spleen (G,H at X400&I at X100); (G): Marked polymorphonuclear cell infiltration (arrows) (non-vaccinated challenged group II), (H): Marked thickening of the splenic capsule (arrows) (vaccinated challenged group III), (I): Almost normal red and white pulps except for mild hemosiderosis (arrows) (vaccinated propolis administered group IV). Lung (J at X400, K&L at X100); (J): Extensive hemorrhage (presence of large numbers of extravasated RBCs in the alveolar lumens)(arrows) (non-vaccinated challenged group II), (K): Pneumonic area (represented by marked vascular congestion, and leukocytic infiltration) (arrows) (vaccinated challenged group III), (L): Almost normal pulmonary tissue except for a focus having mild congestion and few mononuclear cell infiltration (arrows) (vaccinated propolis administered group IV)

Discussion

Propolis is a resinous hive product gathered by bumble bees from different plant sources. It has various biological and pharmacological properties, for example, antimicrobial, anti-inflammatory and immunomodulatory (28,29).The propolis extract may act as an assistant adjuvant substance, potentiating the humoral reaction activated by the antigen (11). Propolis flavonoids are adjuvant with immune enhancement properties, for example, improving the differentiation of T, B lymphocyte cell and expanding neutralizing antibody titers (30).

By evaluation of erythrogram in the present study, non-vaccinated (group II), after 6th week of the experiment showed a macrocytic hypochromic anemia and thrombocytopenia, that may be attributed to hemorrhage induced by *P. multocida* (31-33). Our results were confirmed by the histopathological findings in different organs from rabbits in group (II), where kidneys and lungs showed vascular congestion and extensive hemorrhage with presence of large numbers of extravasated RBCs in the alveolar lumens respectively.

Group (III) showed normocytic normochromic anemia at the end of the 2nd week of the experiment. This anemia may be due to the adverse effect of formalized killed *P. multocida* vaccine on erythropoiesis process through its effect on erythrocyte precursor cells in the bone marrow (34). However, these parameters returned to normal values of the control at the end of 6th week of the experiment. This may be attributed to the transient adverse effect of formalized killed *P. multocida* vaccine on erythropoiesis process. These results agreed with Jasprica et al. (35) and Nassar et al. (36).

Group (IV) did not show any significant changes in erythrocytic parameters during all the experimental periods. This could be attributed to protective effect of polyphenolics, components of propolis, on the red blood cell membrane (37), together with a possibility that RBC act as a natural flavonoid reservoir (38). There is a considerable and reliable evidence for antioxidant activity of propolis in vitro (39,40). Flavonoids and phenolic acids under specific conditions in vitro can act as antioxidant preventing the undesired toxic impacts of free radicals (41).

Regarding the platelets count, groups (III and IV) did not show any significant changes in platelets count during all experimental periods when compared with control group; that suggests the positive effect of propolis and killed *P. multocida* vaccine on the number of platelets. Also, it suggests the effective role of combined propolis in elevating myeloid and megakaryocytic types of colony forming units (CFUs) of hematopoietic tissue of treated animals. This result agreed with Oršolic´ and Bašic´ (42).

Regarding leukogram, there was a leukocytosis with a highly significant neutrophilia at the end of 6th week in group (II) rabbits after challenging with virulent *P. multocida*. This may probably due to the severe inflammatory response induced by *P. multocida* as concluded by Wu et al. (43). Also, Galdiero et al. (44) said that the porins, main polypeptide of the outer membrane of *P. multocida*, affect various biological functions of cells involved in the immune response as well as in inflammation especially neutrophils. On the other hand, group III did not show significant changes in neutrophilic count along all the experimental periods; this may be attributed to the weak neutrophilic stimulation by the killed vaccine. This result agreed with Cho et al. (45). While, this group showed a significant increase in total leukocytic count only at the end of 2nd week of the experiment, mainly due to rise in lymphocytes that suggests the immune enhancing effect of formalized killed vaccine on the lymphocytes. In contrast, the total leukocytic count in this group return to normal values of the control at the end of 6th weeks post-treatment; this may be attributed to that inactivated vaccine enhance mainly humoral immune response for short period (46,47).

Group (IV) showed leukocytosis mainly due to rise in neutrophilic, lymphocytic and monocytic counts. This rise presents in propolis treated rabbits suggesting the immunomo-

dulatory effect of propolis alone or its enhancing effect to immunity when used as adjuvant with killed vaccine (48,49). Propolis can act as an immune stimulant as well as increase the total leukocytes value (50). Also, this rise in WBCs may be attributed to stimulation of multiplication of leukocyte precursors from pluripotent stem cells caused by propolis (42).

Our findings agreed with Orsolić and Basic (51,42) who reported that, water-soluble derivative of propolis (WSDP) given to mice caused a significant elevation of leucocytes in peripheral blood.

Concerning the phagocytic activity, group (IV) recorded the highest significant increase in the phagocytic activity followed by group (III) compared with other groups (I and II). This result may be discussed due to the immuno-modulatory effect of propolis and its components (52). Also, Possamai et al. (53) found that mononuclear phagocytes exposed to propolis adsorbed onto polyethelene glycol (PEG) microspheres showed high levels of superoxide release, phagocytosis, and microbicidal activity.

Propolis flavonoids were reported as adjuvants, that have an immuno stimulation action that accelerate the transformation of T, B lymphocyte cell and augmenting neutralizing antibody titers (30). Group (III) showed a significant increase in the phagocytic activity compared with control, but this rise was not as high as that of the propolis treated rabbits. This result may be attributed to the effect of killed vaccine on induction of only humoral immune response without great effect on cell mediated immunity (45,54).

Regarding the biochemical analysis for evaluation of liver functions, group (II) at the end of 6^{th} week of the experiment showed a highly significant rise in serum level of ALT, AST and ALP that suggests the damaging effect of virulent *P. multocida* on the liver. Similar results previously obtained by Nassar et al. (15). These results disagreed with Thurston et al. (55) who found that rats injected with a sub lethal single subcutaneous dose (0.2 microgram/kg of body weight) of *P. multocida* D toxin showed no significant changes in serum level of these enzymes. This difference with our work may be due to difference in bacterial dose, duration of challenge or physiological difference in the animal species. Group III showed a significant increase in ALT and AST level and total and indirect bilirubin at the end of the 2^{nd} and 4^{th} weeks. This could be interpreted due to mild hepatic irritating effect induced by formalized killed *P. multocida* vaccine injection. The same results were previously recorded by Nassar et al. (15). On the other hand, Group IV showed non significant rise in ALT, AST and ALP level and total, direct and indirect bilirubin when compared with the control (group.I) that could be attributed to hepato-protective and improving effect of propolis on liver functions (56).

Our results were confirmed by histopat-hological changes in group (II), where liver showed multiple focal areas of coagulative necrosis. While, the liver of vaccinated rabbits in groups III and IV have less severe lesion represented by mononuclear cell infiltration in the portal areas and normal hepatocytes with mild portal congestion respectively. These results were in consistent with Mathy et al. (57) and Nassar et al. (37).

Serum urea and creatinine levels in bacterial challenged rabbits (group II) revealed a highly significant rise. In contrast, formalized killed vaccinated rabbits either alone (group III) or with propolis (group IV) showed non significant changes in serum urea and creatinine levels in comparison with control (group I) along the experimental period. These results proved the renal damaging effect of virulent *P. multocida* which can be prevented by administration of killed vaccine alone or in combination with propolis.

Our obtained results were established by the histopathological findings in group (II), where kidneys showed vascular congestion, mononuclear cell infiltration with widening of the glomerular space. While, the kidneys of vaccinated rabbits (group III) have slight vacuolation in renal epithelium. These results confirmed the adverse effect of virulent *P. multocida* on renal histology which can be

prevented by administration of killed vaccine alone or in combination with propolis.

Conclusion

As an evident from the current study, it could be concluded that the use of propolis improved the immune protection of rabbits against pasteurellosis than using the vaccine alone.

Acknowledgment

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Conflict of interest

The authors declare no conflicts of interest.

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Slov Vet Res 2018; 55 (Suppl 20): 73–84 **Original Research Article**
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DIVERSITY OF ARTHRODERMATACEAE COMMUNITIES THAT CREATE HAVOC TO THE OVERALL HEALTH OF HUMAN AND ANIMALS

Mohamed Taha[1], Yasmine H. Tartor[1]*, Safaa A. Abdallah[2], Mona M. Osman[3], Ahmed M. Ammar[1]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Zagazig University, [2]Environmental affairs apparatus, Cabinet of Ministers, [3]Directorate of Veterinary Medicine, Sharkia Governorate, Egypt

*Corresponding author, E-mail: jasmen21@yahoo.com; yasminehtartor@zu.edu.eg

Abstract: Keratinous substance rich soil is most conducive for keratinophilic fungi growth and occurrence. Dermatophytes and other related fungi are potential pathogens causing human and animal dermatomycoses. Herein, this study went for screening the presence of dermatophytes and related keratinophilic fungi in various soil samples collected from different locations of Sharkia Governorate, Egypt. A total of 80 soil samples from roadsides, fields and stables were subjected for mycological analysis using modified hair-bait technique with hair of horse, cattle and goat, sheep wool, and chicken feathers as a keratin source for keratinophilic fungi growth. Keratinophilic fungi were identified according to their phenotypical characterization in combination with PCR amplification and sequencing for internal transcribed spacer (ITS) region of rDNA. Keratinophilic fungi were recovered from 73.75% of soil samples (59/80). Field soils yielded a higher positivity rate for keratinophilic fungal isolates (90%) than roadsides (66.67%) and stables (58.82%). The majority of keratinophilic fungi belonged to dermatophytes (57.47%). *Microsporum gypseum* (50.85%) were detected in the majority of sites followed by *Trichophyton mentagrophytes* (30.51%), *Chrysosporium* species (28.81%), *C. keratinophilium* (23.73%), *C. tropicum, C. zonatum, Arthroderma multifidum*, *Arthroderma benhami*, *Arthroderma fulvum*, *Clonostachys* species, *Simplicillium obclavatum* and *Purpureocillium lilacinum* (1.69%, each). It was found that horse and goat hair were more suitable for isolation of keratinophilic fungi with a percentage of 100% for each, followed by cattle hair (91.66%), sheep wool (87.5%) and chicken feathers (83.33%). This investigation demonstrated that the various soils of Sharkia Governorate might be critical suppliers of certain keratinophilic fungi that may constitute hazards to human and animal health. The genetic-based identification is strongly recommended for a high discriminatory identification of keratinophilic fungi.

Key words: keratinophilic fungi; geophilic dermatophytes; *Arthroderma benhami*; *Arthroderma fulvum;* ITS sequencing

Introduction

Keratinophilic fungi are an exceedingly specific, keratin-corrupting ecological group of filamentous fungi. They live in natural environments, grow and even reproduce on keratin-rich remains in the soil such as outer cornified layers of the skin, hair, nail, fur, feather, horn, hoof and beak (1). They

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comprise fungi known as geophilic dermatophytes and the species of the genus Chrysosporium related to them (2). Majority of soil fungi are potential pathogens to both human and animals causing dermatophytosis (ringworm) and are parasites of keratinized tissues (3). Dermatophytosis is common worldwide superficial fungal infections and is of veterinary and public health significance (4,5), but its prevalence can vary extensively, relying on geographical and other epidemiological variables as (temperature, humidity, pH, climate, environmental light and amount of organic substance in soil, etc.) (4,6). Dermatophytes are classified according to the conidial morphology to Microsporum, Trichophyton, Epidermophyton genera (in their anamorphic phase) and Arthroderma (in their telomorphic phase). Ecologically, dermatophytes have been classified into zoophilic, anthropophilic or geophilic species on the basis of their natural habitat (animals, humans and soil, respectively) (6). Zoophilic and geophilic dermatophytes often incite a more powerful inflammatory response when they endeavor to attack human skin than anthropophilic dermatophytes (7).

Considering the significance of soil saprophytes as potential human pathogens, many researchers have studied the occurrence of dermatophytes and keratinophilic fungi in soils in many territories around the world (2,8–10). So far, data on the frequency and distribution of keratinophilic fungi in various soils of Sharkia Governorate, Egypt are meager. Thus, in this study the variety of keratinophilic fungi in different destinations frequented by human and animals in Sharkia Governorate, Egypt were investigated and identified using conventional phenotypic and molecular characterization. Keratinophilic fungi were also tested for their potential to utilize hairs of different animals and chicken feathers as keratin substrates.

Material and methods

Soil samples

A total of 80 soil samples were collected from different sites where human and animals live (33 roadsides, 30 fields, 17 stables and animal habitats) in Sharkia Governorate. The soil samples were gathered from the superficial layer at a depth not surpassing 3-5 cm in sterile polythene bags, taken to the laboratory, stored at 15°C and processed immediately.

Keratin substrates and hair-bait technique

Samples were cultured on fragments of sterile hairs (approximately 2-3 cm long) from healthy animals such as hair of horse, cattle and goat, sheep wool, and chicken feathers as a keratin source for growth of keratinophilic fungi in modified hair-bait technique (11). For this purpose, five Petri dishes were used for every soil sample, one each for different baits. Each Petri dish was half-filled with soil samples and fragments of sterilized hairs or feathers were scattered on their surface. Soil samples were wetted with about 15 ml of sterile distilled water and 10 drops from Dermasel selective supplement (Oxoid Thermo Fisher Scientific, Basingstoke, Hants, UK) dissolved in 3 ml ethyl alcohol. Plates were incubated at room temperature for 2-3 weeks. Soil samples were examined regularly for the development of mycelium on the baits and moistened with distilled water with some drops of Dermasel selective supplement every four day to prevent the drying till fungal growth appeared.

Isolation and identification of keratinophilic fungi

When mycelial growth was visible on the hair, wool or feather, parts of each with fungal growth were put on a slide and examined microscopically for macroconidia, microconidia or septated hyphae after staining with lactophenol cotton blue. Invaded hair, wool or feather was inoculated onto slopes of Sabouraud's Dextrose Agar (SDA) with Dermasel selective supplement containing cycloheximide 500mg/l and chloramphenicol 50 mg/l) (Thermo Fisher Scientific / Oxoid) for 5-10 days at 25°C. Isolates were further subcultured for 14 days on SDA with Dermasel selective supplement at 25°C and growths were recognized phenotypically to genus and species level based on colony morphology and

microscopic examination. Morphology of dermatophyte isolates was examined from all aspects as regard to texture of colonies, rate of growth, surface and reverse colour on Bromocresol Purple Milk Dextrose Agar (BCP), Rice Lactritmal Agar (RLA) and Rice grain (RG) media (12).

Extraction of genomic DNA

Total DNA was extracted from each isolate using Thermo Scientific Gene JET Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific; Dreieich, Germany). One hundred milligrams of mycelia were weighed, placed into liquid nitrogen and then grinded thoroughly with a mortar and pestle. Grounded mycelia powder was transferred immediately into a 1.5 ml microcentrifuge tube containing Lysis Buffer A of the kits and other steps were performed according to the manufacturer's recommendations. The quality of extracted DNA was evaluated using gel electrophoresis.

PCR amplification, sequencing and Phylogenetic analysis

The internal transcribed spacer region of rDNA encompassing ITS1, 5.8S and ITS2 genes was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer pairs (13). PCR reactions were performed in a final reaction volume of 25 µl containing 5 µl templates DNA, 1 µl from each primer (20 pmol), 12.5 µl of Dream Taq™ Green master mix (2x) (Thermo Fisher Scientific; Dreieich, Germany) and 5.5 µl nuclease free water using Biometra T3 thermal cycler (Göttingen, Germany). PCR Cycling condition: An initial denaturation step at 95°C for 5 min was followed by 35 cycles of denaturation at 95°C for 5 min, annealing at 55°C for 1 min and extension at 72°C for 1 min with a final extension step of 72°C for 10 min. Amplicons were electrophoresed in ethidium bromide-stained 1.5% agarose gels (Gellyphor, Euroclon, Italy) and estimated by correlation with markers in the Gene Ruler^{TM} 100-bp DNA Ladder (MBI Fermentas, Vilnius, Lithuania). Gels were photographed utilizing a digital documentation system (Gel Doc 2000, BioRad, UK).

Amplified products of eight genomic DNA samples were each refined utilizing Gene JET PCR purification kit (Fermentas, Vilnius, Lithuania) and afterward sequenced using the Taq DyeDeoxyTerminator Cycle Sequencing Kit (v.2, Applied Biosystems) in an automated sequencer (ABI-PRISM 377). The ITS1, 5.8S and ITS2 sequences were then determined using ITS1 and ITS4 primers. The nucleotide sequences were additionally compared with published sequences of fungi available in the National Center for Biotechnology Information (NCBI) database using BLAST program (http: // www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequence data for *Arthroderma benhami, Arthroderma multifidum, Clonostachys* species, *Arthroderma fulvum, Chrysosporium tropicum, Simplicillium obclavatum, Purpureocillium lilacinum* and *Chrysosporium zonatum* reported in this paper have been deposited in the GenBank database under accession numbers KR265107, KR265108, KY129987, KY129988, KY129989, KY129990 and KY290545, respectively.

The neighbor-joining method that applied to DNA distance matrices was calculated according to Kimura two-parameter model was used for construction of the phylogenetic tree. The confidence values of branches were determined by a bootstrap analysis using MEGA 6 program version 6 (14).

Data Analysis

Data were expressed as cross tabulation using Statistical Package for Social Sciences version 25.0 (SPSS, IBM Corporation, Armonk, NY, USA); where rows and columns represent the isolated Keratinophilic fungi from the examined soil samples and different soils, respectively.

Results

Isolation and identification of dermatophytes and related keratinophilic fungi from soil samples

Eighty seven keratinophilic fungal isolates were recovered by modified hair-baiting technique from 59 positive soil samples. Within 2-3 weeks, hairs, wool or feathers distributed on the surface of soil samples were overgrown with white or brownish white mycelia as shown in (Fig. 1). The potential of keratinophilic fungi to utilize hairs of different animals and chicken feathers as keratinous substrate for growth were tested. It was found that horse and goat hair were more suitable for isolation of keratinophilic fungi with a percentage of 100% for each, while cattle hair (91.66%), sheep wool (87.5%) and chicken feathers (83.33%). Fungal growth with part of invaded hair was subcultured on SDA supplemented with chloramphenicol and cyclohexamide and subjected for identification by macroscopic and microscopic examination which was differentiated to keratinophilic fungi namely *M. gypseum, T. mentagrophytes* and *Chrysosporium* species and *C. keratinophilum.* Macroconidia of *M. gypseum* were produced in great numbers as broadly spindle shaped with moderately thick walls and 4 to 6 septa with rounded ends. Hyphae are branched, septated and hyaline as in Figure (2a and b). Conidia of *Chrysosporium* species were clavate to ovoid with thick walled as in Figure (2c and d).

Amplification of the entire ITS (ITS1, 5.8S rDNA, and ITS2) region of nineteen representative isolates using ITS1 and ITS4 primers yielded unique amplified products ranging from 590 to 740 bp where *Arthroderma multifidum* is smallest and *M. gypseum* is largest in size as shown in (Fig. 3).

DNA sequence analysis was done for eight amplified products from *M. gypseum, T. mentagrophytes, Chrysosporium* species, *Simpliciluim, Purpureocillium* and *Clonosotychs* species which formally identified by phenotypic methods and confirmed by PCR for ITS region. Nucleic acid sequence revealed 100% identity to the reference sequences in NCBI Genbank database. A phylogenetic tree was developed with the sequences of closest kind strain in light of ITS region of rDNA gene sequences (Fig.4). The phylogenetic tree showed different clusters for each species, indicating variation in their sequence.

Incidence of Keratinophilic fungi in different soils

Keratinophilic fungi were recovered from 73.75% of the examined soil samples (59/80). The most elevated rate of positive samples was accounted for fields (90%) then roadsides (66.67 %), and stables (58.82%).

Dermatophytes were the predominant keratinophilic fungi isolated from soil samples, represented 57.47% (50/87) of the total isolates. *M. gypseum* (50.85%, 30/59 positive soil samples) were detected in the majority of the sites followed by *T. mentagrophytes* (30.51%, 18/59), *Chrysosporium* species (28.81%), *C. keratinophilium* (23.73%), *C. tropicum, C. zonatum, Arthroderma multifidum, Arthroderma benhami, Arthroderma fulvum, Clonostachys* species, *Simplicillium obclavatum* and *Purpureocillium lilacinum* (1.69%, each). An association of two to three fungi was observed in 30.51% of the positive soil samples. The most incessant fungal combination was *M. gypseum* & *C. keratinophilum* (61.11%), *M. gypseum* & *T. mentagrophytes* (22.22%) and *T. mentagrophytes* & *Chrysosporium* species (22.22%). Based on the occurrence frequency, the other species detected by ITS sequencing including *Arthroderma fulvum, Arthroderma benhami, Arthroderma multifidum* (the perfect state of *M. fulvum, T. mentagrophytes* and *Chrysosporium* species, respectively), *C. tropicum, C. zonatum, Clonostachys* species, *Simplicillium obclavatum* and *Purpureocillium lilacinum* were sporadically occurring.

As depicted in Table (1), among the three types of examined soils, roadsides and fields soils have the highest frequency of keratinophilic fungal isolates (41.4%). That is, a large frequency was for *Chrysosporium* species (47.1%) from roadsides and *C. keratinophilium* (42.9%) from fields.

Figure 1: Hair baits culture of roadsides soil showed fungal growth (white or brownish white mycelia) within 3 weeks

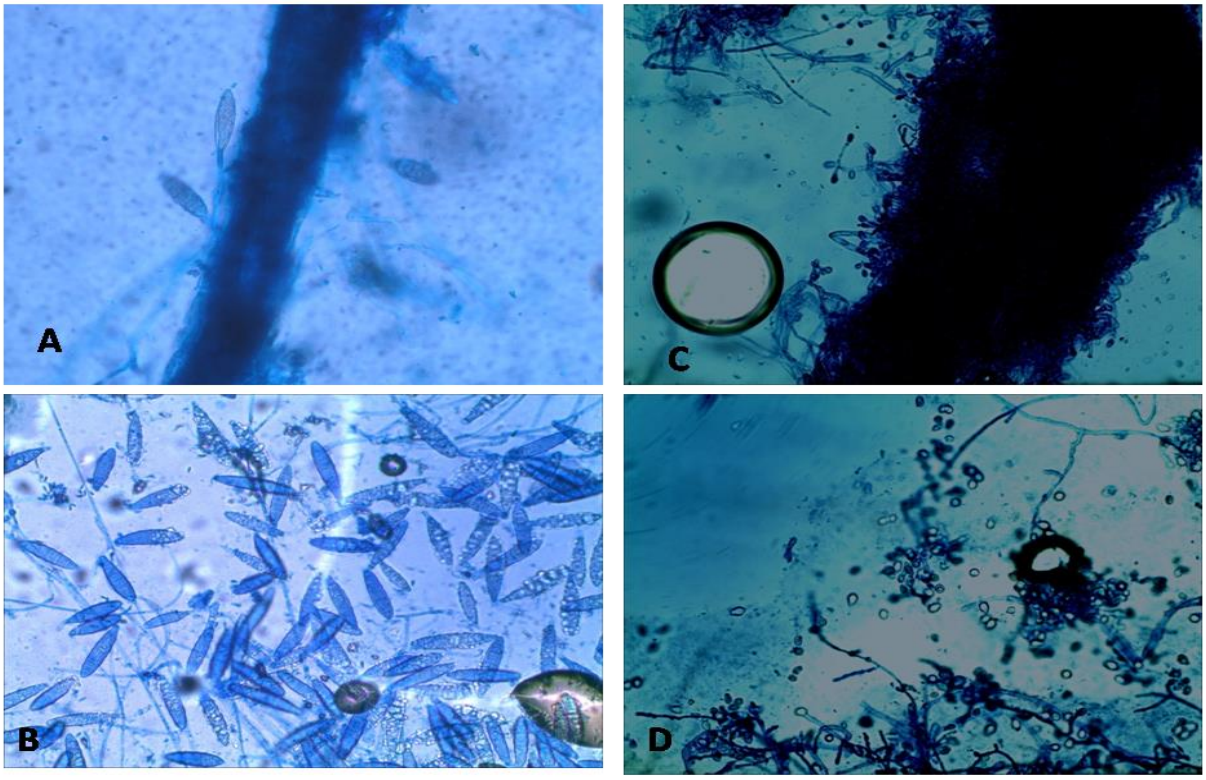

Figure 2: Microscopical examination of *M. gypseum* and *Chrysosporium* species on horse hair from hair baits technique and from culture stained with lactophenol cotton blue (400x). A: *M. gypseum* on hair showing microconidia on short conidiophores. B: *M. gypseum* macroconidia showing: branched septated hyaline hyphae and few microconidia and spindle shaped macroconidia with rounded end. C: *Chrysosporium* species on hair showing one oval cell microconidia on short conidiophores. D: *Chrysosporium* species showing clavate to ovoid thick walled conidia

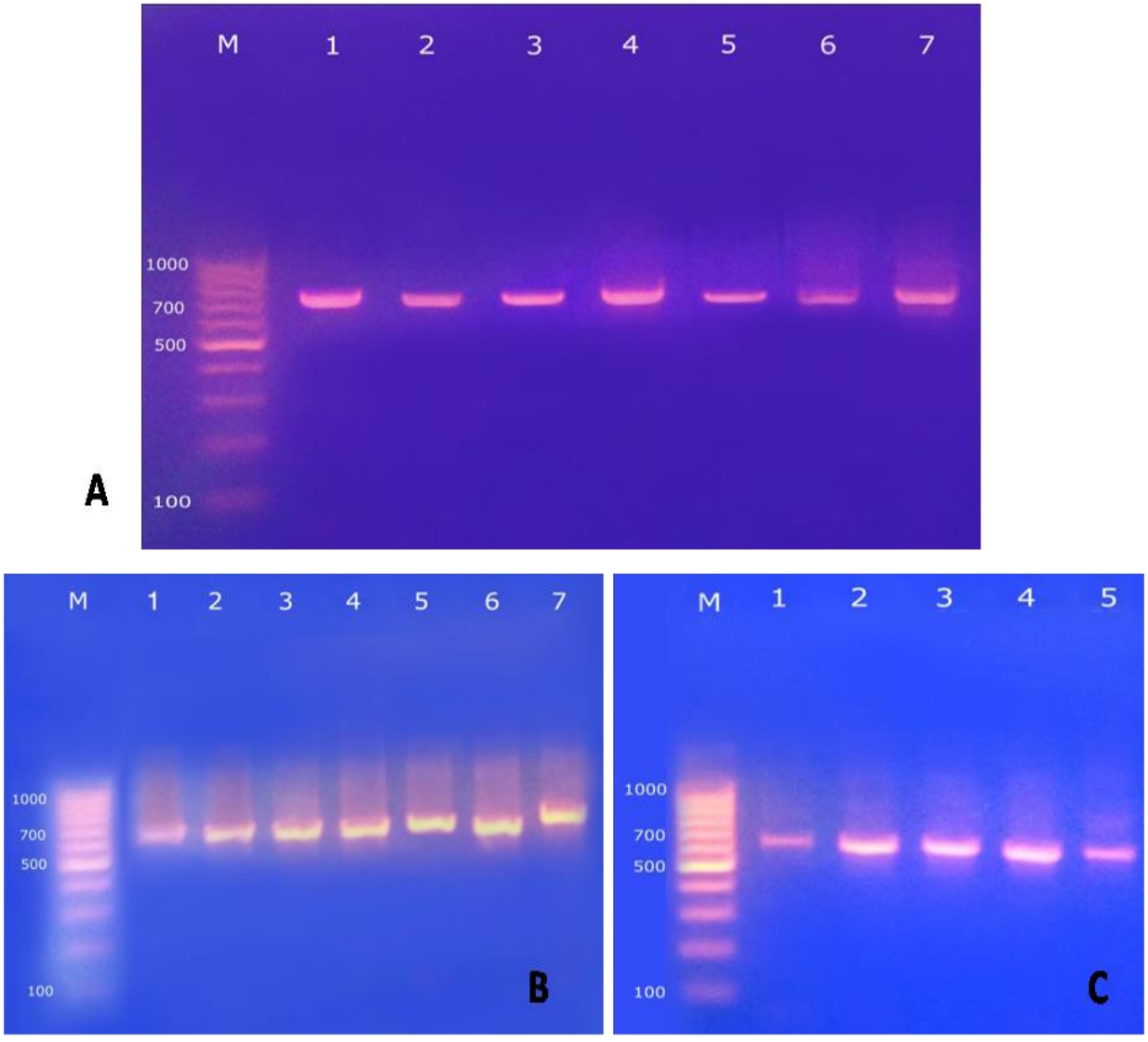

Figure 3: Agrose gel electrophoresis for amplified products of ITS1-5.8S-ITS2 rDNA region of drematophytes and other keratinophilic fungi. A: lane M, molecular size marker, lane 1: amplified products of *Purpureocillium lilacinum* at 770 bp, lanes 2 to 7: *M. gypseum* at 740 bp, lane 7 identified by DNA sequences as *Arthroderma fulvum.* B: Lanes 1-4, 6: *T. mentagrophytes* at 635 bp. Lane 5: *T. mentagrophytes var quinckeanum* at 683 bp and lane 7: *T. mentagrophytes* at 700 bp (identified by DNA sequences as *Arthroderma benhami*). C: lane 1: *Simplicillium obclavatum* at 670 bp, lane 2: *Chrysosporium tropicum* at 620 bp, lane 3: *Chrysosporium zonatum* at 610 bp, lane 4: *Clonosotychs* species at 600 bp and lane 5: *Chrysosporium* species at 580 bp (identified by DNA sequences as *Chrysosporium* state of *Arthroderma multifidum*)

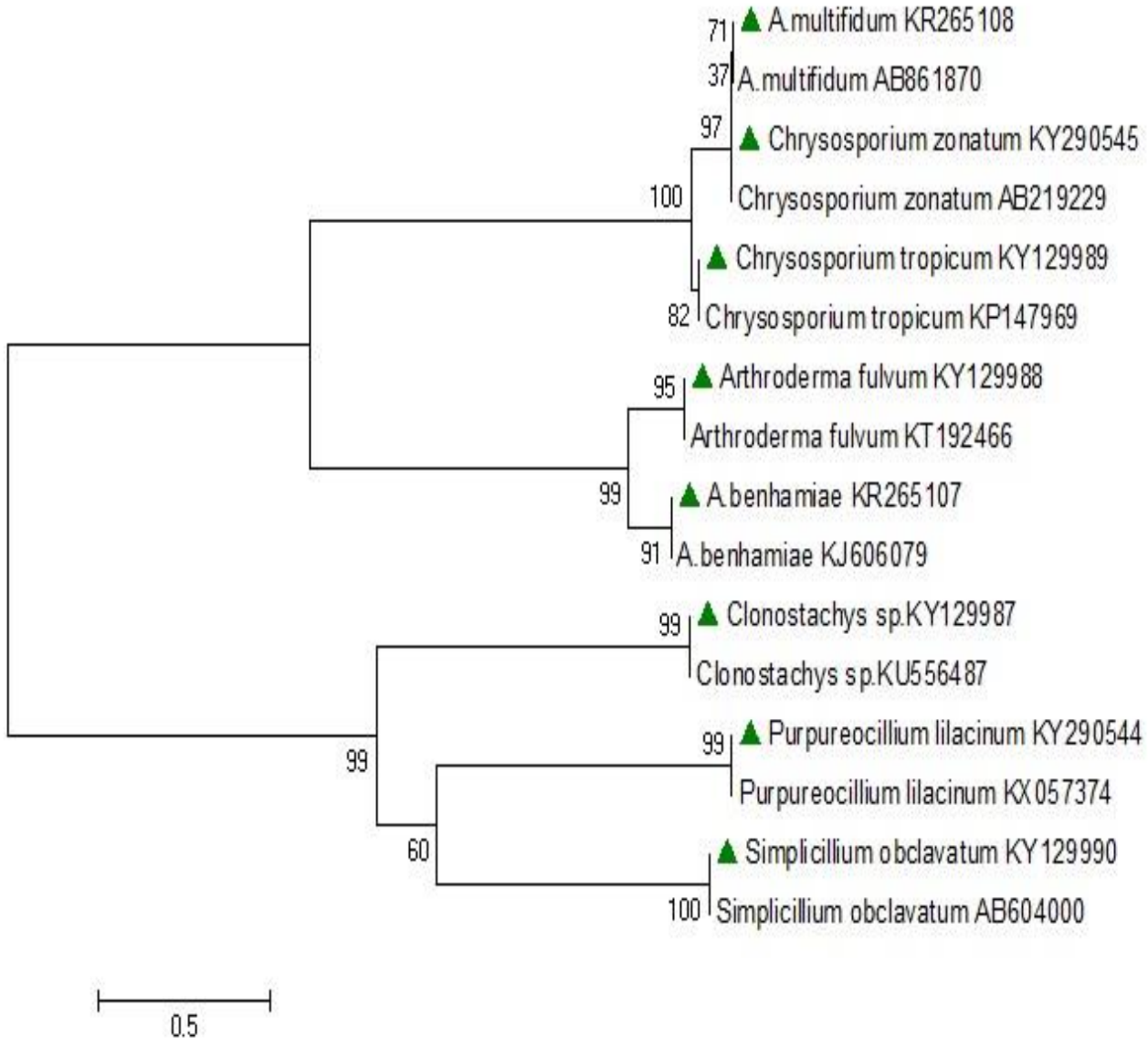

Figure 4: Phylogenetic tree based on ITS region of rDNA gene sequences for Keratinophilic fungi obtained in this study with their reference strains. Bar indicates two base changes per 1000 nucleotide position. Numbers at the respective nodes are percentage of 1,000 bootstrap replicates

Table 1: Frequency and distribution of keratinophilic fungi in different types of soil in Sharkia Governorate, Egypt

Soil fungi * Soil samples Cross tabulation						
			Types of soil samples examined			Total
			Roadsides	Fields	Stables	
Fungal isolates	*M. gypseum*	Number	13	12	5	30
		*Frequency % within spp.	43.3	40.0	16.7	100.0
	†*Arthroderma fulvum*	Number	1	0	0	1
		Frequency % within spp.	100.0	0.0	0.0	100.0
	T. mentagrophytes	Number	7	7	4	18
		Frequency % within spp.	38.9	38.9	22.2	100.0
	‡*Arthroderma benhami*	Number	0	1	0	1
		Frequency % within spp.	0.0	100.0	0.0	100.0
	Chrysosporium species	Number	8	7	2	17
		Frequency % within spp.	47.1	41.2	11.8	100.0
	C. keratinophilium	Number	4	6	4	14
		Frequency % within spp.	28.6	42.9	28.6	100.0
	C. tropicum	Number	0	1	0	1
		Frequency % within spp.	0.0	100.0	0.0	100.0
	C. zonatum	Number	1	0	0	1
		Frequency % within spp.	100.0	0.0	0.0	100.0
	§*Arthroderma multifidum*	Number	1	0	0	1
		Frequency % within spp.	100.0	0.0	0.0	100.0
	Clonostachys species	Number	0	1	0	1
		Frequency % within spp.	0.0	100.0	0.0	100.0
	Simplicillium obclavatum	Number	1	0	0	1
		Frequency % within spp.	100.0	0.0	0.0	100.0
	Purpureocillium lilacinum	Number	0	1	0	1
		Frequency % within spp.	0.0	100.0	0.0	100.0
Total		Number	36	36	15	87
		Frequency % of all isolated sp.	41.4	41.4	17.2	100.0

† The perfect state of *M. fulvum*, ‡the perfect state of *T. mentagrophytes*, §the perfect state of *Chrysosporium* species *Frequency % within spp.= number of isolated spp. from one soil type/total number of spp. from three soil types.

Discussion

Studying of keratinophilic fungi from the environment is of hygienic and epidemiological significance because they constitute a risk of human and animal infection. Therefore, in this study soil samples from different destinations frequented by human and animals in Sharkia Governorate, Egypt were screened for dermatophytes and other related fungi distribution. Dermatophytes and other keratinophilic fungi were recovered from 73.75% of the examined soil samples. In comparison with other soil surveys recorded in the literature, this frequency is similar to those reported in Iran which reached 73.5% (15), India (73.27%) (16) and Mumbai, India (75.0%) (17), but obviously lower than those recorded in the vicinity of Cairo, Egypt (40%) (18), Bahrain (45%) (19) and Assam, India (50%) (10). Moreover, in the soils of Djerba, Tunisia a lowest isolation rate (20.6 %) was reported (20) and a higher rate (100%) was reported in Slovakia (21). This could be ascribed to the climatic conditions and the soils nature.

As affirmed by various investigations, the keratinophilic fungal community varies from that observed for a very populated and strolled territory. With increase in the soils contamination, the indices of fungal occurrence, that correlates with pH, humidity,

organic matter, nitrogen activity, alkalinity and others, are increased (16,20). Subsequently, field soils yielded a higher rate of positive samples for keratinophilic fungi (90%) than from roadsides (66.67%) and stables (58.82%). Furthermore, the highest frequency of keratinophilic fungal isolates (41.4%) was recovered from roadsides and field soils. This is in consistence with Anane et al. (2) who declared a higher rate of keratinophilic fungal isolates from muddy soils than from sandy soil (56.0% and 39.9%, respectively). On the contrary, a lower isolation rate was detected in roadsides and industrial areas in Djerba, Tunisia (20). They attributed that to the high concentration of different pollutants in these regions that could repress the survival of keratinophilic fungi.

Different investigations (22–24) have recognized the internal transcribed spacers region (ITS1 5.8S, and ITS2) of nuclear rDNA as well as the 28S rDNA genes (25) as significant markers for elected yeasts and filamentous fungal species. ITS sequence analysis was conducted for confirmatory identification of eight representative isolates. This approach permitted distinguishing species that were rarely isolated in soils as the geophilic fungal species *Arthroderma fulvum* that causes human dermatophytosis and is one of the *M. gypseum* complex (26). Moreover, the frequent zoophilic dermatophyte *Arthroderma benhamiae*, which gives rise to inflammatory dermatophytosis in human (tinea capitis, tinea corporis, tinea manus and tinea faciei) was identified. Sequencing of the ITS region of rDNA has been affirmed as culture confirmatory test for *Trichophyton* species of *A. benhamiae* (27). Furthermore, *A. multifidum* has been recorded from soil and from bird combs as the dominant species in the Nansei islands in Japan (28).

Distribution of dermatophyte species as *M. gypseum*, *Microsporum fulvum* teleomorph (*Arthroderma fulvum*), *T. mentagrophytes* & its teleomorphs *Arthroderma benhamii* and the other keratinophilic fungi including *C. keratinophilum*, *C. tropicum*, *C. zonatum*, *Chrysosporium* species and its perfect state *Arthroderma multifidum* in various soil samples revealed that they mainly survive on keratinous substrate in soil in all habitats. In addition, the detection of their teleomorphic phase in soil is indicative to long-term survival. Hence, soil acts as store for primary infection, at least for some pathogenic fungi, and furthermore for those which are possibly pathogenic to human and animals. Singh et al. found the same results (29).

Our results revealed the majority of keratinophilic fungi belonged to dermatophytes (57.47%) with *M. gypseum* as the predominating species (50.85%) isolated. It was additionally reported as the first or second most prevalent geophilic dermatophyte recovered from soils in previous literatures (2,20,30,31). Therefore, this is vital in light of the fact that *M. gypseum* causes dermatophytosis in both human (tinea capitis and tinea corporis) and animals. Similar to other studies (2,20), *M. gypseum* was frequently present in soils rich in organic matters.

In this study, Chrysosporium genus was represented by *Chrysosporium* species (28.81%), *C. keratinophilum* (23.73%), *C. tropicum*, *C. zonatum* and *Arthroderma multifidum* (1.69%, each). A large frequency was for *Chrysosporium* species (47.1%) from roadsides and *C. keratinophilium* (42.9%) from fields soils. These well-known soil fungi were isolated with a various recurrence from soils in numerous areas of the world (2,18,20,30,31). Since *Chrysosporium* species occur in different types of soil, it is essential to perceive their pathogenic potential. For instance, *Chrysosporium* species are occasionally isolated from human and animals and have been recorded to cause skin infections or onychomycosis, sinusitis, endocarditis, osteomyelitis, endophthalmitis and pulmonary disease (32–34). Recently, Cook et al. (35) diagnosed a disseminated *Chrysosporium* species infection in a dog. In the human literature, *Chrysosporium* infections either local or invasive are very rare, affect mainly immunocompromised host and can be fatal. To date only thirteen cases were diagnosed (36).

Noteworthy, *T. mentagrophytes* was the second most frequently isolated (30.51%) species from soil samples and it was frequently

recovered from roadsides and fields (38.9%). This is consistent with Jain and Sharma who isolated *T. mentagrophytes* (24.88%) from various soil samples in Jaipur city, India. They found that roadside and garden soils were the most appropriate destinations for almost all keratinophilic fungi (16). As it has been accounted for in past investigations, *Clonostachys* species and *Simplicillium obclavatum* were isolated from soil samples (37,38).

Among the isolated fungal species, *Purpureocillium lilacinum* was isolated from field soils (1.69%), as it has been previously declared (2,9). *Purpureocillium lilacinum* is an emerging, opportunistic pathogen that presents in the soil. It is a new genus for the medically significant *Paecilomyces lilacinus*. It is also known to cause various infections in immunocompromised and immunocompetent hosts and other vertebrates (39,40). Moreover, a case of nasal septal perforation and maxillary sinusitis in immunocompetent patient due to *P. lilacimun* infection (40,41) were reported.

The present work donates the superiority of modified hair-bait technique proposed by Orr (11) in which cyclohexamide was added, for isolation of Arthrodermataceae and other keratinophilic fungi from soil. It was found also that horse and goat hairs are better than cattle hair, sheep wool and feathers of birds when used in hair-bait technique indicating the tendency of Keratinophilic fungi to utilize horse and goat hairs than other sources as keratin bait for fungal growth. Hence the obtained results could be explained with the findings of Suh (42) that each keratinaceous substrates could be utilized as bait, however some were the best and the other were poor baiting materials. Some keratinophilic fungi were well baited on the body hairs of horses, and on the beard and body hairs of goats.

Conclusion

It could be inferred that most of the isolated keratinophilic fungi were either well-known mycotic agents or have been recouped from various animal and human lesions. Therefore, soil acts as a repertoire for primary infection of human and animals, at least for some pathogenic fungi, and furthermore for possibly pathogenic species. The genetic-based identification is strongly recommended to allow a highly discriminatory identification of keratinophilic fungi.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

EFFECT OF THE MEDICINAL PLANT (*AZADIRACHTA INDICA*) ON *Chlamydophila psittaci* INFECTION IN BROILER CHICKENS

Ahmed M. Hegazy[1], Hala M.N. Tolba[1], Lammah K. Abd EL-Samie[2]*, Adel M. Abdelaziz[2], Abdel Moneim A. Ali[3]

[1]Avian and Rabbit Medicine Department, [2]The Veterinary Educational Hospital, [3]Pathology Department, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt

*Corresponding author, E-mail: lamahsamie@gmail.com

Abstract: *Chlamydophila psittaci* is considered one of the important bacterial agents which affecting poultry with zoonotic importance to human health. This study designed to investigate the antibacterial action of aqueous neem leaves extract (*Azadirachta indica*) against experimentally infected broiler chickens with chlamydia. Seventy, one day old Hubbard chicks were randomly selected and divided equally into seven groups, three of the infected groups were treated with neem extract at varied concentration of 4%, 6% and 8% in drinking water compared with the fourth that treated with oxytetracycline. Based on clinical and postmortem examination, growth performance, serum biochemical analysis and histopathological findings the results were evaluated. Chicks received 8% extract at 8 days old for five successive days and control showed normal level of alanine aminotransferase, aspartate aminotransferase, uric acid and creatinine 30 U/L, 26 U/L, 3.90 mg/dl and 0.67 mg/dl respectively, while treated infected groups revealed lower levels unlike infected untreated showed higher levels 68 U/L, 62 U/L, 5.20 mg/dl and 1.62 mg/dl respectively. Moreover treated groups with neem extract of 4%, 6% and 8% recorded significantly better body weights 1130.56 gm, 1135.70 gm and 1254.60 gm respectively, than infected untreated group 963.25 gm at 32 days old as well as feed conversion rate 2.26, 1.94 and 1.77 than 3.80 respectively, at 24 days old. Histopathological examination of infected group showed pulmonary inflammation, myocarditis and hepatic necrotic foci while confirmed that treatment with 8% neem extract resulted in complete recovery of lung tissue and normal myocardium. It is concluded that aqueous leaves neem extract especially 8% concertation had an excellent antichlamydial medication without side effects and recommended in the control of chicken chlamydiosis.

Key words: broilers; *Chlamydophila psittaci*; neem; oxytetracycline; enzymes

Introduction

Chlamydiosis is an infectious disease of zoonotic importance affect humans, animals and birds where it may resulted in outbreaks of in domestic avian, pet and wild birds (1,2). It is caused by obligate intracellular bacteria that responsible for wide range of earnest threats on human and birds (3). The phylogenic analysis of the 16S and 23S rRNA genes showed that order chlamydiae contained two distinguished groups at the family level, *chlamydia* and *chlamydophila* (4).

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Chlamydophila psittaci (C. psittaci) is endemic in birds causing psittacosis or avian chlamydiosis which plays a remarkable role in respiratory infections leading to severe losses in poultry industry (5). The control system of the disease depended on antibiotic treatment commonly oxytetracycline (OTC) , macrolides and doxycycline that inhibit protein synthesis of on chlamydia ribosome**,** but extended exposure of *chlamydia* to antibiotic yielded a greater chance for drug resistance development (6,7). Although the bacteria respond very well to OTC treatment in human and birds (8,9), it has many disadvantages as adverse side effects on birds health and prolonged withdrawal period with residual results (10).

Continuous search for new compounds with antibacterial influences on *chlamydia* and other bacterial species such as *staphylococci* and *lactobacilli* is important (11). Emerging usage of medicinal plants against various diseases is gradually gaining importance. *Azadirachta indica*, known as neem, belongs to the family *Meliaceae* contains various active substances as azadiractin, nimbin, nimbindin, quercetin and others. It has antioxidant, antibacterial, antifungal, antiviral, insecticidal and antiprotozoal properties beside immune-stimulatory actions (10). Therefore the study aspired to assess the therapeutic activity of aqueous extract of neem leaves at different concentrations 4, 6 and 8% against *C. psittaci* infection in chickens.

Material and methods

Experimental birds

Seventy, one day-old Hubbard chicks obtained from EL-Dakahlia poultry Company, were reared in a floor based system at experimental units in Faculty of Veterinary Medicine, Zagazig University. Birds were fed on commercially prepared diet sourced from El-Eman Feed Millers formulated to contain 21% protein and yield 3100 Kcal/kg. Approval of the study was obtained from the Animal Welfare and Research Ethics Committee, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Aqueous neem extract

Leaves were collected from middle aged green trees at agricultural orchards of Faculty of Agriculture, Zagazig University, dried in oven at 37°C for 24 hours and grounded in metallic grinder. Weigh of 40, 60 and 80g of the grind separately soaked, in three nonmetallic jars contain 1 liter of hot boiled distilled water each, for 5-8 hours at room temperature preparing 4%, 6% and 8% neem extract (12).

Oxytetracycline (OTC)

Commercial product contains 200 mg of oxytetracycline HCL (OXYVET-PHARMA) used for treatment of infected chicks with *C. psittaci* in a dose 20 mg/ kg life body weight in drinking water for 5 days (8).

C. psittaci strain

The bacteria, used for challenge was isolated and supplied by Hala (13), prepared in embryonated chicken eggs after Alethea et al. (14) and stored at -80°C. According to Enany et al. (15), briefly 20% suspension of the yolk sac harvest was inoculated into Vero cell, two passages were performed. The SPG buffer (10% fetal calf serum, 10 Ug gentamicin/ml, 100 Ug vancomycin/ml, 2 Ug amphotrecin/ml) was added to the tissue culture flask (1medium:1SPG buffer) undergo subsequent freezing and thawing to release the individual chlamydia from the cells. The harvest was sonicated (1 min) and centrifuged to remove cell fragments (2790 rpm for 10 min). The inoculum was titrated in Vero cell to calculate $TCID_{50}$ with Reed and Muench guidelines (16).

Experimental design

Birds were randomly divided into seven equal groups, 10 birds each, five groups were challenged intranasal with 0.2 ml of 10^6 TCID *C. psittaci* at 7days old (17) while one group received the higher concentration of neem 8% for investigation of possible side effect on liver and kidney function by monitoring (AST, ALT, creatinine, uric acid) and other one left as uninfected not treated control. Four of the infected groups were treated orally with OTC and aqueous neem leaves extract at 4, 6 and 8%

concentration respectively for five successive days started at second day post inoculation.

Samples

Blood and tissues **(**lung – heart - liver) were collected from 2 euthenazed birds from all infected, treated and uninfected not treated control groups at 14 days old, to perform biochemical tests and histopathological examination.

Biochemical tests

Alanine aminotransferase (ALT), aspartate aminotranseferase (AST), albumen, uric acid and creatinine levels in serum were analyzed at Animal Health Research Institute (Zagazig) for detection of liver and kidney function.

Cytological examination

Impression smears from the cut surfaces of the internal organs were fixed in methanol for 5 minutes and dipped in sealed staining jar containing diluted freshly prepared Giemsa stain (1 volume of stock stain + 49 volume of neutral distilled water) and incubated at 37°C overnight. The slides washed by distilled water and alcohol successively then examined microscopically by oil immersion lens for detection of *chlamydia* inclusion bodies (15).

Histopathological evaluation

Collected specimens of lung, liver and heart were fixed in 10% formalin buffered solution, dehydrated in a graded ethanol series, cleared in xylene and finally embedded in paraffin wax. Paraffin sections of 5 µm thickness were stained by hematoxylin and eosin (H&E) and examined microscopically (18).

Performance parameters

Body weight (BW) and feed conversion rate (FCR) for each group were recorded on weekly basis.

Statistical analysis

Data were compiled and analyzed using one-way analysis of variance (ANOVA) with the statistical Package for Social Science version 20.0 (SPSS for windows 20.0 Inc., Chicago, IL, USA) and Duncan multiple range test used to separate means at $P<0.05$ (19).

Results

Clinical and post mortem examination

Signs onset 3 days post infection with depression and anorexia then respiratory signs, rhinitis, sneezing and coughing were developed 10 days post infection with no mortalities. Grossly the scarified birds presented congestion in parenchymatous organs with diffuse thickening of the air sac, fibrinous pericarditis and nephritis. Recorded lesions were milder in birds of treated groups with neem 4%, 6%, 8% and OTC as compared with infected untreated birds.

Performance parameters

Statistical analysis of growth performance results were summarized in two tables. Table (1) clarified that group received neem 8% without infection had significantly ultimate mean BW 177.8±2.70 gm, 1018.60±42.16 gm and 1505.50±59.05 gm than other groups at 8, 24 and 32 days old respectively, whereas the lowest 654.14±26.74 gm and 963.25±33.0 gm noted at 24 and 32 days old respectively, in infected group only. Treated groups with OTC, neem 4%, and 6% exhibited no significant difference in recorded BW 522.5±13.37 gm, 495±9.35 gm and 503.7±12.17 gm, respectively at 16 days old while at 24 days old it was 857.60±25.70 gm, 841.80±14.03 gm and 856.70±17.98 gm in treated groups with OTC, neem 6% and 8%, respectively. At 32 days old control group (1313.73±25.76 gm) had no significant different mean BW than treated groups with neem 8% (1254.60±26.51 gm) whereas significantly different than groups treated with OTC (1198.10±26.19 gm), neem 4% (1130.56±27.19 gm) and neem 6% (1135.70±10.93 gm).

Table 1: Effect of Neem on body weight post *Chlamydia* infection

Age \ group	Control**	8%neem only	Infect+OTC	Infect+4%neem	Infect+6%neem	Infect+8%neem	Infected only
8days	156.8±2.05^{b}	177.8±2.70^{a}	164.6±5.4^{b}	153.9±6.60^{b}	158.7±5.78^{b}	164.6±2.75^{b}	164.4±2.47^{b}
16days	471.9±7.02^{bc}	497.5±5.87^{ab}	522.5±13.37^{a}	495±9.35^{ab}	503.7±12.17^{a}	466.8±6.56^{c}	495.4±10.5^{ab}
24days	806.73±11.87^{bc}	1018.60±42.16^{a}	857.60±25.70^{b}	771.44±16.66^{c}	841.80±14.03^{bc}	856.70±17.98^{b}	654.13±26.74^{d}
32days	1313.73±25.76^{b}	1505.50±59.05^{a}	1198.10±26.19^{cd}	1130.56±27.19^{d}	1135.70±10.93^{d}	1254.60±26.51^{bc}	963.25±33.0^{e}

*Means ± standard error within the same row carrying different superscript were significantly different at P value<0.05, ** Uninfected not treated group

Data shown in Table (2) expressed that groups received neem 8%, control and treated with neem 8% post infection had statistically the same FCR 1.24±0.032^{b}, 1.19±0.025^{b} and 1.16±0.022^{b} respectively, at 16 days old while treated group with neem 6% had significantly higher FCR 1.91±0.077^{a}. Groups infected with *C. psittaci* and treated with OTC, neem 4%, 6% and 8% statistically had parallel FCR 2.04±0.131^{b}, 2.26±0.117^{b}, 1.94±0.102^{bc} and 1.77±0.055^{bc} respectively, at 24 days old, the poorer FCR noted in infected group without treatment 3.80±0.436^{a} while the best recorded in group received neem 8% 1.44±0.091^{c}.

Table 2: Effect of Neem on feed conversion rate post *Chlamydia* infection

Age \ group	Control**	8%neem only	Infect+OTC	Infect+4%neem	Infect+6%neem	Infect+8%neem	Infected only
8days	1.54±0.027^{a}	1.36±0.068^{b}	1.39±0.047^{b}	1.57±0.042^{a}	1.46±0.058 ^{ab}	1.36±0.030^{b}	1.44±0.011^{ab}
16days	1.19±0.025^{b}	1.24±0.032^{b}	1.14±0.032^{bc}	1.05±0.017^{cd}	1.91±0.077^{a}	1.16±0.022^{b}	1.02±0.007^{d}
24days	2.09±0.105^{b}	1.44±0.091^{c}	2.04±0.131^{b}	2.26±0.117^{b}	1.94±0.102^{bc}	1.77±0.055^{bc}	3.80±0.436^{a}
32days	2.09±0.110^{d}	2.47±0.294^{cd}	3.23±0.224^{a}	2.88±0.135^{abc}	3.20±0.108^{a}	2.59±0.090^{bcd}	3.12±0.244^{ab}

*Means ±standard error within the same row carrying different superscript are significantly different at P value<0.05
**Uninfected not treated group

Serum biochemical parameters

Infection with *chlamydia* resulted in optimum levels of ALT, AST, albumin, creatinine and uric acid 68 U/L, 62 U/L, 5.20 mg/dl, 1.62 mg/dl and 5.20 mg/dl respectively, while treated groups with OTC recorded lower levels 22 U/L, 20 U/L, 3.52 mg/dl, 0.52 mg/dl and 2.01 mg/dl respectively, compared with control group 32 U/L, 27 U/L, 3.80 mg/dl, 0.82 mg/dl and 3.40 mg/dl respectively, almost similar to unchallenged chicken which received neem 8% 30 U/L, 26 U/L, 3.20 mg/dl, 0.67 mg/dl and 3.90 mg/dl respectively. Neem treatment efficiently reduced enzyme marker where 4% recorded 20 U/L, 24 U/L, 3.70 mg/dl, 0.40 mg/dl and 3.30 mg/dl respectively, 6% logged 25 U/L, 23 U/L, 3.60 mg/dl, 0.24 mg/dl and 3.20 mg/dl respectively and 8% documented 29 U/L, 23 U/L, 3.40 mg/dl, 0.12 mg/dl and 3.07 mg/dl.

Giemsa stained impression smears

Reflected the presence of elementary bodies within the examined organs mainly lung of challenged birds, which appeared as dense basophilic extracellular bodies (Figure 1A) but not detected in treated groups.

Pathological examination

C. psittaci infection exhibited fibrinous air saculitis and pneumonia with thickened pulmonary septa through intense inflammatory exudate mainly fibrin and numerous leukocytes varied from lymphocyte, plasma cells and heterophiles (Figure 1B and C). Also multiple necrotic areas which margined and infiltrated by numerous leukocytes in the hepatic paren-

chyma (Figure 1D and E) were seen with portal bile duct proliferation or leukocytic aggregations. Intense necrotic myocarditis accompanied with focal hemorrhage and edema was prevalent (Figure 1F).

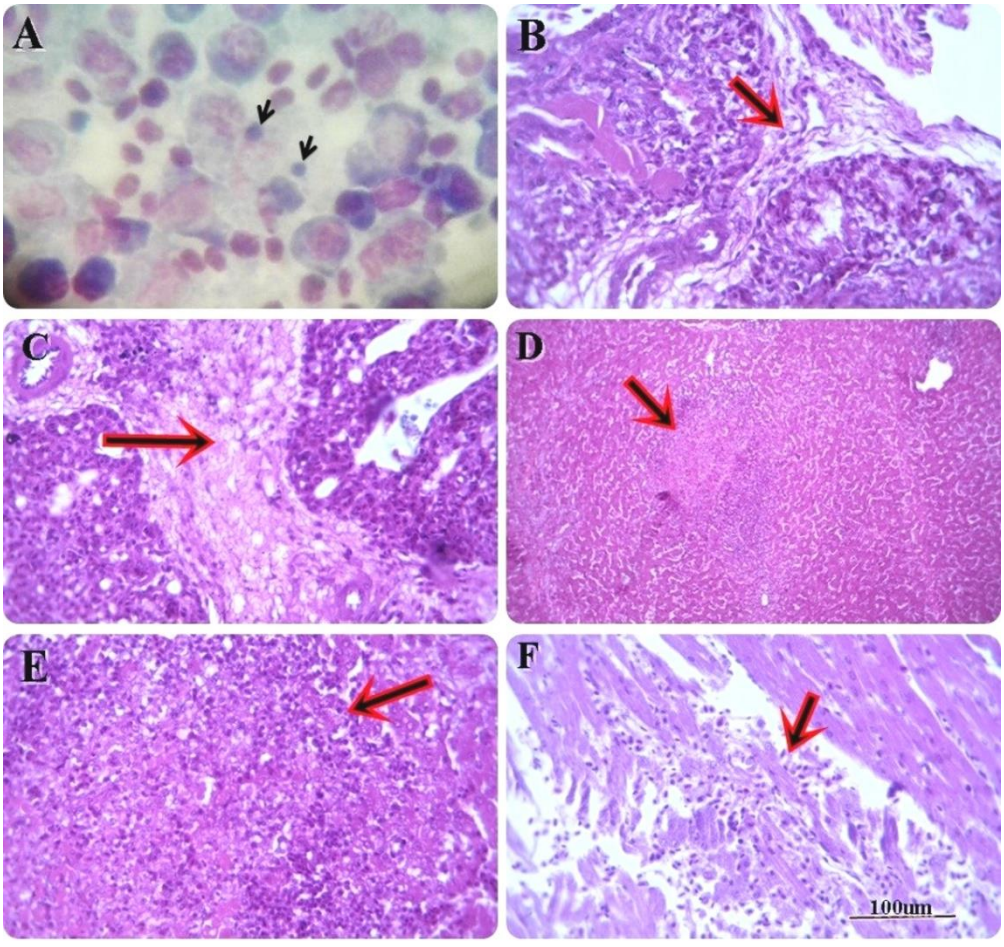

Figure 1: Photomicrograph of infected chickens with *Chlamydia* at 14 days old (A to F). A: Lung impression smear showing elementary bodies stained blue (arrows) Gimesa stain (X1000), B: Fibrinous air saculitis and interstitial pneumonia (arrow) H&E (X100). C: Thickened septa by fibrinous exudate (arrow) H&E (X100). D: Necrotic foci in the hepatic parenchyma (arrow) H&E (X100). E: Lymphocytes, plasma cells and heterophils infiltrate the hepatic necrotic area (arrow) H&E (X100). F: Intense necrotic myocarditis (arrow). H&E (X100)

Treatment with OTC reduced the intensity of the previous lesions in the examined organs, where moderate air saculitis and interstitial pneumonia together with bronchitis and hyperplastic para-bronchial lymphoid aggregation were common (Figure 2A and B). The majority of the hepatic parenchyma was apparently normal meanwhile a few interstitial lymphocytic aggregations could be seen (Figure 2C). Mild myocarditis and edema were encountered (Figure 2D).

Neem 4 or 6 % treatments showed milder lesions of the examined organs meanwhile 8% resulted in complete recovery of lesions

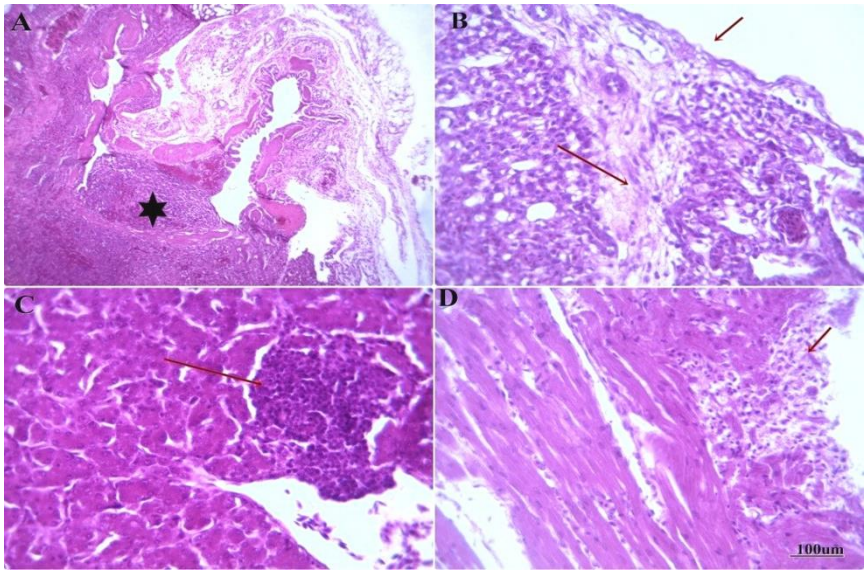

Figure 2: Photomicrograph of challenged chickens with chlamydia and treated with oxytetracycline at 14days old (A to D). A: Bronchitis (star), H&E (X400), B: Moderate air saculitis and interstitial pneumonia (arrow), H&E (X400), C: Focal interstitial lymphocytic aggregation replaced normal hepatic cells (arrow) H&E (X400), D: Focal myocarditis (arrow). H&E (X400)

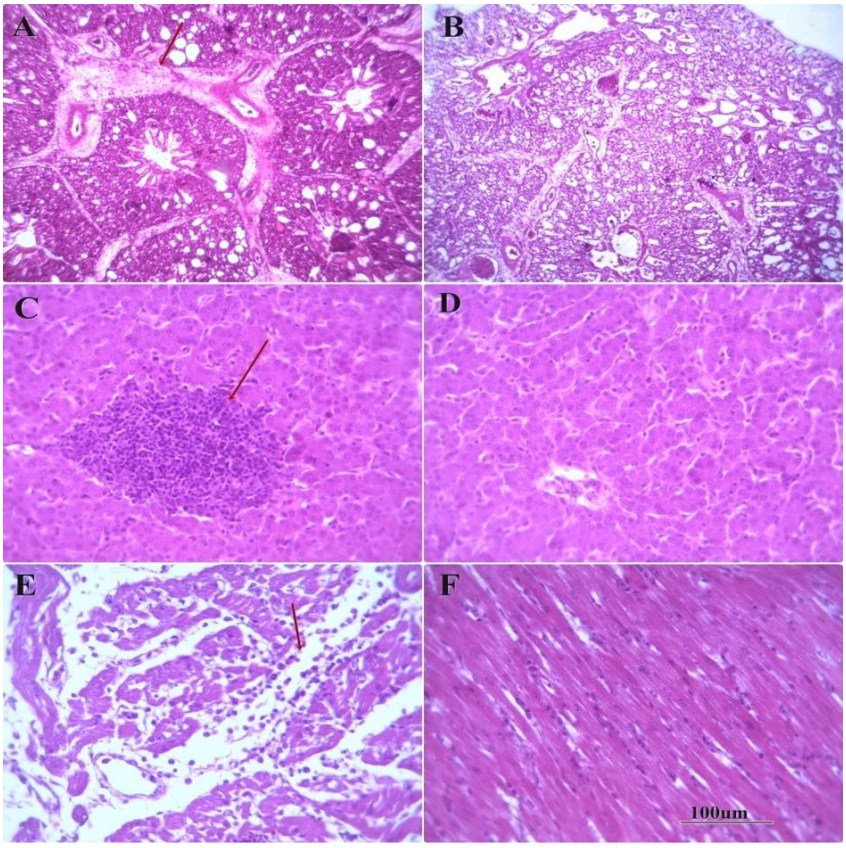

Figure 3: Photomicrograph of treated chickens with different neem concentration post *Chlamydia* challenge at 14days old (A to F), A: Mild interstitial pneumonia (arrow) (6%), H&E (X400). B: Complete recovery of lung tissue with mild congested blood vessels (8%), H&E (X400). C: Focal portal lymphocytic aggregation (arrow) (6%), H&E (X400). D: Apparently hepatic parenchyma with slightly congested hepatic sinusoid (8%). H&E (X400). E: Mild myocarditis and edema (arrow) (6%) H&E (X400). F: Normal myocardium (8%), H&E (X400)

Discussion

Challenged broilers with *C. psittaci* expressed general signs of illness, respiratory symptoms (rhinitis, sneezing, coughing) and greenish diarrhea, meanwhile the recorded gross lesions were septicemia, nephritis and thickening of the air sac which was in accordance with Yin et al, and Zhou et al, (17,20) respectively. Impression smears from liver, lung, heart and spleen that stained with Giemsa elaborate the elementary bodies as explained by Enany et al. (15).

Treatment with aqueous Neem leaves extract showed milder clinical sings and postmortem lesions with no inclusion bodies detection which may be explained by antimicrobial activity of azadirachtin, quercetin and B-sistosterol been in neem leaves with high concentration as reported by Awasthy et al. (21) and Siddiqui et al. (22) who noted that aqueous neem leaves extract suppress many pathogenic bacteria as *staph, salmonella* and *klebsiella*.

Antichlamydial properties of neem was verified where at 16 days old infected groups recorded nearly similar BW after treatment with 6% neem extract and OTC for five successive days furthermore at 24 days old no significant difference of BW for control and treated groups with OTC, neem 6% and neem 8%. At 32 days old, mean BW of the control group were insignificantly different with 8% neem treated group but differently with groups treated with OTC, neem 4% and neem 6%. Parallel to BW, FCR was improved significantly in treated group with OTC and neem extract along the experiment as well as chickens received 8% neem extract without infection. The results were coincided with Chakravarty and Prasad (23) who reported that the highest BW gain and best FCR were achieved in broilers which exposed to neem leave extract when compared with control. The increase of weight gain probably justified by presence of macrominerals as Potassium, Magnesium and phosphorous in addition to microminerals as Iron, copper, Manganese and zinc in *Azadirachta indica* (24), whereas deficiency of these minerals resulted in anorexia and retarded growth (25) or due to diversified effect of Neem therapy on intestinal microflora avoiding stressful conditions (26)

AST also known as serum glutamic oxaloacetic transaminase (SGOT), an enzyme which released in the blood, as a result of injury of certain organs or tissues particularly liver and heart, so its activity varies with age and productive function (27) Hitherto, AST test is more effective than ALT test for detecting liver damage where its level rises faster in hepato-cellular disorders indicating liver damage tumor, kidney or lung damage (28). The presented results showed that the levels of ALT and AST decreased with the higher concentration of aqueous neem leaf extract, proofing no toxic effect of neem within the birds' liver parenchyma and justified by receiving of 8% neem only does not alternate ALT and AST level. It was in agreement with Dkhil et al. (29) who elaborated that hepatoprotective activity of aqueous neem leaves extract lowered ALT, AST. Also serum creatinine and uric acid levels are used mainly as markers of renal function detecting the levels of kidney histological structure damage (30). Over 80% of nitrogen excreated by birds is in the form of uric acid through tubular secretions (31), elevated serum levels of uric acid and creatinine could be considered as indicators of kidney damage (32). Histobiochemical findings highlighted that *C. psittaci* caused high increase of these markers demonstrating severe damage where hyperuricemia associated with injury of tubular endothelial cells. On the other hand, treated groups with neem concentrations showed lower values which indicated by low uric acid and creatinine levels. The hepato-renal protection of neem extract could be due to stabilizing serum level of marker enzymes responsible for this damage or may be due to antioxidant effect of the plant (33). Additionally *Chlamydia* infection revealed fibrinous airsaculitis and pneumonia represented by thickened pulmonary septa with intense inflammatory exudate mainly fibrin and numerous leukocytes varied from lymphocyte, plasma cells and heterophiles clockwise with Martinov and Popov (34) who recorded multiple necrotic areas bordered and infiltrated

by numerous leukocytes allocated in the hepatic parenchyma. Although OTC treatment minimized the intensity of the previous lesions, neem 4 or 6% exhibited milder lesions meanwhile 8% neem resulted in complete recovery of these lesions.

Conclusion

It is concluded that aqueous neem extract has potent herbal anti-bacterial effect against *C. pcittacae* and enhance broiler performance due to posing many minerals that improve digestibility and body weights of broilers. Unlike most of antibiotics it does not affect hepatic or renal tissue; neem could be an efficient substitution of OTC for treatment of *Chlamydia*.

Conflict of interest

The authors declare no conflict of interest.

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Original Research Article

UPREGULATION OF miR-155 IMPAIRS WHITE MATTER SPARING AT THE INJURY AREA FOLLOWING CONTUSIVE SPINAL CORD INJURY IN MICE

Hayam A. Hussein[1], Mohamed Gomaa[1]*, Rochelle J. Deibert[2,3], Lesley C. Fisher[2,3], Stephanie A. Amici[2], Mireia Guerau-de-Arellano[2], Michele D. Basso[2], Ahmed E. Behery[1]

[1]Surgery, Anesthesiology and Radiology Department, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt, [2]School of Health and Rehabilitation Sciences, [3]Center for Brain and Spinal Cord Repair, the Ohio State University, Columbus, OH 43210 USA

*Corresponding author, E-mail: gomaasurgeon@yahoo.com

Abstract: This study was conducted to characterize the effect of miR-155 overexpression on white matter sparing and lesion size following contusion injury of spinal cord in mice. 60 C57BL/6J wild-type and 60 B6.Cg miR-155 knockout mice were used to induce moderate to severe contusive spinal cord injury at T9 segment. All used mice were female, 8–20 weeks old and weighing 18^{+} gm. Mice were divided into two main groups; for Real-time reverse transcriptase polymerase chain reaction (RT-PCR) and histology, then subdivided into five subgroups; 1, 3, 7, 14 and 42 days after the contusion according to time point, each was compared to naive control group. Fresh and fixed tissue were taken from thoracic segments (lesion area) from all time points, dissected and then evaluated using RT-PCR and histology (Eriochrome stain), respectively. We identified significant upregulation of miR-155 at the lesion site by 3 days and continued up to 6 weeks after the injury. Following spinal cord injury, the miR-155 overexpression was accompanied with severe cord damage and less tissue repair while more white matter sparing and smaller lesion size were detected in miR-155 knockout group.

Key words: miR-155; spinal cord injury; sontusion; spicenter; shite matter sparing

Introduction

Traumatic spinal cord injury (SCI) is characterized by primary or acute (seconds to minutes following SCI), secondary (minutes to weeks following SCI), and chronic (months to years following SCI) deterioration phases (1,2). The primary insult to the epicenter area damages spinal cord tissues, ruptures blood vessels, opens the blood-spinal cord barrier (BSCB), and causes edema (3,4). However, secondary events are mainly induced in response to pro-apoptotic signals and severe immune reaction through inflammatory cell trafficking, activation of resident CNS cells, and expression of pro-inflammatory mediators causing neurotoxicity and tissue damage, mainly close to the injury epicenter, and then extends at both directions; rostral and caudal to the injury site. Apoptosis predominately occurs at oligodendroglia, microglia and neurons (5-10).

Micro RNA (miR-155) is a non-coding, short (about 20 nucleotides) and single-stranded RNAs. miR-155 is a negative regulator for specific genes by either impairing

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their translation into protein or inducing mRNA disintegration. A small sequence in the 3′-untranslated region (3′-UTR) of a specific gene is a target area for miR-155 (11-13). miR-155 is involved in the secondary pathogenesis following SCI through controlling inflammation, BSCB permeability, apoptosis and axonal sprouting (14,15). miR-155 plays a major role in axon dieback via downregulating growth-promoting protein and provoking growth inhibitory conditions after SCI. Moreover, genetic ablation of miR-155 enhances axon sprouting and increases neuronal functionality following SCI (14).

Interestingly, miR-155 is one of the highly expressed miRs in various inflammation-related neurological disorders of the brain or spinal cord such as traumatic injury, stroke, multiple sclerosis, amylotrophic lateral sclerosis, encephalomyelitis, and Alzheimer's disease (14,16-20).

miR-155 contributes to the immune effects elicited by blood borne-monocytes, migrated macrophages and microglial cells in CNS. miR-155 mediates hippocampal-associated neuroinflammatory response via microglial activation and IL6 expression. Additionally, microglial miR-155 up-regulation enhances neural stem cells (NSCs) astrogliogenesis (19). On the other hand, miR-155 increases the expression of inflammatory signaling molecules and activates the inflammatory macrophage subtype through upregulating its target genes (e.g. Nos2, Il-1b and Tnfa) and protein in addition to oxidative metabolite production (15,16,18,21-23).

Therefore this study was performed to characterize the effect of miR-155 upregulation on white matter repair after induction of contusive spinal cord injury in mice.

Material and methods

Mice

All animal experiments were conducted in accordance with protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. Two mice strains including 60 C57BL/6J wild-type (WT) and 60 B6.Cg miR-155 knockout (KO) mice were obtained from Jackson Laboratories. These animals were 120 female, 8–20 weeks old and weighing 18^{+} gm. Mice were randomly allocated into two main groups; group I: for PCR and group II: for histology purposes (each group contains 60 mice: 30 from each strain). Each group was subdivided into naïve control subgroup and five subgroups according to time point; 1d, 3d, 7d, 14d and 42d groups (5 mice at each subgroup). Group division was illustrated in Table (1). Mice were housed 3–5 per cage and provided standard food and filtered tap water *ad libitum*.

Table 1: Distribution of 120 mice used in investigation of the effect of miR-155 upregulation on white matter repair after induction of contusive spinal cord injury in mice

Time points	PCR groups		Histology groups	
	Naive control *(n=10)	SCI (n=50)	Naive control (n=10)	SCI (n=50)
1 dpi	5WT, 5miR-155KO mice as control for whole PCR experiments	5WT, 5KO	5WT, 5miR-155KO mice as control for whole histology experiments	5WT, 5KO
3 dpi		5WT, 5KO		5WT, 5KO
7 dpi		5WT, 5KO		5WT, 5KO
14 dpi		5WT, 5KO		5WT, 5KO
42 dpi		5WT, 5KO		5WT, 5KO

*n: total number in each subgroup, SCI: Spinal Cord Injury, WT: Wild-Type, KO: Knockout

Surgical procedures

Spinal cord injury was conducted as previously described by Hansen et al. (9). Mice were generally anesthetized with a ketamine (138 mg/kg body weight) and xylazine (20 mg/kg body weight) cocktail (intraperitoneally-injected) and given prophylactic antibiotics gentamycin (gentocin®, 1 mg/kg, S.C.). Aseptic preparation to the site of injury (at the

dorsal aspect 1-2 cm from the point of shoulder) then putting the animal on stable heating pad (34˚), putting gauze pad under thorax to raise animal and decrease pressure on respiratory muscles during clamping then start surgery under the microscope (Leica MZ8). A ninth thoracic vertebra (T9) laminectomy was performed to expose the dura. After stabilizing the spinous processes of T8 and T10, a moderately severe contusion injury was induced by 75 kilodynes of force using the Infinite Horizon (IH) device. Biomechanics of the injury were monitored on day 0. The incision was closed in standard manner and 2 ml of sterile saline was given subcutaneously. The mice from both genotypes were distributed randomly into two main groups and each group was subdivided into five subgroups.

Postoperative care

During recovery, mice were covered with a warm towel and positioned on a warm heating pad, received antibiotics (1 mg/kg gentocin, S.C.) and subcutaneous injection of saline (2, 2, 1, 1, and 1 ml) for the 5 successive days post injury (dpi). Bladders were manually voided twice daily. They were housed in a temperature controlled room at 37°C for 24h. Food and water were provided *ad libitum*.

Tissue collection in PCR groups

Mice deeply anesthetized and transcardially perfused with 0.1 M phosphate buffered saline (PBS; pH 7.4) at naive, 1d, 3d, 7d, 14d or 42d after SCI. Tissue from injury area was obtained as soon as possible to limit potential degradation of biomarkers in tissue samples. Tissue was placed in Eppendorf tube and then samples were flash frozen into liquid nitrogen. Once frozen, the vial was removed from the liquid nitrogen and stored at –80°C (23).

RNA Isolation

To examine RNA expression at WT samples, cellular RNA was isolated using the miRVana kit (Life Technologies) according to manufacturer specifications. RNA quality/ concentration was quantified using a Nanodrop spectrophotometer (ThermoScientific, Wilmington, DE). Extracted RNA was stored at -80°C until analysis (23).

Real-Time reverse transcriptase PCR

miRNA expression was determined by Taqman Real-Time PCR using miR-155 and sno-202 primer and probe sets (Applied biosystems). TaqMan® Catalog number: 4427975. Assay ID: MI0000177. Gene Symbol: miR-155. Gene sequence: UUAAUGCUAAUUGUGAUAGGGGU. Briefly, after an initial cDNA transcription using specific miRNA primers to generate cDNA using 10 ng RNA as a template, PCR was performed using Taqman universal PCR mix and gene-specific miRNA primers and probe mixture (TaqMan microRNA reverse transcription kit (Applied Biosystems). Reaction mixture was run in an Applied Biosystems 7900 Real-Time PCR machine with denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and primer annealing/extension at 60°C for 60 seconds. miRNA expression was normalized to the small RNA sno202 (23).

Histopathological evaluation

Mice deeply anesthetized and intracardially perfused with 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (pH 7.2) at naïve, 1d, 3d,7d, 14d or 42d after SCI. Spinal cord segments from injury epicenter were post-fixed for 1h in 4% paraformaldehyde, rinsed overnight in 0.2M phosphate buffer (PB, pH 7.4) then cryoprotected in 30% sucrose before being embedded in Optimal Cutting Temperature compound OCT (Thermoscientific) and frozen on dry ice (9).

White Matter Sparing (WMS)

Thoracic tissues were collected from the sites of SCI from naives, 1d, 3d, 7d, 14d and 42d groups and transversely cut 20 um thick on a Microm HM505E cryostat. Using immuno-histochemical procedure, the tissues were stained with eriochrome cyanine (EC) to identify the white matter spared of the lesion epicenter. In addition to EC, a 5% iron aluminum and borax ferricyanide cocktail were

used. Light microscopy measures were used to evaluate the tissue, and tissue images were converted to computer images (MCID-Elite, Imaging Research, Ontario). Using a Cavalerri estimator (In stereology program), a grid was positioned over the tissue at random, and each point within the grid was designated as white matter or grey matter. Per each image area, point totals were combined and calculated to area as follows: estimated area = (ΣP) * (a/p), where ΣP is the total point per section and a/p is the total area per point. Average white matter spared and lesion area was compared between groups to identify differences (9, 24).

Statistical analyses

Group differences for white matter sparing (WMS) were compared using Two-way analysis of variance (ANOVA) with a Tukey's post-hoc analysis. The null hypothesis was rejected at the $p \leq 0.05$ level. All statistical analyses utilized the IBM SPSS program, SPSS Inc. Mean and standard error of the mean (SE) were shown throughout (9).

Results

miR-155 overexpression at the injury epicenter following SCI

Using Taqman RT-PCR, we found significant increase in miR-155 expression at the injured WT epicenter compared to naive WT samples (Relative fold expression=1) starting at 3 dpi and remained upregulated up to 42 dpi following contusion injury. miR155 upregulated at 3 dpi (Relative fold expression=3.4), 7dpi (Relative fold expression=3.8), 14d (Relative fold expression=3.2) and highly overexpressed at 42 dpi (Relative fold expression=4.5) (p=0.001) as shown in Figure (1).

White matter sparing (WMS) is directly affected by miR-155 elevation after SCI

Upregulation of miR-155 after SCI at the lesion area enhances the injury severity and impairs the spinal cord repair after SCI.

White matter sparing at the injured cord, was highly suppressed in WT mice compared to miR-155 KO mice especially at 7dpi (Figure 2). In addition, injury area in WT mice was larger than in miR-155KO mice across the lesion epicenter (Figure 3), indicating that miR-155 deletion enhances tissue repair. Eriochrome stain was used to identify intact myelin in the white matter. Well-differentiated white matter and grey matter were detected at sections of uninjured control. Injured sections from both strains and at all time points were stained with Eriochrome stain to show lesion size and degree of tissue repair following SCI (Figure 4).

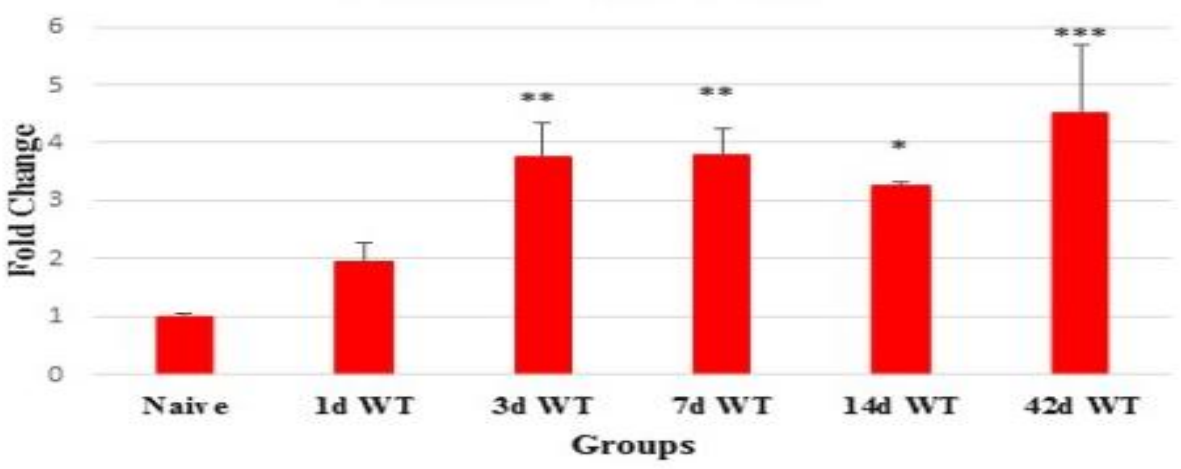

Figure 1: miR-155 upregulation at Injury Epicenter using RT-PCR technique. Contusive spinal cord injury (SCI) upregulates miR-155 at the lesion epicenter of wild-type (WT) mice up to six weeks, compared with WT naive control mice. Means with (*) are significantly different than naïve controls

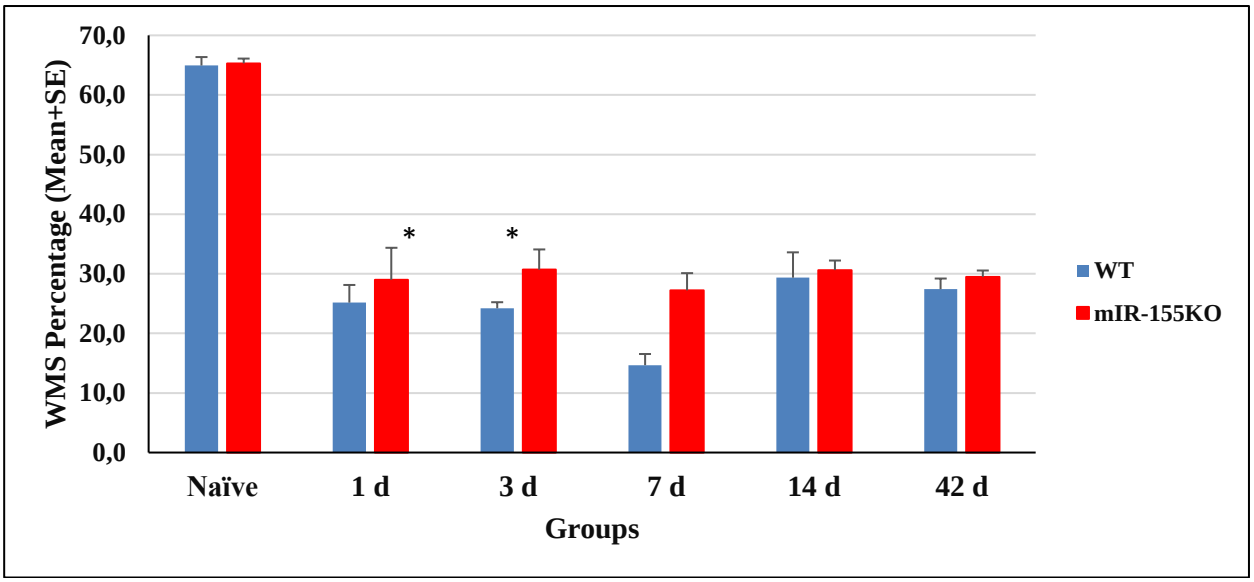

Figure 2: Total WMS Percentage across groups after spinal cord injury (SCI). miR-155 knockout (KO) mice showed more protected white matter than wild-type (WT) control mice at the injury site following SCI. Means of white matter sparing percentage with (*) are significantly different than naïve controls

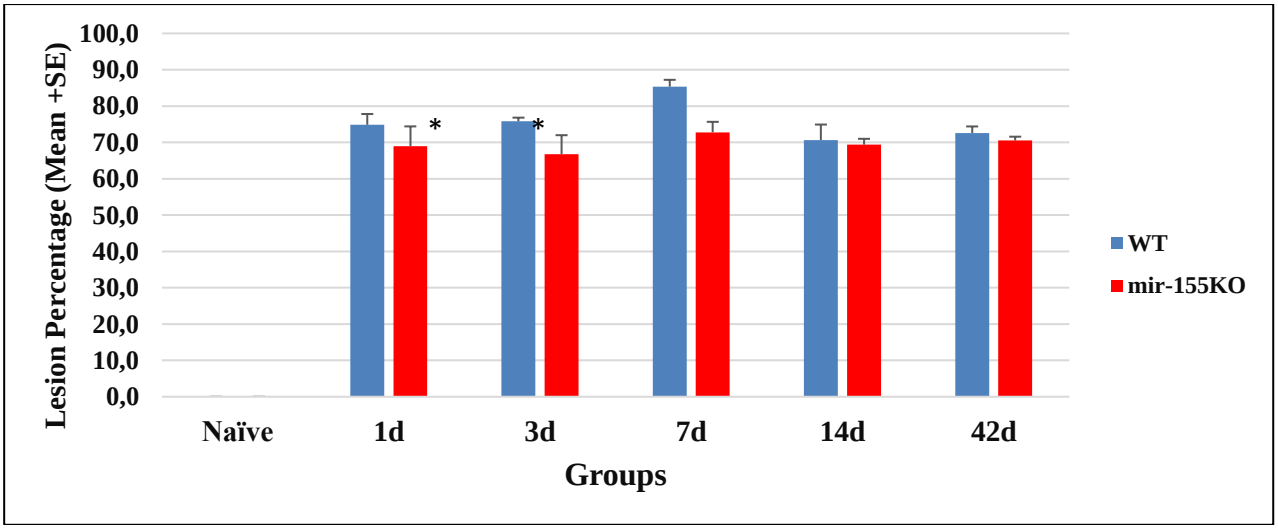

Figure 3: Spinal cord lesion epicenter percentage across groups after spinal cord injury (SCI). WT mice has larger lesion size than miR-155 knockout (KO) mice at the lesion epicenter after SCI. Means with (*) are significantly different than naïve controls

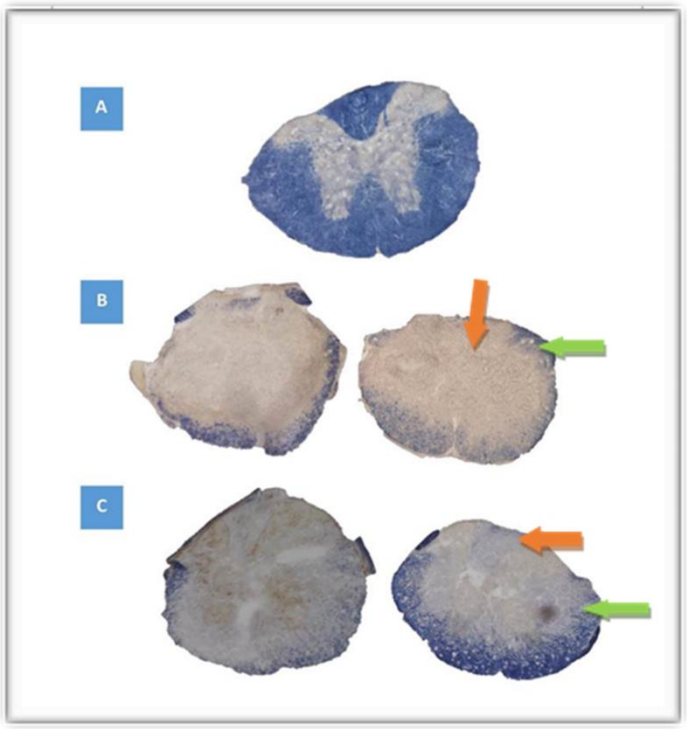

Figure 4: Eriochrome Staining showing the lesion area and degree of tissue repair after spinal cord injury (SCI). Eriochrome was used to identify intact myelin in white matter (bluish colored). A: stained section for naive control mouse showed well-differentiated white and gray matter. B at 3dpi, C at 7dpi: miR-155 knockout (KO) mice (Right side images) had significantly smaller lesions (orange arrow) and more preserved white matter (green arrow) than wild-type (WT) lesions (Left side images) with notable changes evident at injury epicenter. Colored outlines represent lesion size in every animal examined for each genotype at epicenter

Discussion

At the present study, significant overexpression of miR-155 was detected at the injury epicenter starting at 3 dpi and continued high up to 6 weeks following spinal contusion injury. This finding is in agreement with Gaudet et al. (14). miR-155 is markedly produced by microglia (19,22) and macrophages (14). Moreover, inflammatory molecules produced by immune cells stimulate miR-155 overexpression (25,26). Elevated miR-155 upregulates pro-inflammatory signaling while downregulates anti-inflammatory molecules (27,28), which results in activation of inflammatory macrophages. In the CNS, miR-155 is implicated in the pathogenesis of SCI, stroke, multiple sclerosis, amylotrophic lateral sclerosis, encephalomyelitis and Alzheimer's disease (14-17,29,30).

We revealed a novel role for miR-155 in SCI repair and pathology. miR-155 KO spinal cords had reduced lesion size and improved white matter sparing. Prominent neuronal loss is marked by 8 hr post injury in rat SCI, beginning at the gray matter and then extend to the white matter. While apoptosis of glial cells is most distinct by 24 hr post injury, and then peaked again by 7 dpi particularly in the white matter (5). Inflammatory cascade induced post-injury is implicated in the tissue deterioration and loss of spinal cord functionality. Activated macrophages /microglia are primary contributors to secondary pathogenesis after SCI through expression of toxic molecules such as TNF-α, IL-1β, IL-6, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, inducible nitric oxide synthase (iNOS), nitric oxide (NO), reactive oxygen species (ROS) and matrix metalloproteinases (MMPs) which initiate neuroinflammatory reactions at the injury site and induce tissue damage through activation of

astrocytes, loss of oligodendrocytes and neurons, in addition to demyelination (31-33). Caspases, specifically caspase-3, 8, 9 and 10, are good indicators for onset of apoptosis (7, 34-37). Apoptosis can be partially restricted via miR-155-induced suppression of caspase-3 expression in activated macrophages (38). Therefore, the continued upregulation of miR-155 at the lesion area could cause more tissue pathology through promoting the toxic microenvironment.

Conclusion

It could be concluded that miR-155 deletion improved tissue repair by suppressing neuroinflammation and decreasing the secondary damage after the contusion injury.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

HEAVY METAL CONCENTRATIONS AND THEIR RISK ASSESSMENT IN MARKETED SLAUGHTERED ANIMALS IN SHARKIA GOVERNORATE, EGYPT

Alaa Eldin M.A. Morshdy, Rasha M. El Bayomi*, Ghada M. Abd El Galil, Abdallah F.A. Mahmoud

Department of Food Control, Faculty of Veterinary Medicine, Zagazig University, El-Zeraah str. 114, 44519-Zagazig, Egypt

*Corresponding author, E-mail: rmazab_2010@yahoo.com

Abstract: Toxic metals (lead, cadmium and mercury) and essential trace elements (copper and zinc) were analyzed in a total number of 120 samples of muscle, kidney and liver collected from camel, cattle, buffalo and sheep using atomic absorption spectrophotometer after wet digestion. The concentrations of heavy metals ranged from 0.17 ± 0.6 to 0.49 ± 0.09, 0.03 ± 0.01 to 0.12 ± 0.03, 0.39 ± 0.1 to 1.19 ± 0.18, 0.10 ± 0.04 to 8.82±1.01 and 3.25 ± 0.16 to 8.35± 1.33 mg/kg for lead, cadmium, mercury, copper and zinc, respectively. In general, the liver samples had the highest level of lead and mercury, while kidney samples showed the highest content of cadmium as compared with muscle samples. Cadmium (Cd), copper (Cu) and zinc (Zn) levels in all of samples were less than the Egyptian standard limits. The estimation of human health risk for adults revealed an estimated daily intake (EDI) value of muscle and offal below threshold of oral reference dose (RFD) for all metals analyzed. Hazard index (HI) and Hazard quotient (THQ) for all the analyzed metals were below 1, demonstrating that human health risk through consumption of meat and offal is not possible.

Key words: heavy metals; hlaughter animals; EDI; THQ; HI

Introduction

Heavy metals toxicity is highly persistent and widespread environmental health problems owing to the stability and non-degradable properties of these elements (1). They can enter the food chain through natural environmental constituents or human anthropogenic activities for example, an extensive fertilizers and pesticides application in agriculture, fossil fuels burning, waste disposal, atmospheric deposition, and discharge from manufacturing industries (2). These metals can be bioaccu-mulated in the environment and biomagnified in the food and they might reach toxic levels when found even at low concentrations (3). The environment and food contamination are common and constitute the main pathways of heavy metals (4).

In Egypt, camel, cattle, buffalo, and sheep are domesticated animals and live under the same environment with human. These animals have the ability to accumulate various environmental pollutants in their muscle and edible tissues when exposed to such toxic substances (5). Moreover, the edible animal offal (liver and kidneys) have high economic importance and

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gastronomic value although they account for small portion of human diet and very little consumption rate as compared to meat (6). Accumulation of these heavy metals in meat constitute a potential health hazard because they are toxic at low concentrations (7). Lead, cadmium and mercury have no physiological functions in human body and are associated with organs dysfunctions, nervous system and bone diseases (8). Trace essential metals such as zinc and copper are necessary for living organisms at low concentration to maintain normal development and maintenance of human physiology, but the high-level exposure to these element causes human health risks (9).

Monitoring of heavy metal levels in the food by estimation of the heavy metals potential risks is needed. Risk assessment is a method required for human health hazard estimation, in general this method is depend on the target hazard quotients (THQ), a ratio between the contaminant estimated dose and the oral reference dose (RFD) as there is no significant risk below it (10) If the THQ is <1, the meat has no human health risk (11). Therefore, the current study investigated the heavy metals concentrations (Pb, Cd, Hg, Zn and Cu) in muscle and offal of camel, cattle, buffalo and sheep by using of the Atomic Absorption Spectrophotometer and assessed the human risk of these metals.

Material and methods

Sampling

A total number of 120 muscle, kidney and liver samples of camel, cattle, buffalo and sheep (10 of each) were randomly collected from Zagazig city, Sharkia, Egypt. The collected muscle and offal samples were packed in a cold polyethylene bags and then transferred to the Central Laboratory, Faculty of Veterinary Medicine, Zagazig University for the heavy metals analysis.

Heavy metals analysis:

From each sample, one gram was digested overnight in a digestion tube containing 3 ml of 65 % nitric acid (supra-pure, Merk Darmastadt, Germany) and 2 ml of 70 % perchloric acid (extra-pure-Merk, D-6100 Darmastadt, Germany) (12, 13[l]). These tubes were incubated in water bath for 3 h at 70°C with vigorously shaken every 30 minute. Then the tubes were left to cool at room temperature and diluted with 20 ml of de-ionized water and then filtered with Whatman filter paper no. 42. The filtrates were collected in polyethylene films capped glass tubes and kept at room temperature until metals analysis is performed.

Blank solution was prepared from 2 ml of perchloric acid (70 %), 10 ml of nitric acid (65 %) and the standard solutions of Pb, Cd, Hg, Cu and Zn were prepared using pure specialized atomic absorption spectrophotometer standard of these metals. Both blank and standard solutions were treated by the wet digestion method and they diluted using 20 ml de-ionized water. The digested blank and standard solutions were analyzed for heavy metal content using Buck scientific 210VGPAtomic Absorption Spectrophotometer (AAS) (Per Kin Elmer model (spectra-AA10, USA) at the Central Laboratory in Faculty of Veterinary Medicine, Zagazig University, Egypt. The analysis procedure was performed by air/acetylene flow (5.5/1.11/m) flame in case of Pb, Cd, Cu and Zn, while cold vapor atomic absorption spectroscopy technique was used for Hg determination (14). The limits of detection (LOD) were 0.01, 0.005, 0.005, 0.02, 0.02 μg/g for Pb, Cd, Hg, Cu and Zn respectively. The concentrations of Pb, Cd, Hg, Cu and Zn were expressed as μg/g wet weight (ww) which equivalent to mg/kg. Residual metal concentrations in the examined samples were compared with the maximum permissible limits of metals set by Egyptian organization for Standardization (15) and other international standards.

Assessment of human health risk

Risk assessment was determined depend on the metals concentrations detected in the meat samples by means of United States Environment Protection Agency (16) for human health risk assessment. The ingested dose of the contaminant is the same to the adsorbed dose and the cooking not affect the heavy metals toxicity (17). Health risk assessment of heavy

metals level in the examined samples of camel, cattle, buffalo and sheep meat were calculated using the following points: Estimated Daily Intake (EDI), Hazard Quotient (THQ) and Hazards index (HI).

Estimated daily intake (EDI)

The heavy metals estimated daily intake (EDI) was dependent on the heavy metals level in muscle and offal and the consumption rate. The metals EDI was calculated using the following equation: EDI=(Cm x FIR)/BW (11), as Cm = concentration of the heavy metal in the sample (mg/kg wet weight); FIR = bovine and camel meat ingestion rate (35.287 g/day), sheep meat ingestion rate (4.329 g/day) (18) and offal ingestion rate (0.1 g /day) (6,19) BW is the Egyptian adults body weight = 70 kg. Then compared to the metals tolerable daily intakes (TDIs) (20).

Target Hazard Quotient (THQ)

THQ was used to evaluate health risk accompanied with the non-carcinogenic and carcinogenic effect of any toxic metals. Muscle and offal consumption risks of camel, cattle, buffalo and sheep were evaluated according to the THQ. The THQ is a relation between the determined pollutant dose and the level of RFD. The reference dose is the daily exposure of a contaminant estimated which the population exposed along a lifetime continually with no significant hazard (21). If this relation is <1, the population has improbable no noticeable bad effects. The method of estimating THQ was provided. The risk assessment was determined by using the following equation (22): $THQ = \frac{EF \times ED \times FIR \times C}{RFD \times BW \times AT} \times 10^{-3}$, as EF is the frequency of exposure (365 days/year), ED is the duration of exposure (70 years, average duration), FIR is the ingestion rate of meat (g/day), C is the heavy metal concentration in meat (mg/kg); BW is the adult average body weight (70 kg), RFD is the oral reference dose (mg/kg/day) and AT is the exposure time average (365 days/ year × exposure years number, assume 70 years). The oral reference dose value for Pb, Cd, Hg, Zn and Cu is 0.004, 0.001, 0.0016, 0.3 and 0.04 (mg/kg bw/day), respectively (11).

Hazard index (HI)

To assess the potential human health risk among all heavy metal, the hazard index (HI) has been established by the summation of the THQ for all heavy metals in the subsequent equation. HI = Σ HQ = HQ Pb + HQ Cd + HQ Hg + HQ Zn + HQ Cu Where Σ HQ is the summation of hazard quotients of all metals. When HI increases than 1, it poses an alarm for human health concerns (23).

Statistical analysis

The results were planned by using the SPSS computer program, Inc. version 22 (2012). The obtained results were expressed as the mean ± standard deviation (SD). All the statistical analyses were done at the significance level of 0.05 ($P<0.05$).

Results

Mean and standard deviation (Minimum-Maximum) of heavy metals concentrations in the various muscles and organs of camel, cattle, buffalo and sheep analyzed in this study are shown in Table 1. The muscle and organs of the same species have different metal concentration. In the main, muscle and organs were relatively rich in Zn followed by Cu, which are micronutrients followed by levels of Pb, Cd, and Hg which have no biochemical functions and are toxic at minute concentration. Of all detectable toxic metals, Pb in muscle samples and Hg in all muscle and offal were higher than the permissible limit of the Egyptian standards. The estimated daily intake of Pb, Cd, Hg, Zn and Cu in this study ranged from 0.0002 to 0.5646, 0.0001 to 0.2016, 0.0006 to 0.7057, 0.0002 to 0.2319 and 0.0054 to 4.2092 μg/kg BW/day, respectively (Table 2).

THQ for Pb, Cd, Hg, Zn and Cu ranged from 0.00006 to 0.14115, 0.00004 to 0.20164, 0.00035 to 0.44109, 0.00002 to 0.01403 and 0.000057 to 0.057972, respectively (Table 3). The probable health risk of the determined metals was also tested. The HI ranged from 0.0007 in camel kidney to 0.7065 in buffalo muscle and HI<1 means no hazard (Table 3).

Table 1: Statistical analytical results of f lead (Pb), cadmium (Cd), mercury (Hg), copper (Cu) and zinc (Zn) concentrations (mg/Kg wet weight) in the examined samples (No= 10 of each)

Organ	Animal	Lead	Cadmium	Mercury	Copper	Zinc
Muscle	Camel	0.322 ± 0.1^{ab} (0.29-0.65)	0.05 ± 0.02^{a} (0.04-0.09)	0.5± 0.09^{b} (0.63-0.74)	0.14 ± 0.04^{a} (0.02-0.28)	8.35± 1.33^{a} (6.1-12.9)
	Cattle	0.19± 0.02^{b} (0.12-0.23)	0.03 ± 0.02^{a} (0.01-0.09)	1.17 ± 0.28^{a} (0.55-1.88)	0.46 ± 0.17^{a} 0.02-0.28	5.91± 0.34^{ab} (4.87-6.48)
	Buffalo	0.17 ± 0.06^{b} (0.01-0. 3)	0.04± 0.016^{a} (0.01-0.09)	1.4± 0.2^{a} (0.65-1.84)	0.26 ± 0.07^{a} (0.11-0.49)	5.73 ± 0.32^{b} (4.54-6.41)
	Sheep	0.49± 0.11^{a} (0.2-0.66)	0.05± 0.02^{a} (0.001-0.08)	0.81 ± 0.21^{ab} (0.37-1.35)	0.10 ± 0.04^{a} (0.07-0.18)	7.02± 0.69^{ab} (5.26-8.37)
Kidney	Camel	0.31 ± 0.07^{a} (0.13-0.52)	0.12 ± 0.03^{a} (0.01-0.21)	0.39 ± 0.1^{b} (0.02-0.61)	0.16 ± 0.07^{b} (0.03-0.43)	3.8± 0.2^{b} (3.15-4.25)
	Cattle	0.32 ± 0.04^{a} (0.24-0.46)	0.06 ± 0.01^{a} (0.03-0.08)	1.18 ± 0.32^{ab} (0.35-1.98)	1.21 ± 1.10^{a} (0.04-5.62)	3.85 ± 0.08^{b} (3.69-4.16)
	Buffalo	0.17 ± 0.04^{a} (0.09-0. 3)	0.07± 0.01^{a} (0.05-0.1)	1.31 ± 0.25^{a} (0.47-1.83)	0.28 ± 0.06^{b} (0.12-0.40)	3.25 ± 0.16^{b} (2.77-3.6)
	Sheep	0.23 ± 0.08^{a} (0. 1-0.36)	0.07± 0.02^{a} (0.02-0.1)	1.01 ± 0.35^{ab} (0.32-1.42)	0.36 ±0.07^{b} (0.14-0.44)	4.54 ± 0.33^{a} (4.02-5.5)
Liver	Camel	0.40 ± 0.12^{a} (0.08-0.79)	0.11± 0.04^{a} (0.04-0.21)	0.62 ± 0.19^{b} (0.31-1.2)	2.10±0.91^{b} (0.64-5.59)	6.16± 0.67^{a} (4.57-8.51)
	Cattle	0.38 ± 0.08^{a} (0.18-0.55)	0.03 ± 0.01^{a} (0.01-0.07)	1.15 ± 0.27^{a} (0.15-1.76)	2.75 ± 1.42^{b} (0.15-7.91)	5.19 ± 0.38^{ab} (3.82-6.15)
	Buffalo	0.19 ± 0.05^{a} (0.09-0.36)	0.06± 0.02^{a} (0.02-0.11)	1.3 ± 0.18^{a} (0.76-1.77)	2.39 ± 0.67^{b} (1.00-4.64)	4.29 ± 0.24^{b} (3.64-4.92)
	Sheep	0.39 ± 0.09^{a} (0.15-0.6)	0.06± 0.03^{a} (0.02-0.11)	1.19 ± 0.18^{a} (0.84-1.61)	8.82±1.01^{a} (7.09-10.59)	5.36 ± 0.48^{ab} (4.81-6.31)

Mean of the same column for the same tissue carry different superscripted letter are significant different (P<0.05).

Table 2: Estimated daily intake (EDI) µg/ kg body weight of different metals in comparison to the Tolerable daily intake (TDIs) µg/ kg body weight

Animal	Samples	Pb	Cd	Hg	Cu	Zn
Camel	EDI (Muscle)	0.5646	0.0252	0.2521	0.0706	4.2092
	EDI (Kidney)	0.0004	0.0002	0.0006	0.0002	0.0054
	EDI (Liver)	0.0006	0.0002	0.0009	0.0030	0.0088
Cow	EDI (Muscle)	0.0958	0.0151	0.5898	0.2319	2.9792
	EDI (Kidney)	0.0005	0.0001	0.0017	0.0017	0.0055
	EDI (Liver)	0.0005	0.0004	0.0016	0.0039	0.0074
Buffalo	EDI (Muscle)	0.0857	0.2016	0.7057	0.1311	2.8885
	EDI (Kidney)	0.0002	0.0001	0.0019	0.0004	0.0046
	EDI (Liver)	0.0003	0.0001	0.0019	0.0034	0.0061
Sheep	EDI (Muscle)	0.0303	0.0031	0.0501	0.0062	0.4341
	EDI (Kidney)	0.0003	0.0001	0.0014	0.0034	0.0065
	EDI (Liver)	0.0006	0.0001	0.0017	0.0088	0.0077
TDIs		**3.57**	**1.0**	**0.71**	**500**	**300 –1000**

EDI=(Cm x FIR)/BW, as Cm = concentration of the heavy metal in the sample (mg/kg wet weight); FIR = bovine and camel meat ingestion rate (35.287 g/day), sheep meat ingestion rate (4.329 g/day) (18) and offal ingestion rate (0.1 g /day) (19, 6); BW is the Egyptian adults body weight = 70 kg. Then compared to the metals tolerable daily intakes (TDIs) (20).

Table 3: Target hazard quotient (THQ) and Hazard index (HI) of different metals from consumption of the examined samples

Animal	Samples	Pb	Cd	Hg	Zn	Cu	HI
Camel	Muscle	0.14115	0.02521	0.15753	0.01403	0.017644	0.3556
	Kidney	0.00011	0.00017	0.00035	0.00002	0.000057	0.0007
	Liver	0.00014	0.00016	0.00055	0.00003	0.000750	0.0016
Cow	Muscle	0.02394	0.01512	0.36862	0.00993	0.057972	0.4756
	Kidney	0.00011	0.00009	0.00105	0.00002	0.000432	0.0017
	Liver	0.00014	0.00004	0.00103	0.00002	0.000982	0.0022
Buffalo	Muscle	0.02142	0.20164	0.44109	0.00963	0.032767	0.7065
	Kidney	0.00006	0.00010	0.00117	0.00002	0.000100	0.0015
	Liver	0.00007	0.00009	0.00116	0.00002	0.000854	0.0022
Sheep	Muscle	0.00758	0.00309	0.03131	0.00145	0.001546	0.0450
	Kidney	0.00008	0.00009	0.00090	0.00002	0.000843	0.0019
	Liver	0.00014	0.00010	0.00106	0.00003	0.002196	0.0035

THQ values was determined using the following equation: $THQ = \frac{\text{EF x ED x FIR x C}}{\text{RFD x BW x AT}} x10^{-3}$(22). THQ is the target hazard quotient. EF is exposure frequency (365 days/year). ED is the exposure duration (70 years, average lifetime). FIR is the food ingestion rate (g/day). C is the heavy metal concentration in meat (µg/g). RFD is the oral reference dose (mg/kg/ day). BW is the average adult body weight (70 kg) and AT is the averaging exposure time (365 days/ year × number of exposure years, assuming 70 years). HI = Σ HQ = HQ Pb + HQ Cd + HQ Hg + HQ Zn + HQ Cu

Discussion

Concentration of metals in offal and muscles

In general, the liver samples had the highest level of Pb and Hg, while kidney samples showed the highest content of Cd as well as, muscle samples exhibited higher levels of Cu and Zn in all investigated animal. The metal concentration in the organs and muscle followed the order: liver > kidneys > muscle. Liver and kidneys are the target tissues for surveillance metal contamination in animals body since the main function of these organs is eliminating the toxic metals from body and so the metals accumulating in them (24).

Mean Pb concentrations as observed in the sheep muscle showed the highest mean concentration of 0.49 ± 0.09 mg/kg and the lowest concentration of 0.17 ± 0.6 mg/kg were recorded in buffalo kidney. In Egypt, the mean lead level in beef was lower than our study (0.008, 0.109 and 0.042 mg/kg) in muscle, kidney and liver samples, respectively (25). Lower Pb concentrations for bovine, buffalo and sheep muscle, of 0.061±0.03, 0.052±0.02 and 0.010±0.01 mg/kg from Egyptian rural area and 0.093±0.04, 0.081±0.03 and 0.081±0.03 mg/kg from Egyptian industrial areas were reported by Abou-Arab (24). In the same line, Alkmim Filho (26) reported Pb concentrations in Brazilian cattle kidney and liver were 0.226 mg/kg and 0.231 mg/kg, respectively. In addition, Pb concentration of 0.221±0.022, 0.244±0.029 and 0.273±0.034 mg/kg was detected respectively in bovine muscle, kidney and liver from Iran (27). Lead concentrations in the current study appear to be similar to that recorded in cattle liver (0.31±0.04) and in sheep liver from Pakistan (0.22±0.08 mg/kg) (28). However, higher Pb concentration of 2.62±0.08 and 5.48±0.01 mg/kg was recorded in sheep and camel muscle, respectively in Saudi Arabia (29), 0.534 mg/kg in cattle kidney from Iran (30), 21.1±3.30 and 17.05±5.17 mg/kg in cattle and sheep liver, respectively in Iran (31). Muscle, kidney and liver of sheep from control sites in China are obviously higher than those reported in the present study (0.86±0.41, 0.72±0.23 and 0.96±0.40 mg/kg, respectively) (32). The environmental contamination differences are the cause of this variation (27). Lead is one of the most toxic heavy metals. This study indicated that Pb concentrations were above the recommended limits of 0.1 mg/kg for muscle and below 0.5 mg/kg for offal set by Egyptian Organization for Standardization (EOS) (15), European Commission Regulation (ECR) (33) and United States Department of Agriculture (USDA) (34).

The highest Cd concentration (0.12 ± 0.03) was recorded in the kidney of camel while the

lowest value (0.03 ± 0.01) was observed in muscle samples of the cattle. However, Cd concentration in bovine muscle was similar to (0.031±0.02 mg/kg) the concentrations in Egypt (24) and (0.03 mg/kg) South Africa (35). Higher Cd levels for muscle samples of 0.14 mg/kg in beef were reported in Nigeria (36), 1.25±0.02 in sheep and 1.07±0.02 mg/kg in camel from Saudi Arabia, respectively (29). Moreover, high Cd concentrations were reported in liver samples (0.14±0.02 and 0.21±0.4, respectively) and kidney samples (0.93±0.13 and 1.93±0.41 mg/kg, respectively) for cattle and sheep from Iran (31). Lower Cd levels of 0.005 mg/kg in beef muscle in Switzerland (37), 0.028±0.004, 0.114±0.025 and 0.047±0.010 mg/kg in bovine muscle, kidney and liver, respectively in Iran (27) and 0.006, 0.009 mg/kg in sheep kidney and liver, respectively in Kenya (38) were reported. In Egypt, the mean residual levels of cadmium in beef was lower than our study (0.0014, 0.014 and 0.062 mg/kg) in muscle, liver and kidney samples, respectively (25). Meanwhile, Abou-Arab (24) reported that Cd concentrations of animal organs (bovine and buffalo and sheep), respectively collected from Egyptian rural area were 0.010±0.01,0.006±0.004 and 0.011±0.01 for muscle, 0.220±0.11, 0.166±0.11 and 0.880±0.30 for kidney, 0.112±0.07, 0.080±0.04 and 0.082±0.06 mg/kg for liver samples. Generally, in the current study cadmium levels in kidney samples were relatively higher as compared to that found in muscle and liver samples. Camel kidneys had the highest Cd level than other animals as this animal had longer lifetime exposure. The high concentration of Cd in the kidney may be contributed to the excretory function of this organ, as toxic substances excreted from body through the kidney (27). In addition, animals insecure to Cd accumulation in their kidneys because of free protein-thiol groups' occurrence, which strongly join the heavy metals to their construction (39). Cadmium concentrations in the present study were within the permissible limit of 1.0 mg/kg for offal and 0.05 mg/kg for muscle samples (15) and was below the maximum permissible limit of 1.0 mg/kg (40).

Mercury was detected at concentrations ranging between 0.39±0.1 mg/kg in the camel kidney to 1.19±0.18 mg/kg in the liver of sheep. Lower concentrations of (0.00391, 0.0104 mg/kg and 0.00581) were recorded in muscle, kidney and liver of beef, respectively (25) in Egypt. Meanwhile, the concentrations of 0.023±0.006 and 0.039±0.002 mg/kg were observed in sheep and camel muscle from Saudi Arabia (29), and 0.07±0.03, and 0.03±0.03l in kidney and liver of sheep from Ghana (39). However, lower Hg concentrations (0.003, 0.002, 0.003 mg/kg) were reported for cattle muscle, liver and kidney, respectively in Iran (27). The accumulation of Hg was under 0.2 mg/kg in both liver and kidney of cattle (41) in Zambia and 0.00153 ± 0.00005 mg/ kg in liver of veal (42) in Spain. The mean concentration of Hg in bovine kidneys was 0.008 mg/kg in Ireland (43), which was lower than our study.

In the present study the Hg concentrations in muscles and offal were higher than the Egyptian permissible limit (0.2 and 0.5 mg/kg, respectively) (15) and higher than 0.05 mg/kg established by ECR (33) and USDA (34). The high levels of Hg especially in the offal of slaughtered animals could be problematic, as concentration levels exceeded the maximum values permitted in food. The high levels of Hg reflect the increased use of mercury in agricultural and industrial activities in the studied area. Animals' herds are usually seen grazing in and around these contaminated areas. They have the ability to accumulate some residues of these toxic metals. Therefore, the high levels of Hg in meat and offal of slaughtered animals could be due to contamination from soil, feed or water.

The highest Cu value was observed in sheep liver (8.82±1.01 mg/kg), and the lowest concentration (0.10±0.04 mg/kg) was recorded in the muscle of sheep since the liver serves as the main organ for Cu storage and homeostasis maintains in the body (4). In Egypt, Abou-Arab (24) reported higher Cu concentration in bovine, buffalo and sheep samples (muscle, kidney and liver). Higher Cu also found in muscle of sheep (4.60±0.21) and (1.33±0.07 mg/kg) in camel (29). Meanwhile, lower Cu concentration (0.498 mg/kg) in beef (37). The

concentration of Cu in muscle is below their relevant concentrations in liver and kidney, which is coincided with Waegeneers et al. (44). The current study declared lower Cu concentrations than the Egyptian permissible limits of 15 mg/kg (15) and 20 mg/kg set by USDA (34) and ECR (33).

Mean Zn concentrations in all muscle and organs ranged between 3.25±0.16 and 8.35±1.33 mg/kg in buffalo kidney and camel muscle, respectively. Zinc concentrations in kidney and liver samples in the current study were lower than that found in muscle samples and this was in accordance with Abou-Arab (24). The concentration of Zn in this study was less than 17.55±1.097, 8.423±0.620 and 17.04±0.727 mg/kg for muscle, kidney and liver of bovine from Iran (27), 33.85±1.24 and 16.74±0.73 mg/kg for sheep and camel muscle from Saudi Arabia (29). Higher Zn also recorded in kidney (74.91±9.07) and in liver (77.34±13.09) of sheep form Ghana (39), as well as, higher Zn concentration was reported in liver (104.07 ± 5.34 and 105.19 ± 5.11) and kidney (87.94±6.44 and 102.05±5.05 mg/kg) of cattle and sheep, respectively from Iran (31). This difference may be attributed to the difference in the analytical method in other studies, the variations of environmental pollutants type and physiological conditions (27). Copper and zinc concentration in this study were below those reported in Egypt and Belgium (24, 44).

The current study proved that Cd is mostly accumulated in the kidney, Zn in muscle and Cu in the liver that was in agreement with Sedki et al., (45) in Morocco. Zinc levels in this study were below the FAO/WHO (40) permissible limit (50 mg/kg) for edible offal and muscle and below 50 mg/kg for muscle and 80 mg/kg for offal maximum permissible limit of Codex Alimentarium Commission (CAC) (46). The Cd concentrations was uniform in the all tissue of all animals groups in which they were detected and display no significant variation ($p>0.05$). Significant variation was observed for Pb and Zn in samples examined of the four animal groups analyzed ($p<0.05$). Liver of camel was significantly different from liver of cattle, buffalo and sheep in the mean Hg concentrations ($p\leq0.05$). Muscles of different animal species showed no significant ($p>0.05$) for Cu but other offal was significantly different on Cu level ($p<0.05$).

Human health risk assessment

Meat considered as the major sources of vitamins, animal protein, fatty acids and essential trace elements but meat and offal edible for consumption are necessary source for metals exposure (4). The toxicity of heavy metal depends on the daily intake of these metals. The EDI values for non-essential heavy metals Pb, Cd and Hg for adult of 70 kg BW prove that the EDI values obtained were less than the human oral reference dose. These results point to there was no possible human health hazard associated with meat consumed in our city.

The oral reference dose is the daily exposure estimation to the people, which is with no harmful risk through long-term exposure. If the intake is below the RFD (THQ<0.1), indicates no opportunity of any bad risk. The current study results are parallel to those found in other studies on sheep muscle and offal (47) as they found low daily intakes of Pb, Cd and Hg when compared with TDI. However, the EDI was lower than the RFD for Cd, Pb, Zn and Cu but not for Hg in animal tissues and offal examined (4). Chronic exposure to low doses of Pb, Cd and Hg over their safe limit may lead to many diseases as liver malfunctions, headache and neurologic involvement (4). The EDIs of Pb, Cd, Hg, Zn and Hg in muscle and offal of the examined slaughter animals were below FAO/WHO (20) tolerable daily intakes.

Health risk estimation due to muscle and offal consumption from all examined animals was assessed based on the THQ. The THQ value in the current study was below 1 that means improbable population exposure to cause any observable risk (11) from the individual metals intake during the muscle and offal consumption. In the main, the muscle consumption poses less human health risk than liver and kidney, as it accumulates less metal as it not an active place for detoxification. These findings were in agreement with Ogbomida et al. (4), Tyokumbur (48) and Ihedioha and

Okoye (49) who determined THQ value below 1 for cow meat. Additionally, HI of the combination of all metals was calculated by counting all the metals hazard quotients (THQ) together. The probable health hazard of all metals was existed when HI is ≥ 1 (50). The HI for all examined metals was less than 1 and this revealed no undesirable effects on human health from muscle and offal consumption in our city. Our results agreed with Ogbomida et al., (4) who found that all heavy metals analyzed had THQ<1.

Conclusion

It can be concluded that kidney and liver samples had higher metals concentrations than in muscles. The hazard index of these elements are less than 1 and appropriate steps are recommended to reduce the health hazard through consumption of meat as these metals have cumulative effect so as to protect the food chain. Finally, this research provides important data for other ongoing research on the slaughtered animals and their offal in our country.

Conflict of interest

The authors declare no conflict of interest.

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Original Research Article

Aeromonas hydrophila IN FISH AND HUMANS; PREVALENCE, VIRULOTYPING AND ANTIMICROBIAL RESISTANCE

Heba A. Ahmed[1]*, Mohamed E.M. Mohamed[1], Maha M. Rezk[1,2], Rasha M. A. Gharieb[1], Shehata A. Abdel-Maksoud[2]

[1]Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, 44511,Sharkia Governorate, [2]Animal Health Research Institute, Damietta branch, Agriculture Researches Center, Egypt

*Corresponding author, E-mail: heba_ahmed@zu.edu.eg

Abstract: This work aimed to investigate the virulence factors and antibiotic resistance of *A. hydrophila* isolated from different sources in Damietta Governorate, Egypt. The samples comprised of tilapia (*Oreochromis niloticus*, n=150), mugil (*Mugil cephalus*, n=144), human stool (n=102) and fish sellers hand swabs (n=85). A total of 25 *A. hydrophila* isolates were recovered and molecularly confirmed, of which, 2.7% were from tilapia muscles, 2% from tilapia viscera, 6.3% from mugil viscera and 8.8% from stool samples. No isolates were recovered from mugil muscles and hand swabs. Phenotypic determination of heamolysis, lipolysis, proteolysis and gelatinase activity revealed that 60%, 56%, 60% and 100% were positive, respectively. Molecular identification of haemolysin (*hly*), aerolysin (*aer*), lipase (*lip*) and cytotoxic enterotoxins (*act*) virulence associated genes showed that 28%, 68%, 28% and 16% of the examined isolates were positive, respectively. Antimicrobial susceptibility of the isolates to 17 antibiotics was determined, the most resistance pattern was observed for cefixime (88%), while, all of the isolates were susceptible to imipenem. This study revealed that *A. hydrophila* isolated from fish and humans showed several virulence factors and exhibit a wide range of antibiotic resistance which is considered a public health hazard as well as.

Key words: *Aeromonas hydrophila*; virulence; vntibiotic resistance; fish

Introduction

Fish production is one of the most important industrial activities in Egypt (1). Damietta is a littoral governorate with an important role in fish production. However, infection of fish with microbial pathogens is considered a risk factor in aquaculture industry resulting in loss of millions of dollars annually (2). *Aeromonas* infection in fish causes world economic problems because of the high number of fish mortalities particularly in China and India (2). Fish can be bacteriologically contaminated either by polluted water or by handling, processing and unhygienic storage conditions (3). Aeromoniasis is a zoonotic disease caused by different species of *Aeromonas,* the disease occurs due to consumption of contaminated fish, sea foods and drinking water, or direct contact with recreational waters (4). This zoonotic pathogen belongs to family *Aeromonadceae*, it is a facultative anaerobic, non-spore forming Gram-negative bacterium, motile, bacilli or coccobacilli (5,6). *A.*

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hydrophila is considered the most important zoonotic pathogen of concern. It may cause intestinal and extra-intestinal diseases in humans such as septic arthritis, diarrhea (traveler's diarrhea), gastroenteritis, skin and wound infections, meningitis, and fulminating septicemia (7). *Aeromonas* occurs widely in aquatic environments and belongs to the flora of reptiles, amphibians and fish (8). The principal reservoirs are fish in rivers, estuary waters and salt water, it has also been possible to isolate *Aeromonas* from chlorinated water including water supplies. The United States Environmental Protection Agency has incorporated *Aeromonas* species in the contaminant candidate list of emerging water borne pathogens (9). This is attributed to *Aeromonas* capacity of growing and biofilm formation in chlorinated water distribution systems. *A. hydrophila* has been isolated from retail foods including fish, seafood, raw milk, poultry and red meats (10-12).

The pathogenicity of *Aeromonads* has been related to a number of putative virulence factors such as aerolysin, hemolysin, proteases, lipases and DNases. These toxins play a major role in the development of diseases either in humans or in fish (13). The haemolysins produced by *A. hydrophila* are divided into two major groups, extracellular haemolysin and aerolysin based on immunological studies (14).

Traditional methods for bacterial identification are based on the isolation and biochemical reactions (15). However, molecular methods especially polymerase chain reaction has been proven to be more accurate and rapid for the identification. The 16S rRNA gene is a confirmed method which contributes in signature sequencing for molecular identification of *Aeromonas* species (16). Antibiotics play an important role in the control of diseases affecting animals and humans; however this contributes in bacterial resistance. The ability of bacteria to resist wide range of antibiotics helps in enhancing additional virulence features. Multiple antibiotic resistance (MAR) from *A. hydrophila* is a worldwide problem caused by the mis-use of antibiotics (17,18). It was reported that the resistance of *Aeromonas* species isolated from aquatic environment has been increased due to the increased application of antibiotics in aquaculture treatment (19). The antibiotic resistance may be transmitted from livestock products and fish to humans by infection with pathogenic bacteria (19,20).

This study aimed to investigate the prevalence of *A. hydrophila* in tilapia and mugil samples collected from retail markets in Damietta governorate and in stool and hand swab of humans in the same study area. Moreover, the presence of some virulence factors was investigated by phenotypic and genotypic methods. The antibiotic sensitivity test was also applied to evaluate the antibiotic resistance profile of the isolates.

Material and methods

This study was conducted in Damietta governorate, Egypt, and was approved by the Committee of Animal Welfare and Research Ethics Faculty of Veterinary Medicine, Zagazig University, Egypt.

Samples

A total of 294 fish samples comprised 150 tilapia (*Oreochromis niloticus*) and 144 mugil (*Mugil cephalus*) were collected from marketed fish in Damietta Governorate, Egypt. Fish samples were collected in sterilized poly-ethylene bags and transferred to the lab. Viscera and muscles were sampled separately from each individual fish under complete aseptic conditions; samples were collected from each fish after sterilization of the surface by hot spatula according to the international commissions on microbiological specifications for foods (21).

Human samples; 102 stool and 85 hand swabs were collected and directly immersed in sterile alkaline peptone water (APW, Oxoid CM1028). Stool samples were collected from Endemic Diseases Hospital, while, hand swabs were collected from fish sellers. For each collected sample, demographic data (name, sex, age, occupation, residence) were reported.

Isolation and identification

Each sample (fish viscera, fish muscles, human stool and hand swabs) were pre-

enriched in APW at 37˚C for 24 h, then a loopful of the incubated broth was plated onto *Aeromonas* agar (LAB 167) and the plates were incubated at 37˚C for 24 h under aerobic conditions. Suspected colonies were purified by plating on nutrient agar plates and were then subjected to biochemical identification according to Bergey's Manual of Determinative Bacteriology (22). The colonies were examined for morphological characterizations such as shape, Gram stain and motility test. Biochemical characterization was carried out using oxidase, triple sugar iron, pigment formation, esculin hydrolysis, growth at 42˚C and 4˚C, arginine hydrolysis, indole production, Methyl Red, Voges-Proskauer, citrate utilization, urease, hydrogen sulphide production, nitrate reduction, gelatin liquefaction, ornithine decarboxylase, oxidation-fermentation, L-lysine decarboxylase, arginine decarboxylase, β-galactosidase (ONPG), salt tolerance and sugar fermentation (23).

Molecular identification

The extraction of DNA was done by QIAamp DNA Mini kit instructions (Qiagen, Germany, GmbH, Catalog no. 51304) with some modifications. Briefly, 200 µl of the sample suspension was incubated at 56ºC for 10 min after addition of 10 µl of proteinase K and 200 µl of lysis buffer. Then, 200 µl of 100% ethanol were added to the lysate. Washing and centrifugation of the sample were performed following the manufacturer's recommendations. Then, nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

The reaction contained 25 µl of the following PCR master mix; 12.5 µl of Emerald Amp GT PCR mastermix (2x premix), 4.5 µl PCR grade water, 1 µl forward primer (20 pmol), 1 µl reverse primer (20 pmol), 6 µl template DNA. The amplification of 16S rRNA gene for *Aeromonas* genus was performed using primer pair AerF/AerR; 5'-CTA CTT TTG CCG GCG AGC GG-'3 and 5'-TGA TTC CCG AAG GCA CTC CC-'3 amplifying 953 bp (24). The confirmed *Aeromonas* isolates were subjected to PCR for the identification of *A. hydrophila* using the primer pair AH1/AH2; 5'-GAA AGG TTG ATG CCT AAT ACG TA-'3 and 5'-CGT GCT GGC AAC AAA GGA CAG-'3 of the 16S rRNA gene producing a product of 625 bp (24). The used primers were supplied from Metabion (Germany). The amplification cycles (n=35) were performed under the following conditions; 5 min of primary denaturation at 94ºC, secondary denaturation for 30 sec at 94ºC, annealing for 40 sec at 50ºC and extension at 72ºC for 50 sec in first primer pair and for 45 sec in the second pair. The amplified products were resolved by electrophoresis in 1.5% agarose gel (Applichem, Germany, GmbH). The sizes of the amplified product were determined by a gene ruler 1 Kb plus DNA Ladder (Fermentas, Thermo Scientific, Germany). Photographing was done by gel documentation system (Alpha Innotech, Biomedia) and analysis was performed using computer software.

Phenotypic characterization of A. hydrophila virulence determinants

Virulence determinants including haemolysis, lipase activity, protease activity and gelatin liquefaction of *A. hydrophila* isolates (n=25) were evaluated. Haemolysis activity was tested using blood agar plate containing 5% sheep red blood cells (Eiken, Japan) at 37˚C. The haemolysis was examined after 24 h according to Singh and Sanyal (25). The lipase activity was screened by white zones appearance in a selective media based on the hydrolysis of Tween 80 containing (g/l): pepton, 10; NaCl, 5; $CaCl_2.2H_2O$, 0.1; agar agar, 20 and Tween 80, 10 ml v/v (26). Caseinase production capacity was assayed by growing the cultures on LB (HIMEDIA, M575) medium supplemented with 1% of skim milk at 35ºC for 24 h (27). Clear halo production indicated positive reaction. Gelatinase production was determined using Luria-Bertani agar Miller (HIMEDIA, M1151) containing gelatin (30 g/L), cultivation was done at 30ºC overnight and cooled for 5 h at 4ºC. Positive reaction was described by the appearance of a turbid halo zone around the streaked colonies (28).

Molecular characterization of putative virulence genes

Primer pair used for *hly* gene amplification was 5'-CTA TGA AAA AAC TAA AAA TAA CTG-'3 and 5'-CAG TAT AAG TGG GGA AAT GGA AAG-'3 (29). The amplification conditions were 5 min of primary denaturation at 94ºC, secondary denaturation for 30 sec at 94ºC, annealing 1 min at 55ºC, extension at 72ºC for 1.5 min and final extension at 72ºC for 12 min. In case of *aero* gene, the primer pair used was 5'-CAC AGC CAA TAT GTC GGT GAA G-'3 and 5'-GTC ACC TTC TCG CTC AGG C-'3 (30). The conditions were primary denaturation at 94ºC for 5 min, secondary denaturation for 30 sec at 94ºC, annealing for 40 sec at 52ºC, extension at 72ºC for 40 sec and final extension at 72ºC for 10 min. Moreover, the primers used for *act* gene were 5'-AGA AGG TGA CCA CCA CCA AGA ACA-'3 and 5'-AAC TGA CAT CGG CCT TGA ACT C-'3 (31), the conditions of the amplification were primary denaturation at 94ºC for 5 min, secondary denaturation for 30 sec at 94ºC, annealing 30 sec at 55ºC, extension at 72ºC for 30 sec and final extension at 72ºC for 7 min. *Lip* gene was amplified using the primers 5'-ATC TTC TCC GAC TGG TTC GG-'3 and 5'-CCG TGC CAG GAC TGG GTC TT-'3 as described by Sen and Rodgers (32). The reaction conditions were primary denaturation at 94ºC for 5 min, secondary denaturation for 30 sec at 94ºC, annealing 40 sec at 55ºC, extension at 72ºC for 40 sec and final extension at 72ºC for 10 min. Visualization of the products was carried out as described previously.

Antibiogram analysis

A. hydrophila strains were subjected to antibiotic sensitivity testing using Kirby-Bauer disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) instructions. *A. hydrophila* isolates were inoculated in TSB and incubated at 35ºC for 16-20 h, turbid broth was inoculated in Muller Hinton broth (Oxoid, CM0405), the turbidity was adjusted according to McFarland obesity tube No. 0.5. Isolates were streaked on Muller Hinton agar (Oxoid, CM0337) and disks were placed, incubation was done at 37ºC overnight. The used antibiotics were Amoxicillin (AML, 25 µg), Amoxicilline-Clavulanic acid (AMC, 30 µg), Ceftriaxone (CRO, 30 µg), Cephotaxime (CTX, 30 µg), Ceftazidime (CAZ, 30 µg), Cefixime (CFM, 5 µg), Imipenem (IPM, 10 µg), Gentamicin (CN, 10 µg), Tobramycin (TOB, 10 µg), Kanamycin (K, 30 µg), Streptomycin (S, 10 µg), Tetracycline (TE, 30 µg), Ciprofloxacin (CIP, 5 µg), Norfloxacin (NOR, 10 µg), Nalidixic acid (NA, 30 µg), Trimethoprim-Sulfamethoxazole (SXT, 25 µg) and Chloramphenicol (C, 30 µg). Choosing antimicrobials was according to the importance and common use in preventing and treating diseases in both fish farms and human clinics. Interpretation was done according to the followings; Amoxicillin/ Clavulanic acid, Ceftriaxone, Cefotaxime, Ceftazidime, Imipenem, Gentamicin, Tetracycline and Chloramphenicol were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (33). Ciprofloxacin was according to The European Committee on Antimicrobial Susceptibility Testing (34). Streptomycin was interpreted according to CLSI (35). In case of Cefixime, Tobramycin, Norfloxacin and Nalidixic acid, the interpretation relied on French Society of Microbiology (36). The interpretation of Trimethoprime/ Sulfamethoxazole was based on the French Society of Microbiology (37). Moreover, Amoxicillin and Kanamycin assessment was according to Comite de l'Antibiogramme de la Societe Francaise de Microbiologie (38). Multiple antibiotic resistances (MAR) index was evaluated according to the equation described by Krumperman (39). Multidrug resistance (MDR) was defined as resistance of an isolate to at least one agent in three or more antibiotic classes (40).

Results

A total of 481 samples collected from Damietta governorate, Egypt, were examined for *A. hydrophila* contamination. Table 1 illustrates that a total of 34 (7.1%) isolates were identified by morphological and biochemical examination. These isolates were confirmed as *Aeromonas* spp. by PCR (Figure 1A).

Molecular amplification of *A. hydrophila* specific 16S rRNA gene revealed that 25 (5.2%) were positive as shown in Figure (1B).

In case of *Orechromis niloticus*, 7 (4.7%) isolates were recovered; 4 (2.7%) from muscles and 3 (2%) from viscera. Meanwhile, *Mugil cephalus* showed 6.3% isolation rate from viscera. In case of human samples, 9 (8.8%) isolates were recovered from stool samples, while hand swabs revealed no *A. hydrophila* contamination.

The phenotypic examination of putative virulence factors revealed that all the 25 isolates (100%) tested positive for gelatin liquefaction test, while 15, 14 and 15 isolates were positive for heamolysis on blood agar, lipase and protease activity with the respective percentages; 60, 56 and 60%.

The analysis of gene profiles based on the presence of 4 virulence-associated genes (*hly*, *lip*, *aer* and *act*) among clinical and environmental *A. hydrophila* isolates revealed that 7 (28%); 7 (28%); 17 (68%) and 4 (16%) of the isolates were positive for the aforementioned genes, respectively (Figure 1C, 1D, Figure 2A and 2B).

The antibiotic susceptibility assay (Tables 2 and 3) performed on 25 confirmed *A. hydrophila* isolates illustrated that all the isolates were susceptible to imipenem. However, the resistance pattern varied among the other tested drugs; the highest resistance was recorded against cefixime with the proportion of 88% followed by amoxicillin/ clavulanic acid and ceftazidime (80%). The lowest resistance (40%) was against gentamycin, streptomycin, norfloxacin and chloramphenicol. Multiple drug resistance was observed in *A. hydrophila* isolates, out of 25 isolates, four isolates were resistant to 2, 5, 12 and 13 drugs (one isolate, each). Moreover, 6 isolates were resistant to 8, 9 and 10 drugs (two isolates, each), 3 isolates were resistant to 6 drugs, 5 isolates were resistant to 4 drugs and 7 isolates were resistant to 15 drugs. The MAR average was 0.489.

Table 1: Occurrence of *A. hydrophila* and virulence factors in the examined samples

Samples		No. of bacterial-ogically suspected isolates (%)	No. of molecularly confirmed *A. hydrophila*	*hly* gene	*lip* gene	*aer* gene	*act* gene	Haemolysis	Lipase activity	Protease	Gelatinase
Tilapia (n=150)	**Muscles**	5 (3.3%)	4 (2.7%)	0	3	0	0	3	2	3	4
	Viscera	5 (3.3%)	3 (2%)	0	0	3	1	1	2	2	3
Mugil (n=144)	**Muscles**	1 (0.7%)	0	0	0	0	0	0	0	0	0
	Viscera	13 (9%)	9 (6.3%)	0	2	5	3	3	3	2	9
Fish Sellers	**Hand Swabs (n=85)**	0	0	0	0	0	0	0	0	0	0
Patients	**Stool (n=102)**	10 (9.8%)	9 (8.8%)	7	2	9	0	8	7	8	9
Total	**481**	34	25 (5.2%)	7 (28%)	7 (28%)	17 (68%)	4 (16%)	15 (60%)	14 (56%)	15 (60%)	25 (100%)

hly: hemolysin gene, *lip*: lipase gene, *aer*: aerolysin gene, *act*: cytotoxic enterotoxin

Table 2: Antimicrobial susceptibility of *Aeromonas hydrophila* isolates to different antibiotics

Antimicrobials (abbreviation)	***Aeromonas hydrophila* isolates (no = 25)**		
	*R	*I	*S
Amoxicillin (AML 25)	17(68%)	2 (8%)	6 (24%)
Amoxicillin/clavulanic acid (AMC 30)	20 (80%)	1 (4%)	4 (16.%)
Ceftriaxone (CRO 30)	11 (44%)	3 (12%)	11 (44%)
Cefotaxime (CTX 30)	14 (56%)	4 (16%)	7 (28%)
Ceftazidime (CAZ 30)	20 (80%)	-	5 (20%)
Cefixime (CFM 5)	22 (88%)	-	3 (12%)
Imipenem (IPM 10)	-	-	25 (100%)
Gentamycin (CN 10)	10 (40%)	1 (4%)	14 (56%)
Tobramycin (TOB 10)	11(44%)	4 (16%)	10 (40%)
Kanamycin (K 30)	14 (56%)	1 (4%)	10 (40%)
Streptomycin (S 10)	10 (40%)	4 (16%)	11(44%)
Tetracycline (TE 30)	18 (72%)	1 (4%)	6 (24%)
Ciprofloxacin (CIP 5)	12 (48%)	1 (4%)	12 (48%)
Norfloxacin (NOR 10)	10 (40%)	5 (20%)	10 (40%)
Nalidixic acid (NA 30)	19 (76%)	-	6 (24%)
Trimethoprim/sulphamethoxazole (SXT 25)	11 (44%)	-	14 (56%)
Chloramphenicol (C 30)	10 (40%)	4 (16%)	11 (44%)

*R: resistant, I: intermediate, S: sensitive. CIP: Ciprofloxacin; CFM: Cefixime; S: Streptomycin; TOB: Tobramycin; IPM: Imipenem; CN: Gentamycin; NOR:Norfloxacin; AML: Amoxicillin; SXT: Trimethoprim-sulphamethoxazole; C: Chloramphenicol; TE: Tetracycline; CRO: Ceftriaxone; CTX: Cefotaxime; K: Kanamycin; NA: Nalidixic acid; CAZ: Ceftazidime and AMC: Amoxicillin-clavulanic acid.

Table 3: Frequency distribution of multidrug resistant *Aeromonas hydrophila* isolates

Resistance pattern	**No. of *Aeromonas hydrophila* isolates**	**Percentage of *Aeromonas hydrophila* isolates**	**MAR index**
Resistant to 2 antibiotics	1	4%	0.11
Resistant to 4 antibiotics	5	20%	0.23
Resistant to 5 antibiotics	1	4%	0.29
Resistant to 6 antibiotics	3	12%	0.35
Resistant to 8 antibiotics	2	8%	0.47
Resistant to 9 antibiotics	2	8%	0.52
Resistant to 10 antibiotics	2	8%	0.58
Resistant to 12 antibiotics	1	4%	0.70
Resistant to 13 antibiotics	1	4%	0.76
Resistant to 15 antibiotics	7	28%	0.88

Average MAR= 0.489.

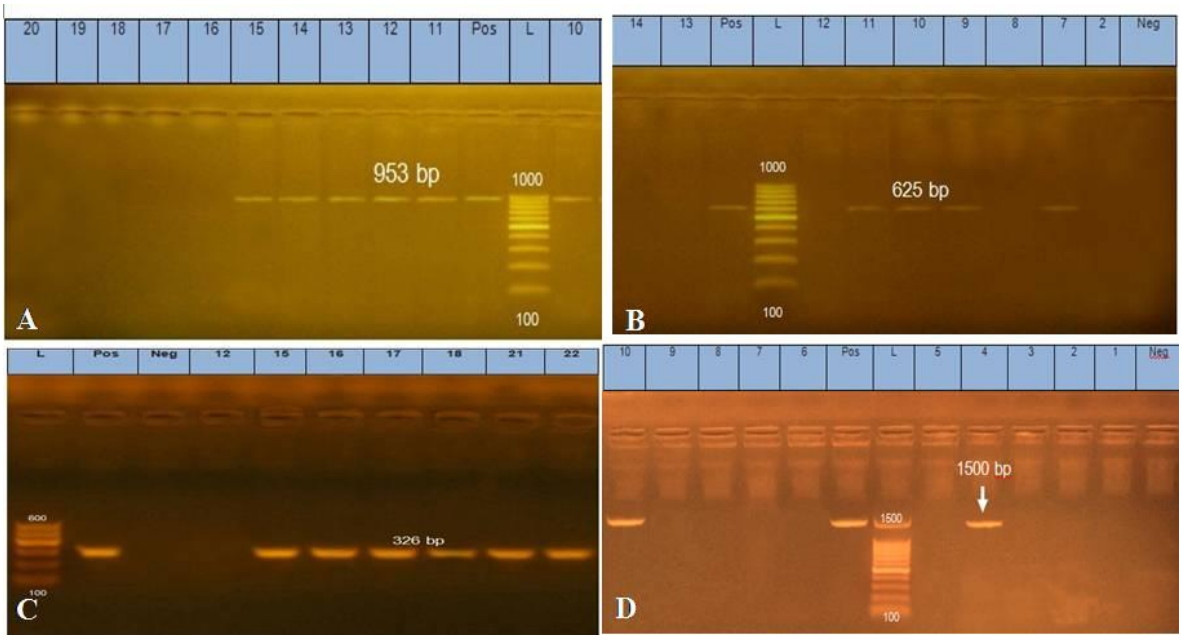

Figure 1: A: garose gel electrophoresis of 16S rRNA gene amplification of *Aeromonas* isolates from different sources with amplicon size of 953bp, L: 100 bp ladder. Lanes (10-15): positive samples. Lanes (16-20): negative samples. Lane Pos:positive control. B: Agarose gel electrophoresis of 16S rRNA gene of *Aeromonas hydrophila* isolates from different sources with amplicon size of 625 bp, L: 100bp ladder. Lanes (7, 9, 10 and 11): positive samples. Lanes (2, 8, 12, 13 and 14): negative samples. Lane Pos: positive control, lane Neg: negative control. C: Examplar of *aero* gene amplification of *Aeromonas hydrophila* isolates. L: 100 bp ladder, Lanes (15, 16, 17, 18, 21 and 22): positive samples. Lane 12: negative sample. Lane Pos: positive control and lane Neg: negative control. D: Examplar of agarose gel of *hly* amplification of *Aeromonas hydrophila* isolates. L: 100 bp ladder. Lanes (4, 10): positive samples, Lanes (1-3, 5, 6-9): negative samples. Lane Pos: positive control and lane Neg: negative control

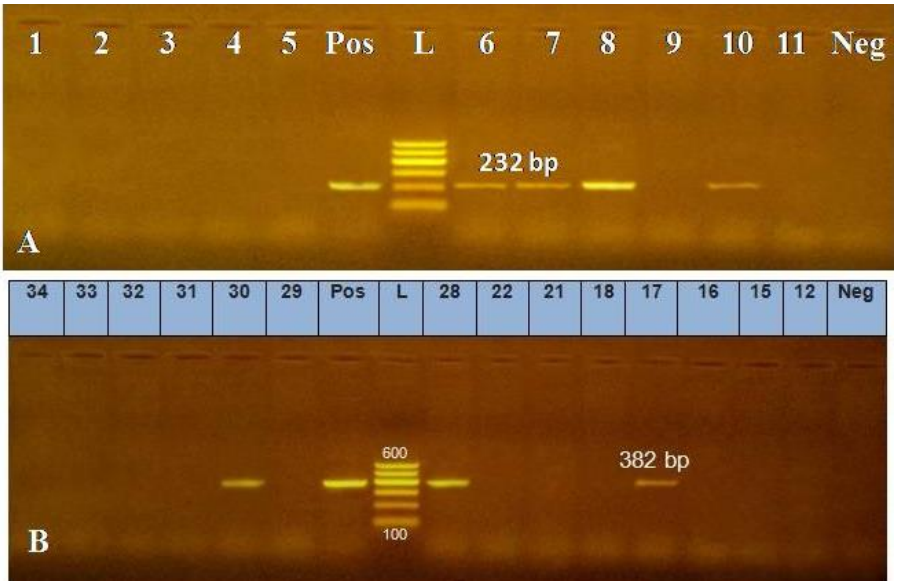

Figure 2: A: Examplar of agarose gel electrophoresis of *act* gene amplification of *Aeromonas hydrophila* isolates. L: 100 bp ladder. Lanes (1-5, 9, 11): negative samples. Lanes (6-8, 10): positive samples. Lane Pos: positive control and lane Neg: negative control. B: Examplar of agarose gel electrophoresis of *lip* gene amplification of *Aeromonas hydrophila* isolates. L: 100 bp ladder. Lanes (17, 28 and 30): positive samples. Lanes (12, 15, 16, 18, 21, 22, 29, 31-34): negative samples. Lane Pos: positive control and lane Neg: negative control

Discussion

A. hydrophila has gained increased attention due to pathogenicity to humans and the ubiquity of the organism in the environment, food and water (41). In this study, the prevalence of *A. hydrophila* in tilapia, mugil, stool and hand swabs was investigated. *A. hydrophila* was isolated from 2.7% tilapia muscles, 2% tilapia viscera, 6.3% mugil viscera indicating that fish in retail in the study area are considered potential source for infection of human consumers.

Ibrahem et al. (42) recorded a prevalence of 3.1% of *A. hydrophila* from the viscera of tilapia in Giza and Sharkia, Egypt. Meanwhile, Ashiru et al. (43) failed to isolate the bacteria from tilapia viscera in Nigeria. Furthermore, tilapia muscles showed isolation rate of 2.7% in our study, this is higher than the results recorded by Castro-Escarpulli et al. (44) who surveyed 250 frozen tilapia samples and reported an isolation rate of 0.8% in Mexico City. Ibrahim et al. (45) reported a higher isolation rate (46.6%) of *A. hydrophila* in fresh tilapia flesh and 53.3% in fresh mugil flesh. Moreover, Ramadan et al. (46) revealed that *Mugil cephalus* rate of infection with *A. hydrophila* was 37% in flesh. This was inconsistent with our study, the inability to isolate the organism from mugil flesh could be attributable to that *Aeromonas* organism is part of intestinal microbiota of either normal or diseased fish and the organism can transport to muscle under stress condition and in case of decrease fish immune state (47), moreover, the difference in individual fish susceptibility is a cause of concern.

The present study showed that *A. hydrophila* was isolated with the percentage of 8.8% from human stool samples, this is nearly similar to the results obtained by Subashkumar et al. (48) who isolated *A. hydrophila* from diarrheic patient stool samples with a rate of 9.7% in Coimbatore, South India. On the other hand, Borchardt et al. (9) reported a lower occurrence of *Aeromonas* species (0.7%) in stool samples in Wisconsin, USA. While, higher isolation rates of 85.7% in the hospitals of India (49) and 18.8% in Sharkia Governorate, Egypt were reported (10). Gastroenteritis caused by *A. hydrophila* is self-limiting watery diarrhea; however, in children and immuno-compromised patients, the disease is more serious (48).

None of the hand swab samples were contaminated with *A. hydrophila* in our study. This might be either because the hand is not suitable for the presence of *A. hydrophila* or due to the traditional habit of fish sellers to frequently wash their hands with water which lead to elimination of *A. hydrophila*. In accordance, another study in Egypt reported that none of the hand swabs from fish handlers were positive (50).

The pathogenesis of *A.* hydrophila is correlated to different factors such as extracellular enzymes, slime production and siderophores (6). *A. hydrophila* can cause cytolytic effect on erythrocytes; this heamolytic activity is a type of enteropathogenicity and may result in diarrhea outbreaks (51). Only 60% of our isolates showed hemolytic activity on blood agar plates. This was lower than other studies that reported high proportions ranging from 72%-95% of *A. hydrophila* isolates with hemolytic activity (42,48,52,53). Meanwhile, Castro-Escarpulli et al. (44) revealed that all the isolates tested positive for hemolytic activity

Lipase activity is another virulence factor which enables *A. hydrophila* to affect the function of the immune system by interaction with human leukocytes (54). Lipase can alter cytoplasmic membrane structure of the host cells and facilitate pathogenicity, the presence of aerolysin gene alleviate this process (31). The lipase activity was observed in 56% of the recovered isolates, while Bhowmik et al. (52) reported a higher prevalence (80.8%). Castro-Escarpulli et al. (44) and Simon et al. (53) reported the lipase activity in 100% of the *A. hydrophila* isolates.

The protease activity has an important role in the pathogenesis of *A. hydrophila* due to the ability of tissue damage and infection establishment by overcoming the host defense (54). Only 60% of our isolates showed protease activity, this is higher than Castro-Escarpulli et al. (44) who reported protease activity in 50% of the isolates, while, Bhowmik et al. (52)

documented 65.4%. Subashkumar et al. (48) reported the protease activity in all the tested isolates (100%). Gelatin liquefaction was observed in 100% of our isolates. This was similar to a study that reported gelatinase activity in 100% of the examined isolates (44).

Aeromonads may express cytotoxic enterotoxins (*act*), which inhibits the phagocytic ability of phagocytes; aerolysin proteins which exhibit enterotoxic properties and hemolysins (*hly*A) which are enterotoxigenic hemolysins (41,54). The haemolysin gene was identified in our study with the percentage of 28%; this is lower than the detection rate of the *hly* gene in different studies with the range from 30-100% (4,10,44,46,52,53).

The aerolysin gene was detected in 68% of the isolates. This is lower than detection rates ranged from 70-100% in different studies (44,46,53,55). While, Tahoun et al. (10) and Yogananth et al. (4) reported lower rates of 66.7% and 50%, respectively.

The detection rate of the lipase gene was 28% in our study. This result is lower than that reported by Younes et al. (56) who reported that 81.8% of *Aeromonas hydrophila* strains isolated from *Oreochromis niloticus* were positive for lipase gene, while, Simon et al. (53) and Castro-Escarpulli et al. (44) reported the gene in all the examined *A. hydrophila* isolates.

The *act* gene is responsible for enterotoxigenicity of *Aeromonas*, 16% of our isolates harbored the gene. This was inconsistent with other studies with higher detection rate of the gene ranging from 23-94% (46,52,53,55,56). The difference in the frequencies of the virulence associated genes between the studies could be attributed to variation in the origin of the samples.

Antibiotic susceptibility profile against 17 antibiotics was performed on 25 *A. hydrophila* isolates. In the present work, 100% of the isolates were susceptible to imipenem. This result is inconsistent with Castro-Escarpulli et al. (44) who reported a resistance of 50% to this drug. The most resistance pattern was recorded against cefixime (88%), followed by amoxicillin/clavulanic acid and ceftazidime (80%). Ramadan et al. (46) reported a resistance of 56% to ceftazidime. The examined isolates showed high resistance rates to both nalidixic acid and tetracycline (76% and 72%, respectively). Tetracycline and nalidixic acid resistance rates of 37.5%, each, were reported by Sarder et al. (57), while 100% of *A. hydrophila* isolates were susceptible to both tetracycline and nalidixic acid as described by Topic Popovic et al. (58) and Castro-Escarpulli et al. (44), respectively. In contrary, Ashiru et al. (43) reported 100% resistance to tetracycline.

Resistance to ceftriaxone, tobramycin and trimethoprim/sulphamethoxazole was 44%, each. Castro-Escarpulli et al. (44) documented 50% resistance against trimethoprim/ sulphamethoxazole, while, Topic Popovic et al. (58) reported 100% susceptibility to the drug.

The resistance of the isolates to cefotaxime and kanamycin was 56%, this is higher than the results of Ramadan et al. (46) who reported 40% and 6% resistance against the two drugs, respectively. Castro-Escarpulli et al. (44) observed 100% susceptibility of *A. hydrophila* isolates to both antibiotics, while, Sarder et al. (57) reported 87.5% susceptibility to kanamycin.

Ciprofloxacin was reported as the most active drug against *Aeromonas* infection (41,59,60). Our isolates showed resistance to ciprofloxacin with the percentage of 48%, this is inconsistent with other studies that reported 100% susceptibility to ciprofloxacin (43,44, 61). Moreover, Sarder et al. (57) recorded a lower resistance rate (6.3%) of the isolates to the drug.

The lowest resistance in our study was reported to chloramphenicol, norfloxacin, streptomycin and gentamicin with the percentage of 40, each. Sensitivity of *A. hydrophila* (100%) to chloramphenicol was reported in different studies (44,53,55). In addi-tion, 100% susceptibility of *A. hydrophila* iso-lates to norfloxacin was reported by Ye et al. (55).

Our isolates expressed 40% resistance to streptomycin this was comparable to 30 and 25% resistance reported by Ramadan et al. (46) and Sarder et al. (57), respectively. While, Ye et al. (55) and Simon et al. (53) reported 100% sensitivity to streptomycin.

The indiscriminate use of antibiotics for therapy in humans and animals and as growth promoters in animal and fish industry resulted in increased resistance and consequently, resistant organisms which can be transmitted to humans and other animals through many routes like food consumption and water contact even contaminated soil, livestock and pets could be considered as sources of resistance transfer (17,19,62).

The multiple antibiotic resistance (MAR) pattern of *A. hydrophila* was calculated and it ranged from 0.11 to 0.88. Krumperman (39) reported that MAR index of more than 0.2 is an evidence of high risk source contamination. Odeyemi and Ahmad (19) reported MAR index range of 0.25: 0.68 in different *Aeromonas* spp isolated from different aquatic sources.

Conclusion

In this study, *A. hydrophila* was isolated from different environmental and clinical samples. Isolation of *A. hydrophila* from fish flesh posed a zoonotic concern to public health. The observed MDR is resulted from the indiscriminate use of antibiotics either in fish farms or in diarrhea treatment. Therefore, a good disease management and control strategy is recommended for both fish farms and treatment assay to deal with antibiotic usage. Further studies are needed on the genetic relatedness among isolates from different sources and the virulence factors of the isolates must be investigated in details.

Conflict of interest

The authors declare no conflict of interest.

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Original Research Article

PREDICTING THE OPTIMAL TIME OF BREEDING AND THE POSSIBLE APPROACHES FOR TREATMENT OF SOME ESTRUS CYCLE ABNORMALITIES IN BITCHES

Faten M. Labib, Hassan H. Mansour, Atef B. Mahdy, Asmaa A. Abdallah*

Department of Theriogenology, Faculty of Veterinary medicine, Zagazig University, Egypt

*Corresponding author, E-mail: asmaa.abdallah2016@yahoo.com

Abstract: Infertility problem is a common syndrome among bitches and its causes are numerous, but the apparent most frequent cause is the mating at incorrect time. The aim of this study was to avoid the possible causes of infertility in bitches by predicting the optimal time of breeding and try to treat disorders in estrus cycles. A total number of 18 bitches of German shepherd breed were used in this study for monitoring phases of estrus cycle and predicting the optimal time of breeding. Those bitches examined by vaginal cytology, progesterone assay, measurement of vaginal PH, vaginal endoscopy and ultrasonographic examination of ovaries from proestrus to diestrus. By vaginal cytology, cornified cells first appeared on Day 4 from the beginning of proestrus, then increased till reached to 85% on Day 12 which is considered as the ovulation day. Measuring of progesterone concentration at the beginning of proestrus was very low (less than 1 ng/ml) then increased in 13 bitches only out of 18 bitches which used in this study till reach to 6.5 ± 1.37 ng/ml within ranges (4.8 to 8.3 ng/ml) on Day 12 from the beginning of proestrus. The other five bitches showed disorders in normal sequence of estrus cycle which was discovered by progesterone assay, vaginoscopy and ultrasonography of ovaries. Abnormalities in estrus cycle were recorded; two cases of anovulation and three bitches of persistent proestrus. As a trial for correcting the deviated estrus cycle, we injected hCG hormone in two anovulatory bitches for occurrence of ovulation, and also used methergine drug in cases of persistent proestrus as a trial for stoppage of continuous bloody discharge. The optimal time of breeding in 13 ovulatory bitches was within 2 to 3 days after ovulation day. Pregnancy rate after breeding by 20 days was recorded by ultrasonographic examination was 92.3 % (12/13 bitches). When applying these applications, we concluded that progesterone hormone assay was the accurate method for predicting the ovulation time and achieving higher pregnancy rate , and also can judge on estrus cycle if normal or abnormal.

Key words: bitch; bvulation; brogesterone assay; bersistent proestrus; hCG administration

Introduction

Estrus cycle in bitches is unusual and differs than in other domestic animals; include four stages as proestrus, estrus, diestrus and anestrum. Estrus period is long with average 9 days in most bitches. Female dog accepts male for such long period and the poor relationship

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between behavioural characteristic and time of ovulation in the bitch can lead to difficulties in identifying the optimal mating time. Anestrum period is very long within a range of 4 to 10 months (1). Fertility in the bitch has a great socio-economic importance. The most common cause of infertility in the bitch is mating at the incorrect time **(**1), so determining the optimal time of breeding is considered the most important aim of routine breeding management (2). The character of spontaneous ovulation and long estrus period lead to difficulty in determining the suitable time of mating and lead to apparent infertility in the bitch (3). Previous studies discussed clinical assessments for estimating the time of insemination as visualization of physical and behavioral signs during estrus cycle, vaginal cytology, and examination of cervico-vaginal secretion and measurement of reproductive hormones (4,5). Progesterone assay has been a valuable tool for following the reproductive events in bitches, and also rise of serum progesterone before ovulation due to the luteinization of preovulatory follicles is one of the distinctive features in canine reproduction (6). Another valuable tool in breeding management program was a vaginal cytology, it can help to demarcate the estrus cycle stages under the effect of reproductive hormones which play role in morphological changes in vaginal epithelium, also is available and inexpensive (4). Another clinical assessment used for predicting ovulation time was ultrasound as mentioned by Renton et al. **(**7) who compared endocrine changes and ultrasound as means for determining ovulation in the bitch and improve fertility. The other workers used assessment of turgidity of vulva and record the changes in vaginal mucosa as the way for identifying the ovulation time (8,9). The vaginal pH in bitch changes during different stages of estrus cycle under the effect of steroid hormones, but very limited studies measure pH as a tool for identifying ovulation time (10,11). "The development of a rapid and reliable method for predicting ovulation in dogs would have a large impact on canine reproduction", as improving the fertility, lowering the cost especially if used frozen semen and more benefit in embryo transfer (12). Following up measurement of serum progesterone concentration from beginning of proestrus till end of estrus period, not only to accurately detect the insemination time and predict parturition date, but also to identify unusual estrus cycles which is mainly caused by ovarian dysfunction (13). This study aimed to improve fertility and find the best accurate facilities for breeding management in bitches throughout comparing the clinical assessments as vaginal cytology, progesterone assay, and measurement of vaginal pH, vaginal endoscopy and ultrasonographic examination of ovaries, and use them in predicting the optimal time of breeding. Also recording abnormalities in estrus cycle and try to treat them.

Material and methods

Study design

A total of 18 German shepherd bitches with different weights ranged from 10 – 20 Kg and with age ranged from 9 months to 3 years. These bitches were housed at the clinic of veterinary hospital, Faculty of Veterinary Medicine, Zagazig University, Egypt from January to July, 2018. The bitches were fed on a balanced diet and provided with water *ad libitum* (14). These bitches were examined from the beginning of proestrus (The first day of serosanguinous vaginal discharges and vulval edema) till the beginning of diestrus period (identified by bitch not accept male dogs and sharp decrease in cornified epithelium cells percent) for predicting the optimal time of breeding by vaginal cytology, progesterone assay, vaginal pH measurement, vaginal endoscopy and ultrasonographic examination of ovaries and recording the phases of estrus cycle if normal or abnormal. All procedures involved bitches has been carried out after approval from Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC/2/F/42/2018).

Vaginal cytology

Vaginal smear was applied day after day from first onset of proestrus till the beginning of diestrus. It was carried out by using a sterile

vaginal swab, which was introduced into the vulva upward cranially to avoid clitorial fossa and was forced into anterior vagina**.**

The swab was rotated inside the anterior vagina, rolled on clean glass slide and then fixed by methyl alcohol for one minute. The slide was stained with modified wrights Giemsa stain for five minute, rinsed by a few drops of water and then dried by filter paper (4). Finally, the slide was examined under high power of microscope (X 400) to determine estrus cycle stages and detect the vaginal cytology cornification index.

Cornification index = Total number of cornified vaginal cells / Total number of vaginal cells in smear × 100.

Measurement of progesterone hormone

Blood samples were withdrawn from bitches day after day from the beginning of proestrus till the beginning of diestrus period. They were collected from cephalic vein and then centrifuged at 1000 round per 15 minutes at room temperature to obtain a serum sample (6). Serum samples were sent to ULTRA lab, Sharkia governorate at the same day of collection for measuring progesterone hormone by chemiluminescence assay (CA) as a quantitative assay, and the results were expressed as ng/ml.

Measuring of vaginal pH

The vaginal pH was measured using pH strip (Gomhoria Company, Egypt) within a range from 4 to 9. This was done by insertion of the pH paper into the vagina by the aid of a glass speculum. The pH paper was left in contact with the vaginal wall and the vaginal secretion for at least 30 seconds (11). The readings were recorded by comparing the changes in the color of pH strip with the appropriate table strip.

Vaginal endoscopy

Vaginal examinations were performed day after day using 1.5 meter long and 8.2 mm diameter rigid endoscope (USA). It was inserted into the vagina, following the natural anatomy of the tract. The moving images appeared on screen were recorded and saved to a memory card. These procedures were applied in the non-sedated, standing bitch but in viscous bitch 0.5-1 ml of 2% xylazine solution was injected intramuscular as a sedative. Vaginoscopic assessment is based upon observation of the mucosal fold contours and profiles, the color of the mucosa and of any fluid present as well as the changes in vagina from the beginning of proestrus to diestrus onset.

Ultrasonographic examination of ovaries

Bitches' ovaries were examined by ultrasound (Mylab esaote, the Netherlands) day after day from proestrus onset till beginning of diestrus. Bitches were secured at lateral recumbancy during examination and examined by curved transducer with frequency 7.5 MHZ or by linear transducer with frequency 8 MHZ. The kidney was considered as a guide during ovarian examination. Ovaries were monitored throughout cycle to record any newly appeared structures and to detect the time of ovulation.

Recording disorders in estrus cycle and dealing with them

By applying the previous clinical methods especially progesterone assay and vaginal endoscopy for judging the status of estrus cycle (normal or abnormal), disorders were recorded as persistent proestrus and anovulation. Methergine (1cm/20Kg BW for three days intravenous) was used as a hemostatic drug for treating persistent proestrus, however, hCG hormone (250 IU/ bitch for once time intramuscular) was used as a trial for helping the occurrence of ovulation in anovulatory bitches.

Laparotomy operation was carried out for one anovulatory bitch which not respond for hCG treatment under general anesthesia as Thiopental sodium 2.5% with a dose of 20 mg/kg BW for exploration of disturbance causing abnormalities in estrus cycle. During laparotomy, 2 uterine horns, 2 fallopian tubes, 2 ovaries were removed. Pieces of right and left ovaries, fallopian tubes, and uteri from bitch with estrus cycle abnormalities were collected, fixed in 20 % buffered neutral formalin (N.B.F.), dehydrated in series of ascending

grade of ethanol followed by clearing in Xylol (three changes) and then embedded in paraffin. Using rotary microtome, sections of 4-6 µm thickness, then slides were stained with Hematoxyline and Eosin (H&E). All stained sections were examined with standard light microscope and then photographed (15).

Statistical analysis

The date characterizing the duration of estrus cycle phases, vaginal PH and progesterone assay was analyzed using the SPSS program, version 22. The average progesterone concentration for cyclic ovulatory bitches and sampling day were calculated as the mean ± SD for normal ovulatory bitches. Analyses were performed using bar chart. The values of vaginal pH were expressed as the mean ± SD and represented in bar chart.

Results

These clinical assessments applied on 18 bitches of German shephered breed with spontaneous normal proestrus, mean ± SD of their proestrus was 10.2± 0.05 days, mean ± SD of their estrus was 8.04 ± 0.12 days and diestrus started between 19 and 20 days after the onset of the proestrus.

Vaginal smear from along estrus cycle

It revealed the appearance of superficial cornified cells on day 4 from the beginning of proestrus and the cornification index was 10%. This cornification index continued to increase till reached 85% on Day 12 from the beginning of proestrus (Figure 1A and B), and then slowly decreased from days 13 to 15. After Day 15, cornification index sharply decreased till reach 30% on Day 20 from the beginning of proestrus, and then continued to decrease until completely disappeared on Day 24 from the beginning of proestrus (Figure 1B).

Progesterone concentration along estrus cycle

The progesterone concentration was low (< 1ng/ml) at the beginning of proestrus then continued in low values till the beginning of estrus that reached 1.55± 0.155 ng/ml. Two days after the beginning of estrus, progesterone concentration was 6.5 ± 1.37 ng/ ml with a range of (4.8 to 8.3 ng/ml) then continued to increase with progression of days to diestrus (Figure 2).

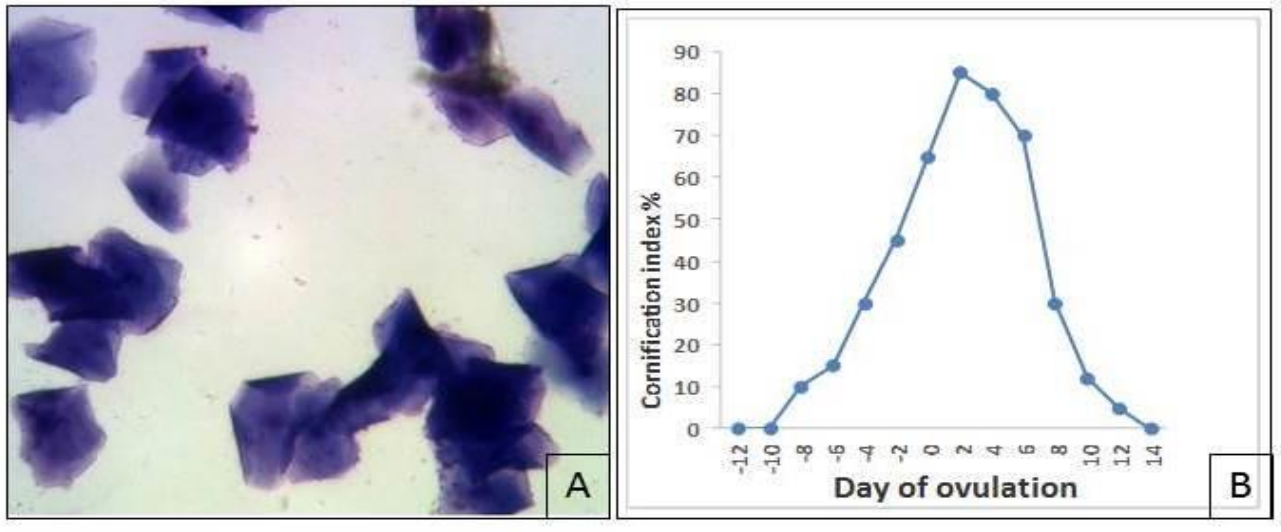

Figure 1: (A) Vaginal smear during estrus of German shepherd bitches showing high percent of cornnified angulated cells; take more staining with Geimsa stain (X400). (B) Cornification index curve from the beginning of proestrus till the beginning of diestrus in German shepherd bitches

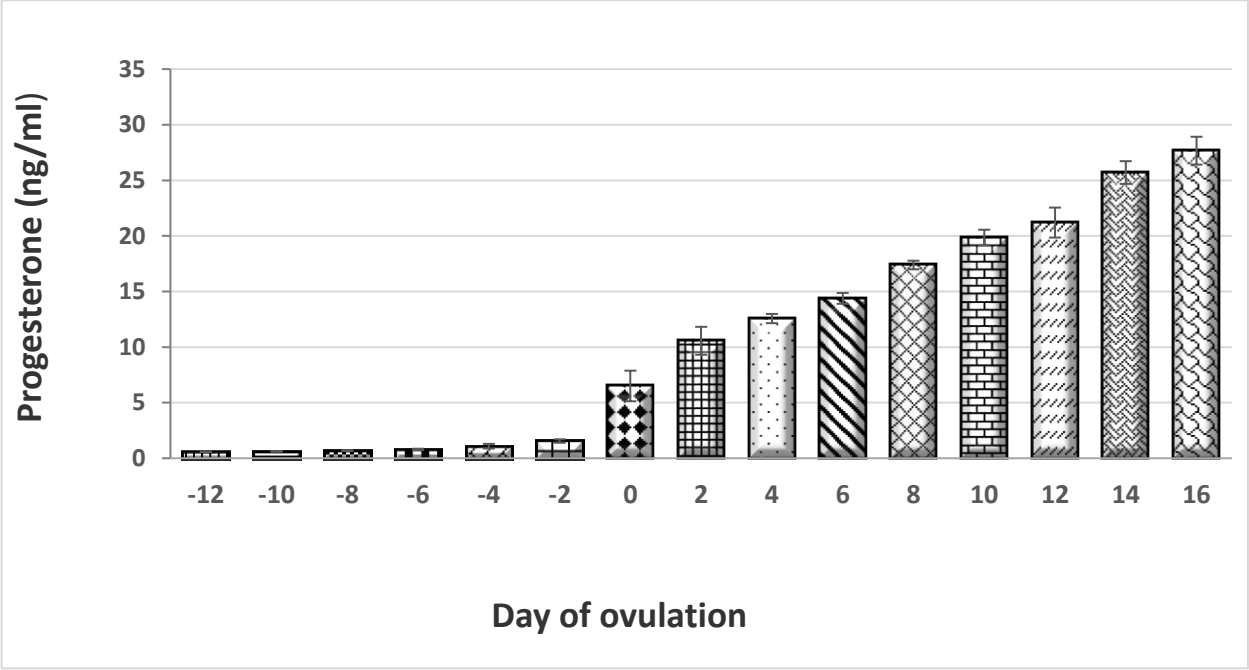

Figure 2: Bar chart showing mean ±SD of progesterone concentration from the beginning of proestrus till the beginning of diestrus in German shepherd bitches

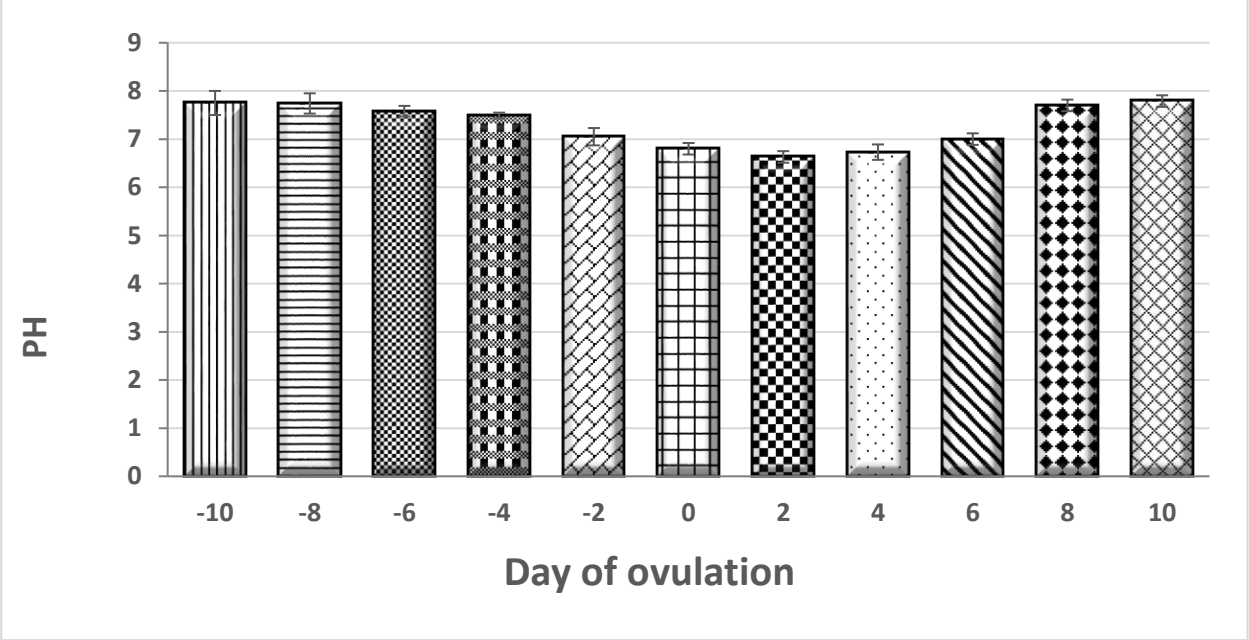

Figure 3: Bar chart Showing mean ± SD of vaginal pH from the beginning of proestrus to the beginning of diestrus in German shepherd bitches

The dynamic of vaginal pH during estrus cycle

Measuring of vaginal pH during proestrus and estrus shown that the pH of vaginal secretions at the second day of proestrus ranged from 7.3 to 8.2 (7.76±0.17) then continued to decrease till reach their low values at the last day of proestrus between 7.4 and 7.6 (7.49±0.06). The trend of a reduction of the vaginal pH continued during the first days of estrus as following; pH ranged from 6.8 to 7.3

(7.05±0.28), 6.5 and 7.1 (6.8±0.27) and 6.5 and 6.8 (6.63±0.12) on the first, third and fifth day of estrus, respectively. After the fifth day of estrus, pH values returned to increase till reach on Day 10 post ovulation to 7.79± 0.12 as illustrated in (Figure 3). The expected ovulation day was day 12 where pH ranged from 6.5 to 7.1 (6.8± 0.27).

Vaginal endoscopy findings

Vaginal endoscopy of bitches during proestrus, revealed that, the vaginal mucosa was edematous with swollen folds, take round shape and red color with oozing of bloody discharges from cervix (Figure 4A). During the first three days of estrus, the edema of mucosal folds decreased, the vaginal folds collapsed and appeared wrinkled, and the color changed into white. During the mid of estrus, mucosa became more crenulated, folds are flattened**.** During diestrus, mucosal folds appeared flat and covered with green mucoid discharges**.** The expected day of ovulation was decided when mucosal folds appeared wrinkled and mating occurred when more wrinkled.

Ultrasonographic results of ovaries

Repeated examination of ovaries using real time B mode ultrasound during proestrus showed the presence of spherical, anechoic follicles with thin walls. The diameter of these follicles was ranged from 2 to 3 mm at the beginning of proestrus and increased with progression of proestrus days till reach 8 to 10 mm at the end of this stage. Two days after the beginning of estrus phase, diameter of these follicles decreased with increasing in echogenicity of ovaries. These changes referred to the time of ovulation. After these changes by 3 days, corpus luteum appeared as echogenic structure with small anechoic cavity. The optimal time of breeding in 13 ovulatory cyclic bitches was within 2 to 3 days after the day of ovulation, pregnancy rate after 20 days from breeding was 92.3 % (12/13 bitches).

Abnormalities of estrus cycle (unusual estrus cycle in bitches)

Deviation from normal progesterone concentration has been recorded from the beginning of proestrus in 5 bitches out of 18 examined. Following up these bitches, it was found the following.

Persistent proestrus

By examination of 3 out of 5 bitches under investigation in current study, there was bloody vaginal discharge continuously for a period ranged from 35 to 40 days as in (Fig.4.B). These bitches accepted male and bred on Day 20 although of continuation of bloody discharge, low serum progesterone concentration (less than 1 ng/ml) till day 30. Later on, serum progesterone increased till reach 4.40 ng/ml. After day 40, bloody discharge stopped and progesterone concentration reached 23.7 ng/ml due to corpus luteum formation. Pregnancy diagnosis of those bitches after mating by one month, appear empty hypoechoic uterus without any pregnancy. To our knowledge, methergine drug (1cm/20 Kg BW for three days intravenous) was used for these bitches on Day 25 from the beginning of discharge as a trial for stoppage these continuous bloody discharges, but this was useless as bloody discharges not stopped and continued for days ranged from 35 to 40 from the beginning in those three bitches.

Anovulation

In the remaining 2 bitches out of the 5 with abnormal estrus cycle, progesterone measured at the beginning of proestrus was 0.878 ng/ml and estrogen was 73.8 pg/ml in both bitches. Four days later, progesterone and estrogen concentrations were 1.43 ng/ml and 41.16 pg/ml, respectively in one bitch. Later on, estrogen decreased into 32.79 pg/ml and progesterone decreased into 0.652 ng/ml in same bitch. Serial measurement of serum progesterone for one month with two days interval revealed a stationary concentration of less than 1ng/ml in spite of stoppage of serosanguinous discharge after 8 days from the beginning.

In the other bitch, estrogen concentration decreased gradually till reach 19.82 pg/ml and continued at this concentration along one month. However, progesterone concentration continued less than 1ng/ml from the appearance of serosanguinous discharge and for a period of

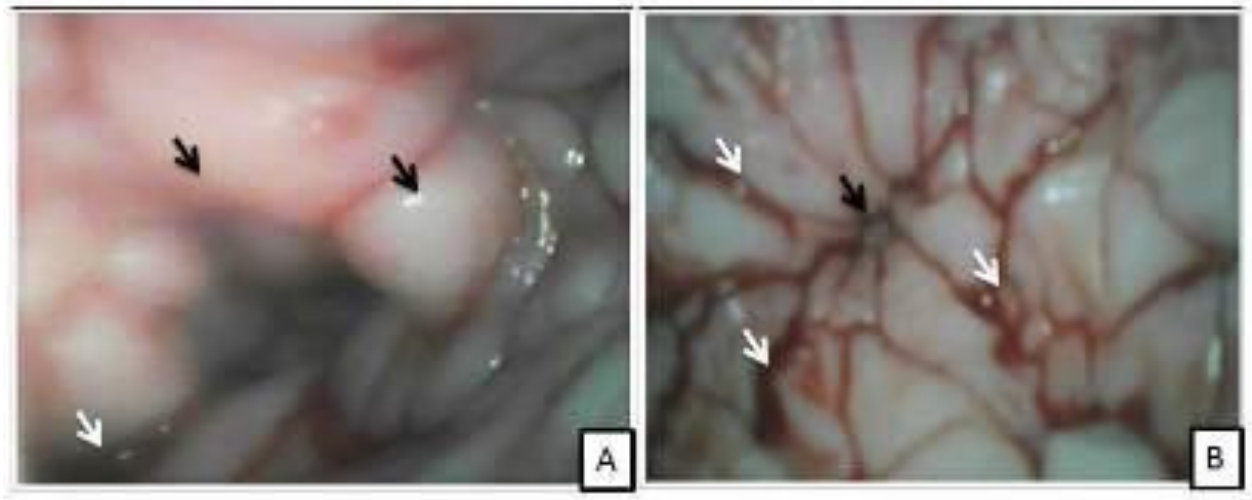

Figure 4: (A) Vaginal endoscopy showing edematous vaginal mucosal folds (black arrow) with presence of bloody discharge (white arrow) during late normal proestrus in German shepherd bitches. (B) Vaginal endoscopy showing opened cervix (black head), vaginal mucosa is fissured and covered with bloody discharge (white arrow) on Day 33 from the beginning of proestrus in abnormal persistent proestrus in German shepherd bitches

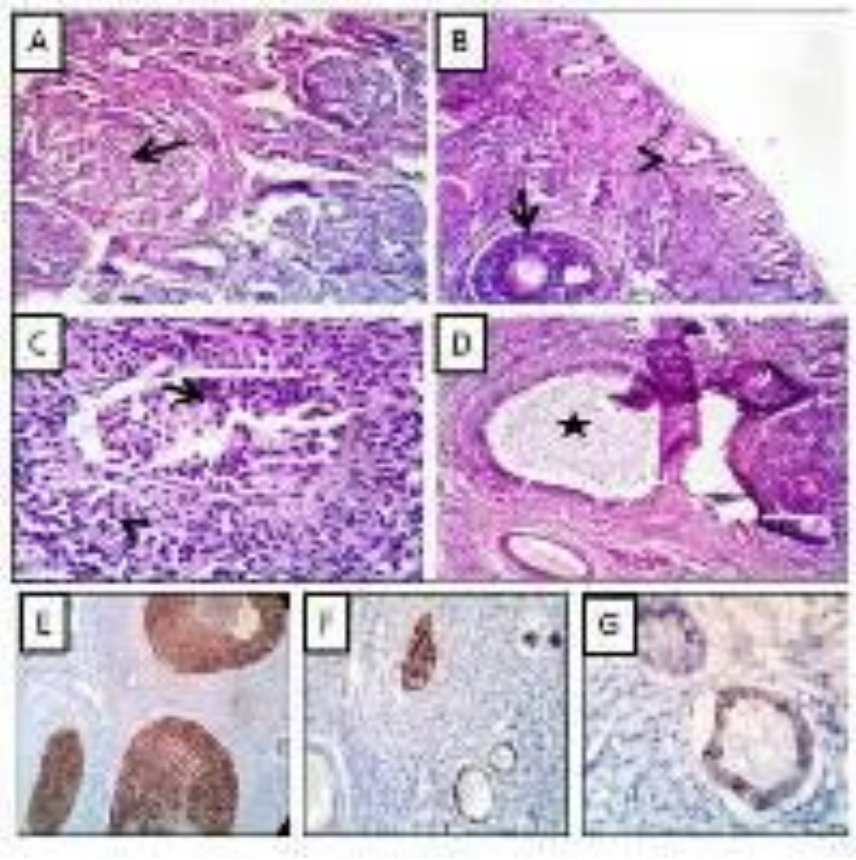

Figure 5: Photomicrograph of L. ovary in a case of anovulation in mongrel bitch (staining with H&E) showing: (A) dysmoplastic changes associated with the neoplastic growth (black arrow). (B&C) mass of globular structure with central radiating cells (black arrow) followed by compacted cell layer with large hyperchromatic nuclei (arrow head). (D) Cystic graffian follicle (star). In lower part of photo showing immunostaining with tumor markers as inhibin: Examined sections revealed diffuse reaction in mature graffian follicle granulosa cells (E) and focal positive reaction in tumor cells (F, G)

one month. In those two bitches, serosanguinous discharge stopped after 8 days, then bitches attracted to male and bred normally day after day for three times although ovulation not occur and progesterone not increase.

Pregnancy diagnosis of these bitches by ultrasonography after the last mating by 20 days revealed that the uterus was empty without pregnancy. The approach with those cases was by injection of hCG (250 IU/ bitch) once intramuscular. In one bitch, progesterone concentration increased after injection by 3 days and reached to 4.56 ng/ml, but in the other bitch serum progesterone did not changed or increased after injection. Histopathology applied on these genitalia after ovariohysterectomy to know the cause of anovulation and abnormal structure on ovaries. Histopathological alterations in this bitch clarified the presence two small follicles, follicular cyst and abnormal structure as suspected tumor on the two ovaries. The ovarian stroma of the right ovary appeared infiltrated by sheets, acini and trabecular structures of epithelial cells, some with dysplastic nuclear changes and dysmoplastic changes associated with the neoplastic growth. The ovarian follicles showed different developmental changes with regular structures. One of the graffian follicles was cystic and filled with fine granular esinophilic fluid; the granulosa cells of such cyst were atrophied. The Left ovary showed the same histomorphological changes beside a mass of globular structure with central radiating cells, followed by compacted cell layer with large hyperchromatic nuclei beside a group of infiltrating small epithelial cells with hyperchromatic nuclei. The Preliminary Diagnosis of such case was ovarian neoplasm with glandular differentiation, possibility of granulosa cell tumor. Immunostaining with tumor markers as inhibin showed the presence of granulosa cell tumor as in (Figure 5).

Discussion

The presence of variability in the day on which ovulation occurs among bitches play a role in difficulty to determine the optimal time of mating, so applying clinical assessments or finding methods for predicting ovulation time is the corner stone for controlling infertility's problems in bitches.

Monitoring the changes in vaginal epithelial cells during proestrus and estrus by vaginal cytology showed first appearance of cornified cells after beginning of proestrus by 4 days, then increased in percent with the progression in days of proestrus till reach 85% or more on the day of ovulation (Day 12 from beginning of proestrus). These results are comparable with previous observations and records **(**16-18). Our results also agreed with Moxon et al. **(**2) who reported a decrease in cornified cells percent post ovulation then followed by sharp decrease at the beginning of diestrus. Appearance of cornified cells within low percent on Day 4 from the beginning of proestrus attributed to increase in estrogen hormone concentration, and then reached a high concentration at the beginning of estrus where cornification index was 70%. Slowdown in cornification index after ovulation was caused by declining of estrogen concentration and increasing in progesterone concentration, and then disappeared completely at the beginning of diestrus due to high concentration of progesterone as a result of corpus luteum formation.

Measurement of progesterone concentration by chemiluminescence assay (CA) showed that progesterone was present in low values at the beginning of proestrus due to high estrogen concentration which secreted from follicles on ovaries, and then increased to 1.5 ng/ml at the beginning of estrus due to partial lutenization of follicular layers. By measuring progesterone, the expected ovulation time was on Day 12 from the beginning of proestrus as progesterone begun to increase within ranges of 4.8 to 8.3 ng/ml. These progesterone profiles for 13 cyclic ovulatory bitches only due to the other 5 bitches shown deviation in progesterone concentration than normal values. The current findings are in accordance with Johnston and Root, Concannon **(**19,20), who noticed that progesterone concentration at the day of ovulation ranged from 4 to 10 ng/ml due to complete luteinization of follicular layers. Consequently, the optimal time of breeding was

within 2 days after ovulation till the occurrence of maturation of primary oocyte into secondary oocyte which is fertilized by sperms (21).

The vaginal pH measurement is an important parameter to show certain biological patterns and also to determine the optimal time of insemination in the bitch. pH values during proestrus which were reported by Antonov et al. (11) Schulz (22) and Ross (23) were ranged from 6.9 to 8.2, then decreased toward the ovulation time within a range 6.5 to 6.8 and continued at these low values till insemination, then rose again on the way to diestrus onset, exactly analogous with our findings of PH value during ovulation time (6.8). However, Vaginal PH values were low during ovulation and in the days after wards when mating occurs to almost coincide with PH of seminal plasma and providing favorable conditions for survival of spermatozoa in the female reproductive tract for long period ranged from 4 to 6 days.

By using vaginal endoscopy for examination of mucosal folds, it was noticed that vaginal mucosal folds were edematous with bloody discharge during proestrus due to high concentration of estrogen hormone which caused water retention and diapedesis of erythrocytes. After that, mucosal folds were wrinkled and appeared white in color due to an abrupt withdrawal of the water retention effect of estrogen. These findings coincide with Lulich (24) and Xavier (25), who reported that the prominent vaginal endoscopic image during ovulation is wrinkled vaginal mucosa as a result of sharp decrease of estrogen. The optimal time of breeding was after appearance of more wrinkled vaginal mucosal folds as a confirmed result of ovulation occurrence.

The last method for examination was ultrasonographic of ovaries for following up the structures on it and progression of them to predict the ovulation time. Using real time B mode ultrasound at the first days of proestrus, it revealed the presence of small anechoic follicles within small diameters then with progression of days, these diameters increase till reach to 8–10 mm at the first day of estrus. These results were similar to Yeager and Concannon (26), who reported that proestrus stage is characterized by presence of small anechoic structures on ovaries. Identifying the ovulation moment by ultrasound is difficult due to presence of similarity in shape of follicles and corpus luteum to some extent as both contained retained follicular fluid. Repeated follow up of ovarian structures may help in identifying its structures. Our study revealed that changing ovarian structures from anechoic numerous structures with large diameter to hypoechoic smaller structure is considered the time of ovulation in parallel with the study of Renton et al. (7) that referred to formation of hypoechoic corpus luteum with small anechoic cavity post ovulation. In two bitches out of 18 bitches used in this study, serum progesterone concentrations did not elevate over 1.43 ng/ml, although, their entrance to the cytological estrus phase and accepting male dog. This was recorded anovulation where corpus luteum has no been formed and therefore progesterone was not elevated. The anovulatory cycles have been previously reported as uncommon **(**27). Meyers-wallen **(**13) stated that the possible causes of anovulation were either, the hypothalamus did not secret sufficient GnRH or pituitary gland did not secret enough LH or ovary did not well respond to these hormones due to problems in it. Interestingly, one German shepherd bitch responded to hCG administration, then after 3 days, progesterone concentration was elevated as may be the possible cause was insufficiency of LH secretion, but another bitch did not respond to treatment with hCG due to the presence of granulosa cell tumor which discovered by histopathological examination. There is no available literature regarding bitches with persistent proestrus that is characterized by continuous bloody discharge for a duration ranged from 35 to 40 days as reported in this investigation. Meyers-wallen (13) reported the occurrence of split heat which was defined as abnormally short period of proestrus or estrus accompanied by low serum progesterone value as in anovulatory cycle. Meyers-wallen (13) mentioned the persistent estrus for a period 2 months with normal proestrus period due to insufficient LH secretion. To our knowledge, this is the first study which referred to persistent proestrus. Secretion of bloody discharge conti-

nuously for a range 35 to 40 days may be caused by low LH surge, so follicles persist on ovaries and secret estrogen for that previous period which leads to continuous secretion of these discharges.

Conclusion

Progesterone assay and vaginal endoscopy are the accurate methods for predicting the optimal time of mating as progesterone hormones concentration increased only after ovulation and mating not occur except after ovulation, and also vaginoscopy can declare vaginal changes during ovulation. So following up cyclic bitches and applying clinical assessments, especially two cited above help detecting the optimal time of breeding and judging the estrus cycle)normal or abnormal) as well as achieving the high percent of fertility in bitches. The early interference in anovulatory cases with hCG administration enables us to correct this disorder and reduce cases of infertile bitches.

Conflicts of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

MOLECULAR INVESTIGATION OF ANTI-DIABETIC EFFECT OF *BALANITES AEGYPTIACA* FRUITS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

Maha M. El Deib[1]*, Haytham A. Ali[1,2]

[1]Biochemistry Department, Faculty of Veterinary Medicine, Zagazig University, 44511Egypt, [2]Biochemistry Department, Faculty of Science, University of Jeddah, Saudi Arabia

*Corresponding author, E-mail, dr.mmeldeib@yahoo.com

Abstract: *Balanites aegyptiaca* (*B. aegyptiaca*) is an anti-diabetic medicinal plant traditionally used in Egyptian folk medicine as alternative therapy for the treatment of type 2 diabetes. No available studies revealed the mechanism(s) of the associated anti-diabetic effect especially at the molecular level. This study aimed to explore the possible molecular mechanism(s) that underline *B. aegyptiaca* fruits aqueous extract administration in diabetic and non-diabetic rats. Four equal groups (n=10) of albino rats were used. The prepared aqueous extract of *B. aegyptiaca* fruits was given orally (80 mg/kg body weight for 4 weeks) to normal control and streptozotocin (65 mg/kg BW, i. p.) -induced-diabetic rats. Administration of *B. aegyptiaca* fruits aqueous extract in diabetic rats significantly elevated the serum insulin (91%) and reduced serum glucose (54%), cholesterol (26%), triglycerides (16%) and LDL cholesterol (25%) compared to the diabetic control. Produced hypoglycemia in treated diabetic rats simultaneously accompanied at least by significant improving (p<0.05) of pancreatic insulin and α-amylase; hepatic insulin receptor A, glucose transporters (GLUT-2 and GLUT-4), and adipocyte leptin gene expressions. In conclusion: The anti-diabetic effect of *B. aegyptiaca* fruits aqueous extract was achieved by increasing insulin level as well as stimulating endogenous insulin secretion and enhancing its action at the target tissues. So it covered at least most of the main therapeutic strategies of diabetes. More studies are needed for preparation of a standardized dose and dosage regimen of active constituents of this promising fruit that can play a significant role in the management of type 2 diabetes and related complications.

Key words: *Balanites aegyptiaca*; streptozotocin; α-amylase; insulin receptor A; GLUT-2; GLUT-4; leptin

Introduction

Diabetes mellitus (DM) is one of the most common chronic diseases that cause serious damage to many different body systems, especially nerves and blood vessels**.** The second type of diabetes (type 2) is the most public form of diabetes due to defects in the secretion of insulin or insulin resistance**.** More than 80% of people living with diabetes are present in low- and middle-income countries as reported by WHO and the mortality is expected to double between 2005 and 2030 (1).

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Side effects and limited efficacy associated with the oral anti-diabetic drugs leads to increased demand of research on natural anti-diabetic products. Herbal remedies have been used as alternative medicines for the treatment of many diseases, including DM due to their remarkable effectiveness, relatively low costs, and the low side effects of their use (2). One of the most widely used in this field is *Balanites aegyptiaca* (*B. aegyptiaca)*.

Balanites aegyptiaca (L.) Delile, belongs to the family Zygophyllaceae probably called the 'desert date' (Heglig in Arabia). It is an evergreen tree present in wild areas and savannah areas in Africa and South Asia. Mesocarp of fruit contains 1.2 to 1.5% protein, 35 to 37% sugars, 15% organic acids and other constituents. Phytochemical studies on *B. aegeptiaca* isolated several classes of metabolites many of which possess biological activities such as cumarins, flavonoids and steroidal saponins. Balanitoside (furostanol glycoside) and 6-methyldiosgenin, balanitin-3 (spirostanol glycoside) and Balanitin-6 and -7 have been reported from fruits (mesocarp) of *B. aegyptiaca*. The different parts of the plant (leaves, bark, fruit or root) characterized by multipurpose medicinal application as antidiabetic, hypocholesterolemic, hepatoprotective, cardioprotective**,** antioxidant, antiviral, antibacterial, anti-inflammatory, anti-nociceptive, analgesic and anthelmintic (3).

In Egyptian folk medicine, its fruits mesocarp extract is traditionally used as a hypoglycemic agent **(**4-6) and as an anti-diabetic (7). The exact mode of action of this extract is still unclear. Some previous *in vitro* studies suggested that *B. aegeptiaca* may produce its hypoglycemic action through an intestinal reduction of glucose absorption by inhibiting α-amylase activity that considered as the first line therapy in diabetes treatment (5). Another hypothetical possible mechanism suggested that the hypoglycemic action might be produced through potentiation of insulin secretion from β-cells or due to glucose transport enhancement to peripheral tissue **(**6).

To our knowledge, there are no available studies revealed the possible anti-diabetic molecular mechanism(s) of *B. aegyptiaca* fruits extract on normal or diabetes-induced experimental animals. For this reason, the current study was planned to explore, *in vivo*, the possible mechanism (s) by which *B. aegyptiaca* fruits aqueous extract may succeed to produce hypoglycemic or anti-diabetic effect in streptozotocin (STZ) - induced diabetic (hyperglycemic) and normal (normoglycemic) rats.

Material and methods

Preparation of B. aegyptiaca fruits aqueous extract

The *B. aegyptiaca* fruits were purchased from a commercial source; local markets in Sharkia Governorate. According to the commercial produced company (Al Tahhan company to fill the dates), the fruits were obtained from Heglig trees that grow in the southern desert of Egypt; New Valley. The dried fruits (1 Kg) were soaked in distilled water for 24 h and filtrated after the seeds were discarded. This freshly prepared filtrate was freeze dried (using labcono, freeze dryer, model 18) to give thick dark brown extract. The dosed extract was prepared (100 mg extract dissolved in 10 ml distilled water) immediately before administration. The prepared aqueous extract of *B. aegyptiaca* fruit was given orally in a dose of 80 mg/kg body weight (BW) (4) by an orogastric tube daily for 4 weeks.

Animal housing and management

Forty male albino rats were used. Their age and weight at the beginning of the experiment were approximately 6 months and 120±20 gm. Rats were acclimatized for two weeks under standard laboratory conditions including good aerated room with suitable temperature, provided *ad libitum* with food and drinking water. The experimental procedures were conducted according to the guidelines for experimental animal care of the Faculty of Vet. Med. Zagazig University, Egypt and approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary.

Animal grouping

Rats were divided into four groups (n=10): the first group served as normal control,

received saline, the second group received *B. aegyptiaca* aqueous extract in a dose of 80 mg/kg BW. The 1st and 2nd groups assigned as normoglycemic groups. Diabetes type 2 was induced in the 3rd & 4th groups by a single intraperitoneal injection of STZ (Sigma-Aldrich Co., USA) freshly prepared, dissolved in 0.01 M cold sodium citrate buffer (pH 4.5) immediately before use, in a dose of 65 mg/kg BW (8). The third group used as diabetic control while the fourth group treated with *B. aegyptiaca* aqueous extract in a dose of 80 mg/kg BW. To avoid the circadian rhythm, each of the drug or saline was administered between 7:00 and 8.00 am. The dosing was for 4 weeks using orogastric tube. STZ-injected rats were fed with glucose solution (5%) next 24h to avoid drug induced hypoglycemia. After three days, rats with fasting blood glucose levels >200 mg/dl were considered as diabetic rats.

Sampling

At the end of experimental period, over-night-fasted rats were scarified. Blood samples were individually collected and the sera were separated and stored at -20°C until used for biochemical investigation. Liver, pancreas and adipose tissues were rapidly taken on liquid nitrogen for molecular analysis.

Biochemical analysis

Calorimetrically serum glucose, total lipids (TL), triacylglycerol (TAG), total cholesterol (TC) and HDL-c levels were determined by SPINREACT kit according to manufacture instructions (kit obtained from Girona, Spain). Serum insulin levels were determined by specific ELISA kit for rat insulin according to manufacture instructions (Cat. No. ezermi-13 kelisa, Billerica, MA, USA). LDL-c was estimated by Friedewald formula (9).

Relative quantitative polymerase chain reaction (RQ-PCR)

Hepatic, pancreatic and adipose tissue total RNA were extracted from liquid nitrogen saved samples, grinding occurred in sterilizing mortars and 30 mg were used for extraction using RNeasy Mini Kit (Qiagen, Germany, cat. no. 74104). One µl of extracted total RNA was checked for purity and quantity at OD260/OD280 by NanoDrop® ND-1000 Spectrophotometer, (NanoDrop Technologies, Wilmington, Delaware, USA). Only samples with purity more than 1.8 were used for frequent steps. First strand cDNA was synthesized using Revert Aid™ First Strand cDNA Synthesis kit (Fermetas, life science, Pittsburgh, PA, USA). Relative quantitative real-time PCR using cDNA, QuantiTect® SYBR® Green PCR kit (Qiagen, Germany) and designed primers for insulin, IRA, GLUT2, GLUT4, α-amylase and leptin genes using a Rotor-Gene Q cycler (Qiagen, Germany). The conditions of PCR were 95℃ for 5min, 30 cycle (95℃ for 45sec, 60℃ for 30sec and 72° C for 45sec) for insulin 1 and Glut-4; 95℃ for 4min, 35 cycle (95℃ for 30sec, 58℃ for 30sec and 72° C for 30 sec) for IRA, Glut-2 and β-actin; 94℃ for 4min, 35 cycle (95℃ for 30sec, 56℃ for 30sec and 72° C for 30 sec) for amylase and leptin genes. β-actin gene was used as a constitutive control for normalization. Primers were designed according to references listed in Table (1). $2^{-\Delta\Delta Ct}$ were estimated and the relative fold changes were calculated according to the method of Litvak and Schmittgen (10).

Statistical analysis

All data are expressed as mean ± SD. Data was compared among groups using One-way analysis of variance (ANOVA) with statistical package for social science (SPSS, 21 software, 2015). For testing the inter-grouping homogeneity, the Duncan's multiple rang test was applied. *P* values of less than 0.05 represent statistically significant difference.

Results

Induction of diabetes type 2 by STZ caused a significant reduction in the relative mRNA expression of pancreatic α-amylase and insulin; hepatic insulin receptor A (IRA) and GLUT-2; adipocyte GLUT-4; with significant increase of leptin hormone in comparison with the normal control rats. Molecular investigations in normoglycemic rats treated with *B. aegyptiaca*

Table 1: The primer sequences used in this study

Target Gene	Primer Sequence (5'→3')	Product size(bp)	Gene bank ID	References
Insulin1	F: ATGGCCCTGTGGATGCGCTT R:TAGTTGCAGTAGTTCTCCAGCT	331	3630	(11)
IRA	F: TTCATTCAGGAAGACCTTCGA R:AGGCCAGAGATGACAAGTGAC	222	24954	(12)
GLUT-2	F: TTAGCAACTGGGTCTGCAAT R: TCTCTGAAGACGCCAGGAAT	243	25351	(11)
GLUT-4	F: GAGCCTGAATGCTAATGGAG R: GAGAGAGAGCGTCCAATGTC	187	442992	(13)
α-amylase	F: TTGCGTTCAGGAGACCAAC R: CATAGGTTTGTGAGGCGGT	158	24203	(14)
Leptin	F:ATCAAGACCATTGTCACCAGGATC R:CTGGTCCATCTTGGACAAACTCA	129	25608	(15)
ß-actin	F- TCACTATCGGCAATGTGCGG R- GCTCAGGAGGAGCAATGATG	260	81822	(12)

IRA = insulin receptor A; GLUT2 = Glucose transporter 2; GLUT4 = Glucose transporter 4.

watery extract showed no significant differences with the normal control. In the opposite, STZ diabetic rats treated with *B. aegyptiaca* (group 4) produced a significant improvement of all relative gene expressions of α-amylase, insulin, IRA, GLUT-2 and GLUT-4 in comparison with diabetic non treated rats but they still significantly less than that of the control. Relative leptin mRNA expression significantly decreased under the effect of *B. aegyptiaca* treatment in comparison with diabetic non treated rats but it still higher than control level (Figure 1).

Treatment with *B. aegyptiaca* significantly decreased serum levels of TL, TC in non-diabetic rats than control group. While STZ-diabetic rats showed significant elevation of serum fasting blood glucose, TL, TAG, TC and LDL-c, whereas serum insulin and HDL-c levels were significantly decreased. Treatment with *B. aegyptiaca* fruits successfully ameliorated these bad effects through decreasing the serum fasting blood glucose (54%), TL (40%), TAG (16%), TC (26%) and LDL-c (25%) and increasing the levels of serum insulin (91%) and HDL-c (43%) compared to diabetic rats but they did not reach to the levels of control group (Table 2).

Table 2: Blood glucose, insulin levels and lipid profile in STZ-diabetic and non- diabetic rats treated with *B. egyptiaca* aqueous extract (80 mg/kg BW)

Parameters	Control	Non diabetic+*B.egyptiaca*	Diabetic control	Diabetic+ *B.egyptiaca*
Blood glucose (mg/dl)	103.32±5.76^{c}	89.21.8c ± 1.7^{c}	418.4±12.43^{a}	193 ± 3.5^{b}
Insulin (µIU/ml)	0.963±0.004^{a}	0.97 ± 0.006^{a}	0.4± 0.002^{c}	0.763 ±0.08^{b}
Total lipids (mg/dl)	308.4 ± 4.28^{c}	299.4 ± 1.9^{d}	824.4± 10.5^{a}	493.7± 5.5^{b}
Triacylglycerol (mg/dl)	105.42±3.21^{c}	96.07 ± 3.4^{c}	138.2± 4.32^{a}	116.6 ± 1.53^{b}
Total cholesterol (mg/dl)	164.5± 3.42^{c}	156.4 ± 1.7^{d}	226.8 ± 4.3^{a}	197.9 ± 1.6^{b}
HDL-c (mg/dl)	65.4±3.25^{a}	63.13 ± 1.7^{a}	36.8±0.87^{c}	52.56±0.98^{b}
LDL-c (mg/dl)	74.43±2.48^{c}	71.17 ± 1.7^{c}	109.3±3.2^{a}	81.5 ± 0.43^{b}

All data are expressed as mean ± SD.

Means which have different superscript in the same row are significantly different from each other at $p<0.05$ and vice versa.

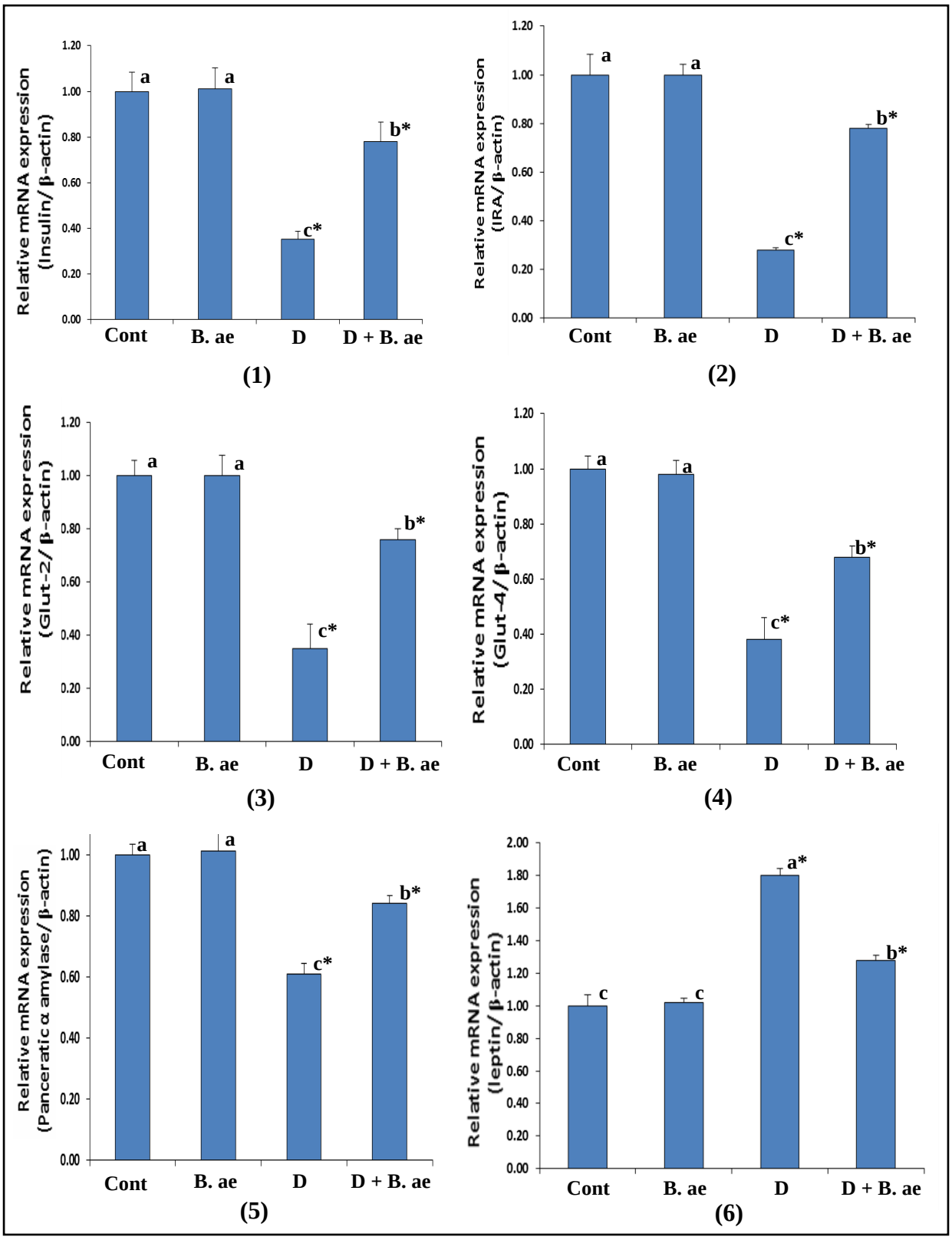

Figure 1: Effect of *B.aegptiaca* fruits extract (B.ae) (80 mg/kg BW) on the relative mRNA expression of insulin 1 (1), insulin receptor A (IRA) (2), glucose transporter 2 (GLUT2) (3), glucose transporter 4 (GLUT4) (4), α-amylase (5) and leptin (6) in STZ diabetic and non- diabetic rats. Cont = control=group 1, B.ae=group 2 treated with *B. aegyptiaca*, D= Diabetic= group 3, D+ B.ae =Diabetic rats treated with B.ae=group 4

Results

Induction of diabetes type 2 by STZ caused a significant reduction in the relative mRNA expression of pancreatic α-amylase and insulin; hepatic insulin receptor A (IRA) and GLUT-2; adipocyte GLUT-4; with significant increase of leptin hormone in comparison with the normal control rats. Molecular investigations in normoglycemic rats treated with *B. aegyptiaca* watery extract showed no significant differences with the normal control. In the opposite, STZ diabetic rats treated with *B. aegyptiaca* (group 4) produced a significant improvement of all relative gene expressions of α-amylase, insulin, IRA, GLUT-2 and GLUT-4 in comparison with diabetic non treated rats but they still significantly less than that of the control. Relative leptin mRNA expression significantly decreased under the effect of *B. aegyptiaca* treatment in comparison with diabetic non treated rats but it still higher than control level (Figure 1).

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Discussion

B. aegptiaca succeeds to induce hypoglycemic effect in the hyperglycemic diabetic rats but not in normo-glycemic ones that is a desirable feature as hyperglycemia was found to cause a cascade of adverse effects (16).

The observed hyperglycemia and hypo-insulinemia in STZ diabetic rats in the present study are consistent with several previous studies (17,18). STZ in a single low dose induced type 2 diabetes characterized by partial destruction of pancreatic β-cells (19) which induced hyperglycemia and leaved many of surviving β- cells can be regenerated (20). Enhancement of such regeneration is flourished by administration *Balanites* extract as it is rich in flavonoids (21). Flavonoid genistein (a soy derived isoflavone) was found to reduce β-cell apoptosis, preserve islet mass and promote islet β-cells survival (22). In support Helal et al. **(**23) reported that aqueous extract of *B. aegyptiaca* (seeds) was able to ameliorate beta-cell dysfunction in alloxan induced diabetic rats. Those stimulate insulin synthesis and release by the β-cells of the islet of Langerhans expressed as elevation of insulin gene expression and raising its level in the blood. Consequently, insulin decreased the elevated blood glucose level by 54%, as it accelerates the rate of glucose uptake from blood vessels into peripheral tissues (24). *Balanites* fruit extract and *Fenugreek* extract were able to reduce blood glucose level by 24% and 58% respectively in STZ-diabetic rats (5). That was through induction of the gene expression of insulin receptor (IRA) and glucose transporter (GLUT2) in hepatic cells. Both of them form a receptor-transporter complex on the rat hepatocyte membrane that forms a mechanism of insulin-mediated hepatic glucose regulation (25). Studies on GLUT2-null mice proved that GLUT2 orderly the function of central glucose sensors (26). The GLUT4 transporter is functionally characterized as an insulin-responsive glucose transporter. It was redistributed from an intracellular location to the plasma membrane following insulin stimulation (24). Glucose transporters; GLUT2 and GLUT4 play key roles in the control of blood glucose concentrations (27). Induction of these transporters with *Balanites* treatment impressive an anti-diabetic effect of *Balanites*.

The increment of insulin under the effect of *B. aegptiaca* fruits aqueous extract in our *in vivo* study ran in parallel with an *in vitro* study (28) which demonstrated that *B. aegyptiaca* stimulates insulin secretion and increases the number and affinity of insulin receptors in β-cells. Recently in 2016, *B. aegyptiaca* fruit aqueous extract was found to increase plasma insulin and liver

pyruvate kinase (L-PK) levels in STZ induced diabetic rats (29). L-PK responsible for increased liver glucose utilization and catalyzes the conversion of phosphoenolpyruvate to pyruvate, the last irreversible steps of glycolysis in the liver (30,31).

Inhibition of carbohydrate–hydrolyzing enzymes, α-glucosidase and α-amylase considers the first line therapies in diabetes treatment by reducing post-prandial glucose levels. Pancreatic α - amylase play of an important role for breakdown of α (1,4) glycosidic linkages of starch and other glucose polymers (32). The inhibition of α-amylase activity determines the anti-diabetic potency. Pancreatic α- amylase gene expression in our *in vivo* study behaves like insulin; it reduced in diabetic rats and induced under the effect of *B. aegyptiaca* fruit aqueous extract treatment. It may be because of 5'-flanking region of the pancreatic amylase gene that contains an insulin-dependent element mediates the loss of expression in diabetic animals (33). It was found that insulin administration *in vivo* has a stimulant effect on amylase activity in the pancreatic acinar cells of STZ diabetic rats, which confirm this relation (34).

In contrast in *in vitro* studies, it was found that *B. aegyptiaca* bark aqueous extract have α-amylase inhibitory activity between 45-75 % at 200 mg/ml concentration (35). And it was able to inhibit intestinal alpha-amylase activity in dose - dependent manner (5). Recently in 2018, Gawade and Farooqui (36) reported that the bioactive phytochemicals present in *B. aegyptiaca* leaves ethanol extract shows *in vitro* alpha amylase inhibition activity.

The observed gap between *in vivo* and *in vitro* studies revealed that the anti-diabetic effect of *Balanites* fruit, *in vivo*, is not dependent on pancreatic α-amylase gene expression. This gap raises an interesting question; whether the increase of pancreatic α-amylase gene expression in the treated animals was a compensatory response to α –amylase inhibition? To answer this question the enzyme activity should be estimated before that must be taken into account in the future. To date there is no experimental indication of pancreatic amylase enzyme and adipose leptin hormone gene expressions in *Balanites* treated diabetic rats. On the other hand, the differences of the used doses, the extracted different parts of the plant and the used methods of extraction may increase this gap.

Leptin is a polypeptide hormone releases from adipocytes. Its production is controlled by the ob/gene. Leptin play a role to control food suppressing and stimulates energy expenditure which lowers body weight (37,38). Hyperin-sulinemia increased serum leptin as they correlated to each other (39).

Hypoglycemic and insulinomimetic effect of the extract together with the elevation of leptin hormone in adipocytes, herein, and probably in serum (4) may be the cause of decreased serum TL and TAG. Consequently they improved lipid profile by decreasing TC, LDL-c and increasing HDL-c. The enhancement of lipase activity in the pancreatic acinar cells by insulin (34) may increase over mobilization of lipid from blood vessel to liver and decrease hepatic lipogenesis mechanism that result in decrease lipid mobilization to blood.

In harmony, anti hyperglycaemic and anti - hyper lipidemic activity of *B. aegyptiaca* were previously reported (4,40). Morsy et al. (6) suggest this fruit as a factor for diabetes and hyperlipidemia control.

Conclusion

This investigation provides new evidence about the possible molecular mechanisms of anti-diabetogenic effect of *B. aegyptiaca* fruit aqueous extract in STZ induced diabetic rats. The extract succeeds to reduce insulin demand as well as to stimulate endogenous insulin secretion and enhances its action at the target tissues. So it covered at least most of the main therapeutic strategies of diabetes treatment. Reduced blood lipid could be an additional benefit in diabetes management. The promising obtained result encourages the using of *B. aegyptiaca* fruit aqueous extract in traditional folk medicine for the management of type 2 diabetes and its related complications. Complementary studies are needed for

preparation of a standardized dose and dosage regimen of active constituents.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

PREVALENCE AND PATHOLOGICAL FEATURES OF OVINE LUNGWORM IN NILE DELTA

Abd El-Monem A. Ali[1], Mohamed M.M. Metwally[1]*, Nosseur M. El-sayed[2]

[1]Department of Pathology, Egypt, [2]Department of Parasitology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt

*Corresponding author, E-mail: metywally@gmail.com

Abstract: A cross-sectional study was conducted from March 2017 to March 2018, to determine the prevalence of ovine lungworm infection, and to describe the related pathological lesions in naturally infected local breed sheep. A total of 200 whole lungs were collected from fifteen slaughter houses in Nile Delta, Egypt. The overall prevalence for ovine lungworm infection was 4.5%. The prevalence rates in Ossimi, Rahmani, and Barki breeds were 5.55, 4.28, and 2.5%, respectively. The only identified species of lungworms was *Dictyocaulus filaria* (*D. filaria*) (100%). Grossly, the fundamental lesions were multiple patchy consolidated pulmonary tissues with the presence of adult parasites mixed with foamy and sometimes mucous exudate in the lumens of trachea and bronchi. Histopathologically, alveolitis, obstructive bronchiolitis, bronchiolar hyperplasia, emphysema, granulomatous pneumonia, pleural thickening, peribronchiolar lymphoid hyperplasia and trapped different developmental stages and adult parasites besides degenerated and necrotic parasites in the pulmonary tissue were the hallmarks of the histopathological features. In conclusion, ovine lungworms were prevalent in sheep at Nile Delta, Egypt, causing significant damage to the lung and produce characteristic gross and microscopic lesions.

Key words: sheep; lung worm; Nile Delta; *D. filarial*

Introduction

Sheep are multipurpose animals, but in Egypt, they are raised principally for meat production and they participate by about 6.5% of local red meat production (1). Lungworms (dictyocaulosis or husk) had a worldwide distribution with important economic impacts, especially in temperate regions (2-4). Although *Dictyocaulus filaria* (*D. filaria*) is not the most ubiquitous ovine lungworm but it represents an important cause of respiratory disease in sheep particularly in Mediterranean countries (5). Geographically, the prevalence of lungworm infection in ruminants varies greatly (6). Their prevalence ranges between 46.0 to 66.3% in Ethiopia (6-8), 89 to 95% in France (9,10), 26.6% in India (11) and 4.72% in Egypt (12). The clinical signs and pathological lesions of lungworm infestations are dependent principally on the number of invading larvae, host immunity and stage of infection (5). Mild infections may have no clinical signs and pass unnoticed, but, in general, the most associated clinical signs include coughing, tachypnea, nasal discharge, depression, weight loss, and rarely death (5,13). The main histopathological lesions in foreign breeds were interstitial

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pneumonia, granulomatous pneumonia, chronic catarrhal bronchitis, bronchiolitis associated with presence of adult worms, eggs and other developmental stages in the bronchi and large bronchioles (5,11,14). However, the detailed gross and histopathological changes in native breeds present in Nile Delta were not previously described. Therefore, this study was designed to determine the prevalence of ovine lungworm infection, and to describe the related pathological lesions in naturally infected local breed sheep.

Material and methods

Study protocol

A cross-sectional study was conducted in the period from March 2017 to March 2018, on 200 native breed sheep, their ages were over 6 months, slaughtered in fifteen Nile Delta abattoirs. A pre-slaughter inspection for each animal was done where the case history, age, breed, sex and general health condition were recorded. Animals were divided into 2 age groups; young (6 months to one year) and adults (more than one year). The age of animals was determined by dentation (15) and owner's information. The body condition of the slaughtered animals was categorized into 4 groups; obese, fat, average and thin (16). There were no Ossimi (90 animals), Rahmani (70 animals) and Barki (40 animals).

Parasitological and pathological examination

Immediately after slaughter, the whole lungs were collected, and any detectable gross lesions were recorded. Dissections were made along the trachea and bronchial tree and searched for the presence of adult worms and all visual parasites were collected. The recovered nematodes were cleaned with physiologic saline, fixed in 70% ethyl alcohol, then cleared in lactophenol and mounted in emaciated slaughtered animals. Geographically, the study was conducted in Nile Delta region in northern Egypt, between 31°44'N altitude and 30°42'E longitude. The sheep breeds under study were polyol (Sigma-Aldrich Chemie GmbH, Cat. No, 68133-07-3, Kappelweg 1, 91625 Schnelldorf, Germany). All worms were identified according to morphological and morphometric characteristics of parasites (17,18). For histopathological examination, representative tissue specimens from grossly affected lungs were collected and immediately fixed in 10% neutral buffered formalin. Paraffin sections of 5-micron thickness were prepared, stained with Hematoxylin and Eosin (19) then examined microscopically**.**

Statistical analysis

All statistical operations were performed using statistical analysis software, SAS statistical system Package V9.1. Univariate logistic regression model was fitted through Maximum Likelihood (ML) procedure to assess the effect of independent variables [age, sex, breed, season and body condition], on the dependent dichotomous variable (incidence of lung worms). Reference category for the comparison of crude odds ratios (CORs) was nominated as R. P-values of less than 0.05 considered to have a statistically significant association with studied traits.

Results

Physical Examination

Pre-slaughter physical examination revealed that all animals were apparently healthy, but 10.5% (21/200) of sheep had mild to minor respiratory signs (tachypnea, nasal discharge, and cough), 17 (80.95%) out of the 21 sheep which had respiratory signs had a thin body condition.

Parasitological examination

Nine out of the 200 (4.5%) native sheep examined in the Nile Delta abattoirs were positive for *D. filaria* infection. The worms (*D. filaria*) were recovered from bronchus and bronchioles of lungs of the slaughtered sheep. They are thin slender, creamy white in color and up to 8-10 cm long. Microscopically they have very small four lips and very small shallow buccal capsule. The posterior end of the adult male *D. filaria* has copulatory bursa having short, stout, dark brown spicules (Figure 1).

Prevalence of lungworm infection

Sheep of all ages were susceptible, but animals less than one year showed lower infection rate (3.33%) than adult (5.45%). Sex had no influence on the prevalence of infection with *D. filaria* in the examined sheep as the prevalence in females was 4.44% and in males was 4.51%. Breed variation was clear where the prevalence of infection in Ossimi sheep was 5.55%, while in Rahmani sheep was 4.28%, and in Bakri sheep was 2.50%. The influence of seasons on the prevalence of *D. filaria* in sheep was significant ($p < 0.05$) (as the prevalence fluctuated from low in summer (2%) and autumn (2%) to moderate in spring (4%) and high in winter (10%). On the basis of body condition, higher rate of infection with *D. filaria* was observed in animals with thin body condition (23.52%) compared to that having average (6.25%), fat (2.08%), or obese (0.0%) body condition. The prevalence results were summarised in Table (1).

Table 1: Prevalence of ovine lungworms in Nile Delta (March 2017-March 2018)

Risk factors		Examined cases	Positive cases	Prevalence (%)	OR	95 % CI	p-value
Age	Young (≤ year)	90	3	3.33%	1	-	-
	Adult (>year)	110	6	5.45%	1.67	0.41-2.88	0.476
Sex	Male	155	7	4.51%	1	-	-
	Female	45	2	4.44%	0.98	0.15-2.69	0.914
Breed	Ossimi	90	5	5.55%	1	-	-
	Rahmani	70	3	4.28%	0.76	0.18-2.58	0.752
	Barki	40	1	2.50%	0.44	0.05-2.85	0.342
Season	Winter	50	5	10%	1	-	-
	Spring	50	2	4%	0.56	0.21-1.64	0.242
	Summer	50	1	2%	0.18	0.05-0.86	0.049*
	Autumn	50	1	2%	0.18	0.05-0.86	0.049*
Body condition	Obese	39	0	0.0%	1	-	-
	Fat	96	2	2.08%	1.23	0.71-2.73	0.262
	Average	48	3	6.25%	3.46	1.08-4.26	0.034*
	Thin	17	4	23.52%	6.89	3.41-12.60	0.016*

OR= Odd ratios; R= reference value and * Significant differences.

Gross and histopathological examination

Grossly, lungs of affected animals displayed thin cylindrical creamy white worms in a frothy material within the bronchi, pleural adhesions, and patchy consolidated areas, particularly in the caudal lobes. Microscopically, the lesions of verminous pneumonia with variance in the degree of severity were seen in all examined infected lungs. These lesions involved the parenchymal and interstitial tissue. There was alveolitis represented by thickening of the alveolar wall by edema, hyperplasia of type II pneumocytes and mononuclear cell infiltration. The alveolar lumens contained pale eosinophilic material and inflammatory cells mainly eosinophils, with fewer lymphocytes, plasma cells and macrophages (Figure 2A). The lumens of other alveoli were stuffed with a cluster of embryonated eggs or coiled newly hatched larvae accompanied with interstitial mononuclear cell infiltrations (Figure 2B). Focal emphysematous regions with opening of alveoli to a common space accompanied with thickening of the alveolar walls by fibroplasia were seen adjacent to the areas of alveolitis (Figure 2C). Diffuse perialveolar mononuclear cell infiltrations accompanied with obstructive bronchiolitis due to occlusion of the bronchiolar lumen by parasites, eggs, larvae and desquamated epithelial cells admixed with mucous and cellular exudate comprised of eosinophils, lymphocytes, macrophages and plasma cells were seen (Figure 2D and E). Massive numbers of *D. filaria* filled the bronchial lumens were observed in few cases

(Figure 2F). Marked hyperplasia of the lining epithelium of the bronchioles and bronchi accompanied with peribronchial leukocytic infiltrations were consistent findings (Figure 3A). Goblet cell hyperplasia was seen in some lung specimens (Figure 3B). Peribronchiolar lymphoid hyperplasia with peribronchiolar emphysema was common findings (Figure 3C). Granulomatous pneumonia was frequently detected around dead larvae or eggs with centrally located caseated material surrounded by macrophages, lymphocytes and giant cells with a delineated layer of fibrous connective tissue at the periphery (Figure 3 D). Occasionally, granulomas coalesced together and replaced large portions of the pulmonary tissue (Figure 3E). Nodular aggregations of mononuclear cells without detected eggs or larvae were also observed. The majority of these aggregations were found subpleural which showed thickening due to edema, mild leukocytic infiltration and fibrosis (Figure 3F).

Figure 1: Dictyocaulus filarial detected in lungs of sheep (January, 2018); (A&B) Anterior end, scale bar: 0.4mm, 0.1mm, (C&D) male bursa, lateral and dorsal views, scale bar: 0.3mm. (The posterior end of the adult male *D. filaria* has copulatory bursa having short, stout, dark brown spicules)

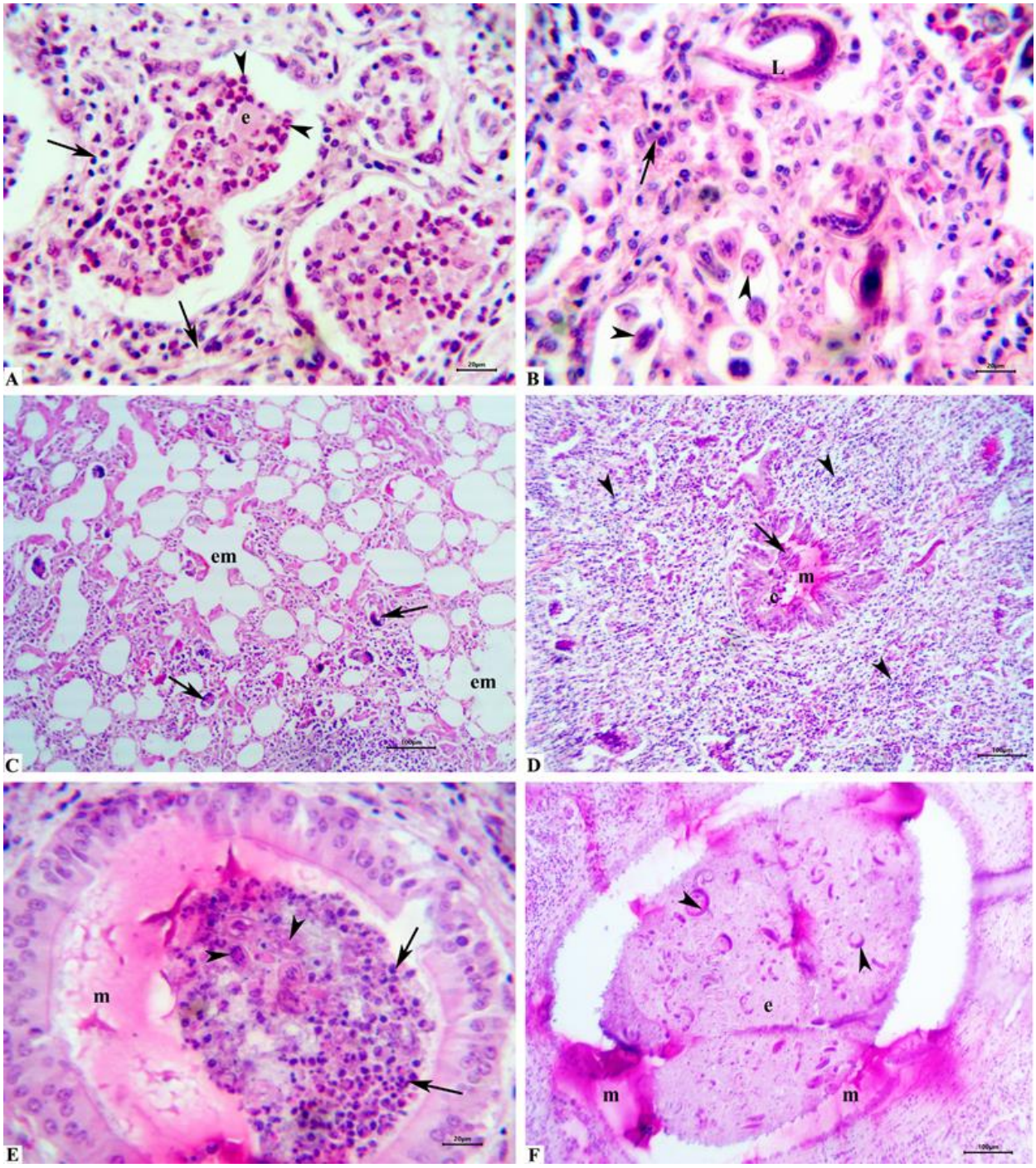

Figure 2: H&E stained sections of lungs of sheep showing; (A) thickening of the alveolar wall by edema, and mononuclear cell infiltration (arrow) and filling of the alveolar lumens with pale eosinophilic material (e) and inflammatory cells predominantly eosinophils (arrowheads), X400, (B) cluster of embryonated eggs (arrowheads) and coiled newly hatched larvae (L) in alveolar lumen accompanied with interstitial mononuclear cell infiltration (arrow), X400, (C) alveolar emphysema (em) adjacent to the areas of alveolitis with presence of eggs and developmental stages in the alveolar lumens (arrows), X100, (D) diffuse mononuclear cell infiltrations (arrowheads) and obstructive bronchiolitis by desquamated epithelial cells (arrow), mucous (m) and cellular exudate (c), X100, (E) occlusion of the bronchiolar lumen by parasites, eggs, larvae (arrowheads), mucous (m) and cellular exudate (arrow), and X400, (F) massive numbers of *D. filaria* developmental stages (arrowheads) admixed in mucous (m) and necrotic eosinophilic material (e) filling the bronchial lumen, X400

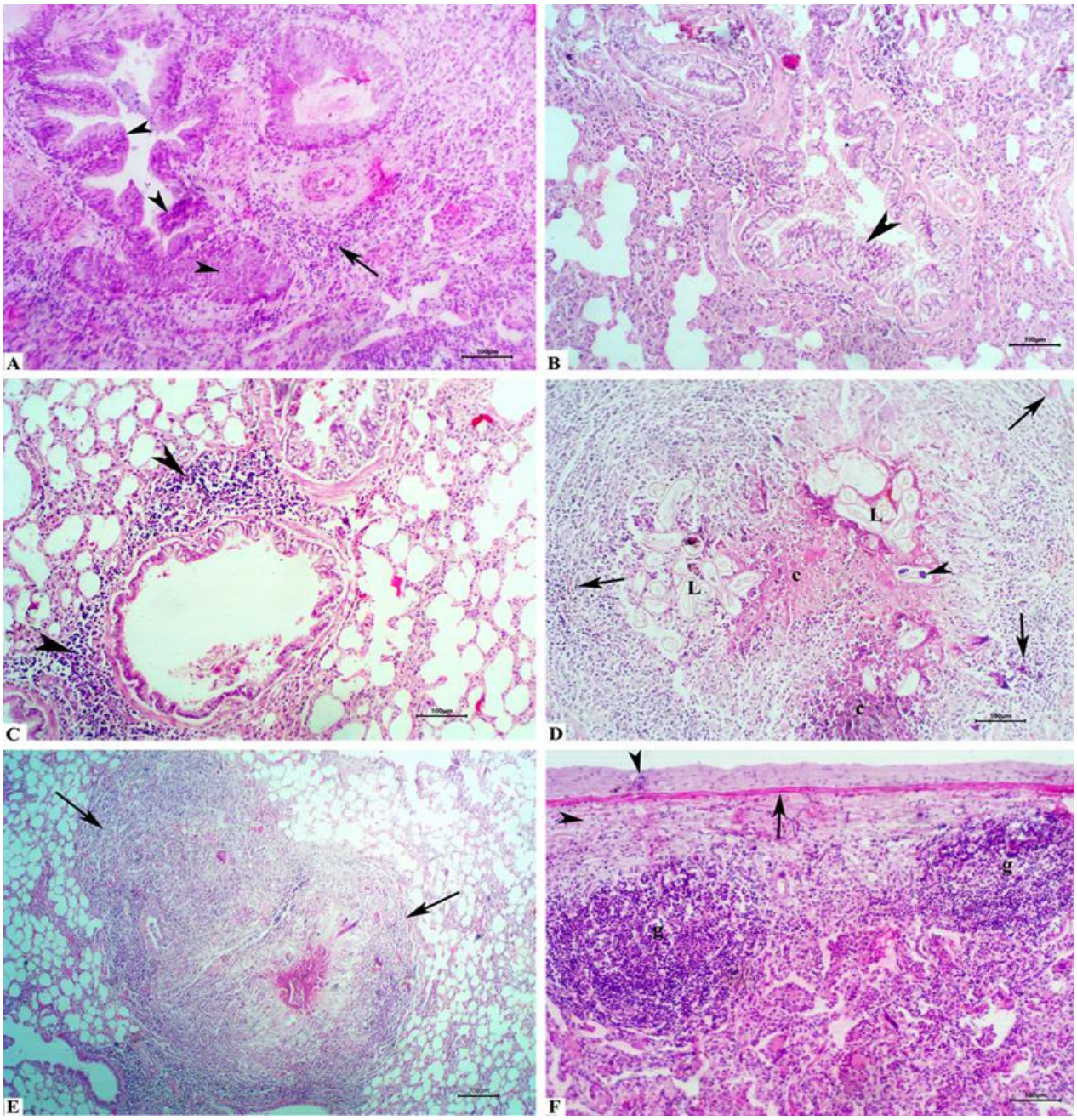

Figure 3: H&E stained sections of lungs of sheep showing; (A) marked hyperplasia of bronchiolar epithelium (arrowheads) and peribronchial leukocytic infiltrations (arrow), (B) goblet cell hyperplasia (arrowheads) (C) peribronchiolar lymphoid hyperplasia (arrows) and peribronchiolar emphysema (em), (D) granulomatous pneumonia consisted of centrally located caseated material (c) with dead larvae (L) and eggs (arrowhead) surrounded by mononuclear cells aggregations and giant cells intermingled with fibrous connective tissue (arrows), (E) Lung showing coalesced granulomas replacing large area of the pulmonary tissue (arrows), (F) Lung showing subpleural granulomas (g) and thickened pleura by edema, mild leukocytic infiltration (arrowheads) and fibrosis (arrow), X100

Discussion

The current study emphasizes that infection with *D. filaria* in sheep caused pulmonary damage and influenced the animal's body condition. The results revealed that the overall prevalence of *D. filaria* infection in Nile Delta among native breed sheep was 4.5% which is accordant with the prevalence in native breed sheep in upper Egypt "4.72%" (12), in Jordan "3.8%" (20), and Bangladesh "3.3%" (21). Few

reports showed lower infection rates than our findings; 0.19% in Iraq (22), and 2.0% in northeastern Ethiopia (23), while the majority of cross-sectional studies showed higher prevalence rates; 48.8% in Canada (24), 9.3% in Syria (25), 23.5% in Turkey (26), 9% in Iran (27), 26.6-34.09 % in India (28), 46.0-66.3% in Ethiopia (6-8), and 89-95% in France (9,10). The prevalence variations among these reports were multifactorial including geographical location, climate conditions, feeding system, and animal's breed, age, and immunity (29-31). The most important factors for *D. filaria* development were climate conditions and body conditions because a damp and cool environment and low immunity are essential (32), so high infection rates present in countries with mild temperatures (33). The lower prevalence in the current study could be attributed to native sheep are mostly raised in closed farms or farmer's houses and fed on dry ration for long period during the year. Our results indicated that there was insignificant increase ($p<0.05$) to the infection rates with age. This might be related to increasing the chance of exposure to infection by *D. filaria* with increasing the age. However, many studies mentioned a higher infection rates in young sheep (6,34,35), while others recorded that infection rate of lungworms increased with increasing the age (23,36). The prevalence rate in females was nearly the same in males (18,37), but a higher infection rates in females were also reported (6,23,38), which might be attributed to immune suppression of the female at the time of parturition and during early lactation (39). Our results declared that the highest prevalence of *D. filaria* infection was in winter (10%) related to the cool-environment which are suitable for development of *D. filaria*. The influence of body condition was highly significant ($p<0.05$) as the highest infection rate (23.52%) was found in animals with thin body condition. Similar findings were reported by many authors (37,40). This could be due to immunosuppression, malnutrition and/or emaciation (41,42). All the identified parasites were *D. filaria* which may be due to absence of intermediate hosts for other lungworms species (29).

Our detected gross and histopathological findings for ovine verminous pneumonia were consistent with previous reports (5,14). Collectively, the histopathological findings could be attributed to the immunological response of the pulmonary tissue to the adult parasite, eggs, and larvae (5). The alveolitis and interstitial leukocytic infiltration were due to an allergic reaction in response to *D. filaria* eggs and larvae in the lung tissue (26). The obstructive bronchiolitis and bronchitis with hyperplasia and desquamation of the lining epithelium were due to the persistent chronic irritation of the respiratory mucosa (13,14), with increased mucous secretion (11,43). The granulomatous pneumonia around the larvae and eggs was due to prolonged existence in the pulmonary tissue with resistance to phagocytosis and failure of acute inflammatory response to remove them (6,44).

Conclusion

In conclusion, the overall prevalence of verminous pneumonia to *D. filaria* infection in the examined native breed sheep in Nile Delta was 4.5%. These pneumonic cases suffered a significant damage to the lung and had a characteristic gross and microscopic lesion.

Conflict of interest

The authors have no conflict to declare.

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Original Research Article

EVALUATION OF *SPIRULINA PLATENSIS* ENRICHED DIET ON GROWTH PERFORMANCE, BIOCHEMICAL PARAMETERS AND IMMUNE RESPONSE OF *OREOCHROMIS NILOTICUS*

Abd-El Alim F. Abd-El Alim[1], Hesham A. Khalifa[1], Abdelhakeem El-Murr[2]*, Menna Allah Magdy[1]

[1]Department of Pharmacology, [2]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Zagazig University, 44511 Zagazig, Egypt

Correspondence author, E-mail:* hakimelmor@hotmail.com

Abstract: Two hundred and twenty five *Oreochromis niloticus* with average body weight 36±1 g, were distributed randomly into five groups in triplicates. The control group was fed on basal diet, while the other groups were supplemented with various levels of Spirulina platensis (0.5, 1, 1.5 and 2%) for 90 days. The growth performance, biochemical parameters (Alanine aminotransferase activity, Aspartate aminotransferase, Creatinine and cortisol), antioxidant effect (Reduced Glutathion enzyme), IgM and lysozyme were determined. Also, investigating the differences between fish fed the control diet and diet supplemented with Spirulina (1%) against the infection with pathogenic strain of *Aeromonas hydrophila*. The final body weight, gain percent and specific growth rate was significantly (P<0.05) increased in fish fed on supplemented diet with Spirulina comparable with that fed on the control diet. The highest final body weight (49.60±0.52g), body gain (11.70±1.04g) and body gain (30.87±4.10%) were recorded in fish fed on diet supplemented with 2% Spirulina. Biochemical, immunological parameters and antioxidant enzyme (GSH) were improved in the group supplemented with Spirulina in comparing with fish fed on basal diet. The best result was recorded in-group fed on Spirulina supplemented diet by level of 1% at which the levels of IgM was 42.40 ±0.66 µg /ml, lysozyme level was 31.33 ±0.44 µg /ml and the level of GSH was 11.7 ±0.39 mg/g tissues. Moreover, Spirulina supplemented with (1%) in diet enhanced fish protection against *A. hydrophila* infection. The survival rate was (80%) in fish fed on diet supplemented with 1% Spirulina.

Key words: Spirulina; *Oreochromis niloticus*; growth; immunity

Introduction

Recently, aquaculture is one of the fastest growing in our world as it has a high level of protein, which is considered the lowest price in comparing with other sources of protein (1). For human, fish is one of the important animal proteins. Nowadays, we all encourage the culturing of fish through many facilities to provide protein all over the world. fish protein is a very excellent source of protein in replacing of red meat .fish flesh contains all of minerals , iodine ,potassium ,iron copper , and vitamin D, A with favorable levels (2).

Vaccines, antibiotics and chemical therapy are used to protect fishes against the infection.

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The excretive use of antibiotics will cause severe damage to fish, disease resistant, severe environmental problems and hazards in food safety (3). also , presence of different causes of infection to fish will interfering with vaccine manufacture (4) so, it is important to use natural plants to improve immunity of fish and to enhance the production to the highest level. The response of fish to infection is occur through two mechanisms specific and nonspecific also, it determined through immune response of fish to infection (5) .To protect fish against the infection , the best way was done by improving of its immunity . Many of natural plants and algae including spirulina used to increase the immunity of fish (6).

Spirulina (*Spirulina platensis*) is marine algae, which its color is green and blue. It grows in lakes, which is rich with carbon. Cyanobacterium is traditionally produced for human, as it is rich with high quality protein and a lot of nutrients product as vitamins, minerals and essential fatty acids (7). Nowadays *S. platensis* are widely used for improving nutrient composition and physiological response to stress in many fish species (8). Recently, *S. platensis* has a powerful effect in improvement the immunity of Carp fish (9). Therefore, the aim of the current study was to investigate the effects of dietary supplementation with dried *S. platensis* at different doses on the immune parameters, growth performances and the resistance to the pathogenic *Aeromonas hydrophila* of *O. niloticus*.

Material and methods

Fish and the experimental design

Two hundred and twenty five apparently healthy live *Oreochromis niloticus* with an average body weight 36±1.0 g acquired from Abassa Fish Farm at Sharkia Governorate. They were kept in glass aquaria (80X60X30 cm) provided with 90 L de-chlorinated fresh water and aerator with 27 ± 2°C water temperature, 5.4 mg/l dissolved oxygen, 7.2 pH, 0.20 mg/l ammonium (NH_4) and 0.02mg/l nitrite. Fish were separated into five equal triplicate groups, each included 15 fish. All fish were fed their respective diets at a point of 3.5% of body weight four times daily for 3 months. The study was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University.

Fish of the control group were fed on basal diet (the basic dietary requirements of *O. niloticus* (10) (Table 1). Other groups were fed on diet supplemented with different level of spirulina (0.5, 1, 1.5 and 2%) (Pure dried *S. platensis* (*Arthrospira platensis*) tablets, Lake Heath Products Co., Ltd. Liyang, Jiangsu, China; each tablet was grounded into powder before used). The diet was analyzed for dry matter, crude protein, ether extract and crude fiber (11). Isocaloric and isonitrogenous diets were prepared at Fish Research Center, Faculty of Veterinary Medicine, Zagazig University, Egypt. The calories level is 2940 kcal/kg ME and crude protein is 30.80% in the form of dry pellets.

Table 1: The chemical composition of the experimental diets used in feeding of *O. niloticus*

Ingredients (%)	Experimental diet				
	Control	Spirulina enriched diets			
		0.5%	1%	1.5%	2%
Yellow corn	35	35	35	35	35
Spirulina[1]	-	0.5	1	1.5	2
Wheat flour	10	10	10	10	10
Soybean meal, %44	18	18	18	18	18
Fish meal, %60	16	15.5	15	14.5	14
Poultry by-product meal	14	14	14	14	14
Vegetable oil	5.5	5.5	5.5	5.5	5.5
Vitamin and mineral mixture*	1.5	1.5	1.5	1.5	1.5
Calculated composition					
DM, %	84.28	83.81	83.33	82.86	82.39
CP, %	30.79	30.79	30.78	30.78	30.77
EE, %	9.92	9.90	9.87	9.84	9.81
CF, %	2.40	2.41	2.42	2.43	2.45
Ash, %	7.09	7.04	6.98	6.93	6.88
NFE, %	38.99	39.10	39.20	39.30	39.40
DE, Kcal/ kg diet**	2944.41	2946.48	2949.01	2951.62	2954.23

* Vitamin and Mineral mixture (alfakema):- Each 1 kg contains:-Vit. A 580000 I.U, vit.D3 8600 I.U, vit.E. 720 mg, vit. K3 142 mg, vit C 0.1 mg, vit B1 58 mg, vit B2 34 mg, vit. B6 34 mg , vit.B12 58 mg , Folic acid 86 mg , Pantothenic acid 8 mg , Manganese sulfate 65 mg , Zinc methionine 3000 mg , Iron sulfate 2000 mg , Copper sulfate 3400 mg , Cobalt sulfate 572 mg , Sodium selenite 25 mg, Calcium iodide 25 mg, Calcium carbonate (Carrier substance) till 1000 gm.
** digestible energy calculation based on values of protein 3.5 kcal/gm, fat 8.1 kcal/gm, NFE 2.5 kcal/gm **(12).**
(DM= Dry matter, CP= Crude protein, EE= Ether extract, CF= Crude fiber and NFE= Nitrogen free extract).
[1] Analyzed composition for *Spirulina platensis* includes (94.80% DM, 62.10% CP, 3.20% EE, 3% CF, 10% Ash and 21.70% NFE).

Growth performance and health status

Fish were weighted at the start, every 14 days and the end of the experiment. The final body weight, body gain (g), body gain percent, specific growth rate %, food consumption (g), feed conversion ratio and condition factor were determined (13-16). Regarding the assessment of fish health status during the experimental period, different reflexes was detected (escape, defensive, tail and ocular) were regularly observed (17).

Blood and liver samples collection

Caudal blood vessels were the source of collection of blood. The collected blood was put in plastic Eppendorf tubes for serum samples preparation without anti-coagulant in syringe then centrifuged (3,000 r.p.m. for 15 min). The collected serum were stored immediately in deep freezer (-20ºC) until use (18). At the start and end of the study one and half gram of livers were taken and homogenized in 5ml cold 20 mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210 mM mannitol and 70mM sucrose per gram tissue. Homogenates were centrifuged at 10.000 x g for15 minutes at 4 °C and the supernatant was collected and stored at-80°C until further determination of reduced Glutathione enzyme (GSH).

Immunological and biochemical parameters

Immunoglobulin M (IgM) was determined using ELISA Kit (Catalog No. CSB-E12045Fh (96k test). CUSABIO BIOTECH CO., Ltd). The lysozyme activity was measured using the turbidity assay (19). Alanine Aminotransferase Activity (ALT) and Aspartate Aminotransferase (AST) were determined (Spectrum Kits, Egyptian Company for Biotechnology, Cairo, Egypt (REF: 265 002 and 261 002, respectively) (20). Creatinine was carried out according to method mentioned by Husdan and Rapoport (21). The **s**erum cortisol was determined by ELISA using Microtiterstrip DIAMED CORTISOL ELISA kit. Reduced glutathione (GSH) was evaluated in the liver homogenate (kit, Cayman, Cat. No. 703002, Cayman, USA) (22).

Challenge test

After 3 months, fifteen fish, which fed on 1% Spirulina enriched diets, and other fifteen fish from control group were collected then challenged with pathogenic strain of *Areomonas hydrophila* (10^8 cfu mL^{-1}) obtained from animal health institute (23). Fish were inoculated by intraperitoneal injection with 0.1 ml of pathogenic strain of *Areomonas hydrophila* according to Collins et al. (24). Infected fish were observed for any changes and the mortalities of all replicates were calculated for a period of 15 days. The mortality was verified by re-isolating the microorganism from internal organs of dead fish. The fish were counted at the end of experiments to determine the survival percentage according to the following formula:

Survival % = (Final number of tested fish/Initial number of tested fish) x 100.

Statistical analysis

Data were statistically analyzed using one way ANOVA, LSD (Least significant difference) based on Snedecor and Cochran (25). Comparing differences among different means were detected with Duncan's multiple range tests (26). Data were showed as mean ± SE and the significance was considered at ($P < 0.05$).

Results

Growth performance and health status

Regarding the growth performance**,** both of total final body weight**,** body gain (g), body gain %, specific growth rate %, feed consumption and feed conversion ratio were determined. The results were demonstrated in Table (2) which revealed that fish fed on supplemented diet with Spirulina showed significant ($P<0.05$) improve in growth performance in comparing with fish which fed on basal diet. There were no significant ($P<0.05$) changes in both of condition factor and survival rate among the fish during the experimental period (Table, 2).

Table 2: The effect of dietary supplementation with different levels of Spirulina on growth performance, condition factor and survival rate of *O. niloticus*

Ingredients (%)	Experimental diet					
	Control	Spirulina enriched diets				P value
		0.5%	1%	1.5%	2%	
Initial body weight, g	36.71±0.77	36.15±0.69	37.98±0.88	37.99±0.70	37.90±0.71	0.545
Final body weight, g	44.71±0.39^{c}	45.85±0.29^{b}	49.21±0.22^{a}	49.58±0.54^{a}	49.60±0.52^{a}	0.011
Body weight gain, g	8.00±0.66^{b}	9.70±0.85^{ab}	11.23±107^{a}	11.59±1.14^{a}	11.70±1.04^{a}	0.022
Body weight gain, %	21.79±2.60^{b}	26.83±3.11^{ab}	29.57±4.15^{a}	30.51±4.17^{a}	30.87±4.10^{a}	0.020
Specific growth rate, %	0. 33±0.03^{b}	0.40±0.04^{b}	0.43±0.05^{a}	0.44±0.05^{a}	0.45±0.04^{a}	0.002
Feed consumption, g	30.25±0.26^{b}	31.70±0.28^{b}	32.08±0.23^{b}	33.85±0.30^{a}	34.00±0.30^{a}	0.002
Feed conversion ratio	3.78±0.29^{a}	3.26±0.29^{ab}	2.85±0.22^{b}	2.92±0.26^{b}	2.90±0.25^{b}	0.002
Initial body length, cm	11.22±0.66	11.35±0.69	11.44±0.88	11.35±0.70	11.44±0.65	0.935
Final body length, cm	13.21±0.39	13.55±0.29	13.95±0.22	13.75±0.54	13.94±0.33	0.432
Condition factor (K)	1.85	1.8	1.73	1.83	1.72	--
Survival rate, %	95.20± 0.0	96.30± 0.14	97.30± 0.12	97.20± 0.14	96.21±0.22	1

^{abc} Mean in the same row with different superscripts are significantly different at ($P < 0.05$).

Immunological and biochemical parameters

Table (3) showed the different level of IgM and Lysozyme to fish fed on dietary supplements with Spirulina and basal diet. Both of IgM and Lysozyme were significantly ($P < 0.05$) improved in all fish fed on dietary supplements with Spirulina compared to the control group. Dietary supplementation with 1% Spirulina showed significantly increase in both of IgM and Lysozyme in comparing with other groups.

Regarding to the impact of dietary supplementation with different level of Spirulina on different biochemical blood parameters of *O. niloticus*, there were no significant variation (P<0.05) between the control group and other groups.

From Table 3, it was clear that significant (P<0.05) increasing level of GSH enzyme in all group fed with supplemented diet with Spirulina in comparing with fish fed on basal diet.

Challenge experiment

The highest survival (80%) rate was recorded in fish fed on 1% Spirulina enriched diets comparing with fish fed on basal diet group (33%).

Table 3: The effect of dietary supplementation with different levels of Spirulina on immune status, blood parameters and liver GSH of *O. nilioticus*

Ingredients (%)	Experimental diet					P value
	Control	Spirulina enriched diets				
		0.5%	1%	1.5%	2%	
Initial IgM value (µg /ml)	22.53±0.28	22.16±0.33	22.32±0.44	22.00±0.76	22.16±0.33	0.692
Final IgM value (µg /ml)	28.50±0.57^{d}	31.20±0.15^{c}	42.40±0.66^{a}	39.41±0.01^{b}	39.23±0.02^{b}	0.011
Initial lysozyme value (µg /ml)	15.00±0.25^{a}	14.50±0.57^{ab}	13.88±0.38^{ab}	13.26±0.69^{b}	13.85±0.52^{ab}	0.026
Final lysozyme value(µg /ml)	18.83±0.33^{d}	21.00±0.26^{c}	31.33±0.44^{a}	28.50±0.22^{b}	27.95±0.24^{b}	0.002
Initial ALT value (µg /ml)	17.21±0.36	16.96±0.26	16.8±0.35	17.23±0.30	16.95±0.16	0.945
Final ALT value (µg /ml)	16.53±0.06^{a}	15.80±0.65^{a}	15.74±0.24^{a}	16.71±0.14^{a}	16.46±0.54^{ab}	0.011
Initial AST value (µg/ml)	17.65±0.42	17.22±0.55	17.33±0.32	17.14±0.46	17.02±0.26	1
Final AST value (µg/ml)	16.95±0.22^{a}	17.01±0.35^{a}	17.12±0.61^{a}	16.96±0.45^{a}	17.01±0.35^{a}	0.017
Initial liver GSH (mg/g tissue)	9.27±0.35	8.97±0.67	9.01±0.48	9.17±0.53	8.84±0.73	0.547
final liver GSH (mg/g tissue)	9.05±0.55^{c}	8.63±0.52^{c}	11.7±0.39^{a}	10.52±0.55^{b}	10.94±0.64^{b}	0.002
Initial Creatinine (mg/dl)	0.22±0.32	0.21±0.41	0.19±0.52	0.22±0.12	0.21±0.22	0.390
Final Creatinine (mg/dl)	0.18±0.12^{a}	0.18±0.32^{a}	0.17±0.42^{a}	0.19±0.27^{a}	0.17±0.35^{a}	0.303
Initial cortisol value (µg/ml)	6.22±0.25	6.34±0.35	6.55±0.51	6.42±0.43	6.21±0.22	.501
Final cortisol value (µg/ml)	5.21±0.27^{a}	5.33±0.21^{a}	5.21±0.31^{a}	5.12±0.52^{a}	5.22±0.15^{a}	0.112

^{abcd} Mean in the same row with different superscripts are significantly different at (P < 0.05).
IgM= Immunoglobulin M, ALT= Alanine Aminotransferase Activity, AST= Aspartate Aminotransferase and GSH= Reduced Glutathione enzyme

Discussion

Growth performance and health status

The current study revealed an improvement of the growth performance of fish supplemented with *Spirulina platensis*. These results are in accordance with Takeuchi et al. (8) who stated that the feed conversion ratio and growth rates were improved with *S. platensis* powder supplementation in diet of striped jack. Also, larval tilapia body weight was improved by feeding on raw *S. platensis* with 30% (dry basis) of uni-feed (27). *O. niloticus* were showed maximum growth performance and better feed utilization with 5g fresh culture of *S. platensis* /kg feed (28) or at 10 g/kg diet of dry culture of *S. platensis* (29). The growth promoting effects of Spirulina may be due to its content of high levels of vitamins and minerals that provide a superior protein used for animal feeding (30). The positive immunostimulating effect and the low amount of cellulose in spirulina cellular structure make its digestible simply, improving both of appetite, feed intake; nutrient digestibility; the health and immunity of fish against infections (31). In contrast, the growth performance of tilapia weren't affected by S. *platensis* supplementation (32). The current results of the health status were matched with Jun et al. (33), who confirmed the high beneficial effect of using Spirulina enriched diets for feeding of *O. niloticus*.

Immunological and biochemical parameters

The findings of this study were similar to Ibrahim et al. (29) who recorded 10% spirulina in fish fed showed improving in its hematocrit

values. White shrimp *Litopenaeus vannamei* (*L. vannamei*) fed on hot-water extract of *S. platensis* improved innate immunity (lysozyme) and reduce the frequency of infection by *Vibrio alginolyticus* (34). The existence of *C-phycocyanin* which was found in Spirulina alga may be the reason of immunity capacity (35). The detected levels of plasma AST and ALT is of significant diagnostic importance in fish. They reflect the general nutritional condition and the integrity of vascular system and liver function. The current findings were matched with Upasani and Balaraman (36) who stated that using of Spirulina in feeding of fish have a powerful effect in decreasing liver and kidney lipid peroxidation. In addition, it makes increasing of antioxidant enzymes in fish as it contains antioxidants substance as carotene, minerals, vitamins, protein, carbohydrates and lipids.

Challenge experiment

This indicted the high beneficial effect of fed fish with supplemented diet with1% Spirulina in raising the immune of the fish to a great extent that make fish can withstand the infection. The results are completely agreed with Ibrahim et al. (29) who observed that *S. platensis* supplementation significantly reduced the mortality rate post challenge infection with *Pseudomonas fluorescens.* Better survival rate could be mentioned that Spirulina algae has carotenoids that improved fish health and capability to resist infections through lessening the stress (30) or improved immune status, such as phagocytosis, producing superoxide and cytokines in common carp (37).

Conclusion

From our study, it could be concluded that the using of 10g/kg dried *S. platensis* in diet for 3 months as it have an excellent effect in improving of growth rate and immunity of *O. niloticus* . Also, feeding *O. niloticus* with diet supplemented with S. *platensis* (1%) will strength it against the infection with pathogenic strain of *A.hydrophila* through improving immunity.

Conflict of interest

The authors declare no conflict of interest.

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Original Research Article

COMPUTED TOMOGRAPHIC, LAPAROSCOPIC AND SECTIONAL ANATOMY OF THE LIVER AND SPLEEN IN GOATS (*CAPRA HIRCUS*)

Ahmed A. Hassan*, Hamed M. Nossir, Khaled Z. Soliman, Esraa M. El-Skeikh, Mervat M.H. Konsowa

Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Zagazig University, Egypt

*Corresponding author, E-mail: aabdelfattah@vet.zu.edu.eg

Abstract: The present study was carried out on nine apparently healthy goats of both sexes, females were non-pregnant and non-lactating. Their weight and age ranged from 28-35 kg and 17 to 24 months, respectively. These goats were divided into three groups; 1) Three were subjected to frozen sagittal section technique, 2) Three underwent computed tomography followed by frozen cross section techniques, and 3) Three were used for laparoscopy. With the aid of these techniques, this study focused on liver and spleen to detect their position, shape and relation with other abdominal organs. Sagittal sectional anatomy was done using right paramedian, left paramedian and midline sagittal sections on the abdomen of goats. The frozen cross sections were compared with CT images at the same level. Laparoscopy was accomplished at three positions; dorsal recumbency, right flank and left flank laparoscopy. The obtained results were presented in plates and anatomical structures of clinical importance were identified and labelled to establish a comprehensive guide for specialists in anatomy, surgery, radiology and veterinary education.

Key words: frozen sections; CT; faparoscopy; *hepar*; *lien*; small ruminants

Introduction

Goats have been established as a model for biomedical research, teaching and surgical training. Because, they are relatively clean and small sized they can be maintained in small areas and handled with ease (1,2).

Computed tomography (CT) images are created by using both X-rays and computer processing (3). It provides a higher contrast and resolution of soft tissues than normal radiography, with the ability to reconstruct images of areas under investigation (4). It is now established as good standard diagnostic procedure in human medicine, as it is non-invasive and can be performed frequently. However, only in the last decade, it became available in veterinary profession, but with limited use as it requires anaesthesia and high costs (5).

Laparoscopy represents a technique for visual examination of the abdominal cavity and its contents. It is a minimally invasive technique that requires the distension of the abdominal cavity with gas (a process called: induced pneumo-peritoneum), this results in improved visualization and facilitated instrumental and visceral manipulation (6). In addition, it provides live visual examination of the abdominal contents using an illuminated tele-

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scope (7). Therefore, it is very useful to display the organs in a natural colour, and so it offers an advantage over other techniques such as ultrasonography or radiography (8,9).

Laparoscopy allows surgeons to access the abdomen without making large incisions in the abdominal wall, an advantage over laparotomy. in addition to reducing tissue trauma, reducing postoperative adhesions and infections, fast recovery, stimulation of the immunity, better cardiovascular stability and lower pain scores (10,11).

Literature lacks adequate information on goat's liver and spleen anatomy using both CT and laparoscopy; besides formalin fixed samples are becoming less and less used in anatomical education. Therefore, this study was conducted to provide the normal anatomy, topography and morphometry of liver and spleen in goats, using laparoscopy and computed tomography compared to standard sectional anatomy procedure. In addition, we aimed to provide photographs that will be of essential importance for veterinary anatomists, radiologists, surgeons and clinicians.

Material and methods

The present study was performed on nine apparently healthy goats of both sexes; females were non-pregnant and non-lactating. Their weight ranged from 28 to 35 kg and their age ranged from 17 to 24 months. The study was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University.

Three techniques were carried out to describe the liver and spleen in goats; Frozen sections technique, Computed tomography and Laparoscopy. Study design and methodology described herein followed the guidelines of Faculty of Veterinary Medicine Research Ethics Committee. The nomenclature used in this study was adopted following *Nomina Anatomica Veterinaria*, 2012 (12).

Frozen sections technique

The goats were sedated with intravenous injection of Xylazine HCl 2% (0.1 mg/kg, Xyla-Ject®, ADWIA, Egypt), followed by slaughtering and complete bleeding. The two fore limbs were removed, and the cadavers were put in a deep freezer (-20°C) in sternal or lateral recumbency position.

After complete freezing of the cadavers (3 days or more), three cadavers, were longitudinally sectioned using electrical band saw through midline, right para-median and left para-median sections (supplementary plate 1/A). The paramedian sections passed through the edge of the first lumbar transverse process parallel to the midline on each side. While, another three cadavers (after CT scanning) were transversely sectioned into serial transverse sections (approximately three cm apart; to cut in each vertebra) starting from the level of the 5^{th} thoracic vertebra and perpendicular to the longitudinal axis of the goat's trunk (supplementary plate 1/B). Each cut section was immediately photographed, and the obtained photos were compared with the corresponding CT images.

Computed tomography (CT)

The CT scan was carried out at a private CT centre, El-Sharkia Governorate, Egypt. A third-generation multi-slice helical CT scanner (GE LightSpeed Ultra 8 system, General Electric Company, USA) was used with eight body slices per rotation and soft tissue window width of 500 HU (Hounsfield unit); its features were 53.2 KW generator, 6.3 MHU tube size and rotation time of 0.5 seconds (supplementary plate 1/C). The CT scan was performed following previously described protocol with slight modifications (13). For this part of the study, three goats were used (after that, the same goats were used for frozen cross sections technique described in point 2.1.).

The CT images were obtained without using contrast material and only soft tissue window was used to detect the location, homogeneity, shape and morphometry of both liver and spleen. After fasting for 24 hours, the goats were sedated with intravenous injection of Xylazine HCl 2% (0.1 mg/kg, Xyla-Ject®, ADWIA, Egypt), then the goats were positioned in sternal recumbency during scanning time. A scout view of the goat was obtained before beginning of the scanning as shown in (supplementary plate 1/D). The

abdomen was scanned with a layer thickness of one mm interval with an exposure of 120 kV and 270 mA. The captured images were compared with their corresponding cross sections at the same level.

Laparoscopic technique

Laparoscopy was carried out using SOPRO-COMEG laparoscope (Germany, supplementary Plate 1/F) at the laparoscopy unit of the Surgery Department, Faculty of Veterinary Medicine, Zagazig University, Egypt. The procedure followed published guidelines (6,10,14).

Results and discussion

The present work focused mainly on the liver and spleen in goats, using different anatomical techniques. With a final goal of improving our understanding and interpretation of clinically important information, provided either as CT scans or laparoscopic images, for a broad range of research and education professionals.

Frozen sectional anatomy

Sagittal sectional findings: Three sagittal sections were obtained; the midline, right paramedian and left paramedian sections.

Liver (*Hepar*): The liver was present in the right paramedian sagittal section (Plate 1/A). It extended from the level of the sixth to last thoracic vertebrae. Its shape resembled a narrow elongated triangular strip. Both right (*Lobus hepatis dexter*) and caudate (*Lobus caudatus*) lobes of the liver were identified. The latter lobe was seen capping the cranial pole of right kidney and its perirenal fat (Plate 1/A). Parietally, the liver was related to diaphragm. Its visceral surface was related to the reticulum, omasum, pancreas, portal vein and dorsal ruminal sac. While, the liver was seen in midline sagittal section as a small narrow slice related dorsally to the ruminal atrium (Plate 1/B). These findings are similar to previously reported findings in sheep and goat (15).

Spleen (*Lien*): The longitudinal section of the spleen appeared as a small fusiform elongated mass in the left paramedian sagittal section. In this aspect, it prolonged from the level of the ninth to last thoracic vertebrae. It was dark red in colour and rested on the dorsal surface of the dorsal ruminal sac and related to the vertebral column and the diaphragm (Plate 1/C). These findings are in accordance with previous descriptions in small ruminants (15–17).

Cross sectional findings: In this part of study, each cross section was identified and described within the topography of the main regions of the abdomen. Appearance of the liver and spleen in each section is tabulated in (Table 1).

Table 1: Liver and spleen in studied goats appearing in frozen cross sections (FCS) and in computed tomography (CT) studies, in relation to thoracic vertebrae number

Organs \ No. of vertebrae	Thoracic vertebrae															
	6th		7th		8th		9th		10th		11th		12th		13th	
	FCS	CT	FCS	CT	FCS	CT	FCS	CT	FCS	CT	FCS	CT	FCS	CT	FCS	CT
• Liver	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
○ Gallbladder	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
○ Caudal vena cava	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
○ Portal vein	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
• Spleen	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-

(+) = visible, (–) = not visible.
Highlighted cells in grey indicate differences between FCS & CT

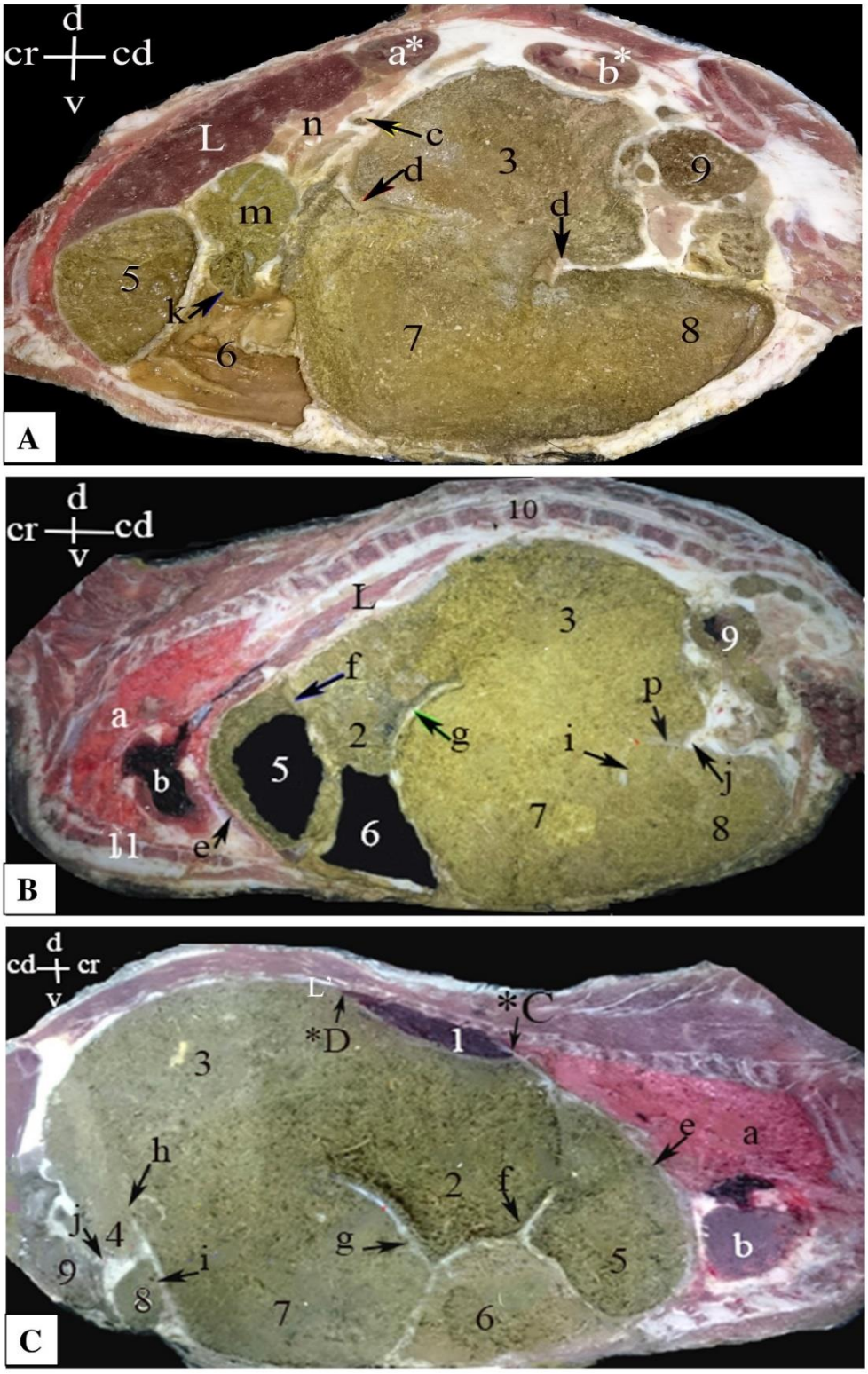

Plate 1: Photomacrographs showing representative frozen longitudinal sections of goat aged 21 months, right paramedian sagittal section (A), midline sagittal section (B) and left paramedian sagittal section (C). 1- Spleen, 2- Ruminal atrium, 3- Dorsal ruminal sac, 4- Dorsal caudal blind sac, 5- Reticulum, 6- Abomasum, 7-Ventral ruminal Sac, 8- Ventral caudal blind sac, 9- Intestine, 10- Vertebral column, 11- Sternum, a- Left lung, b- Heart, c- Portal vein, d- Right longitudinal pillar, e- Diaphragm, f- Rumino-reticular fold, g- Cranial transverse pillar, h- Dorsal coronary pillar, i- Ventral coronary pillar, j- Caudal pillar, k- Omaso-abomasal orifice, L- Liver, L'- Caudate lobe of the liver, m- Omasum, n- Pancrea, p- longitudinal pillar, a*- Right kidney, b*- Left kidney and *C, *D – the extension of the spleen

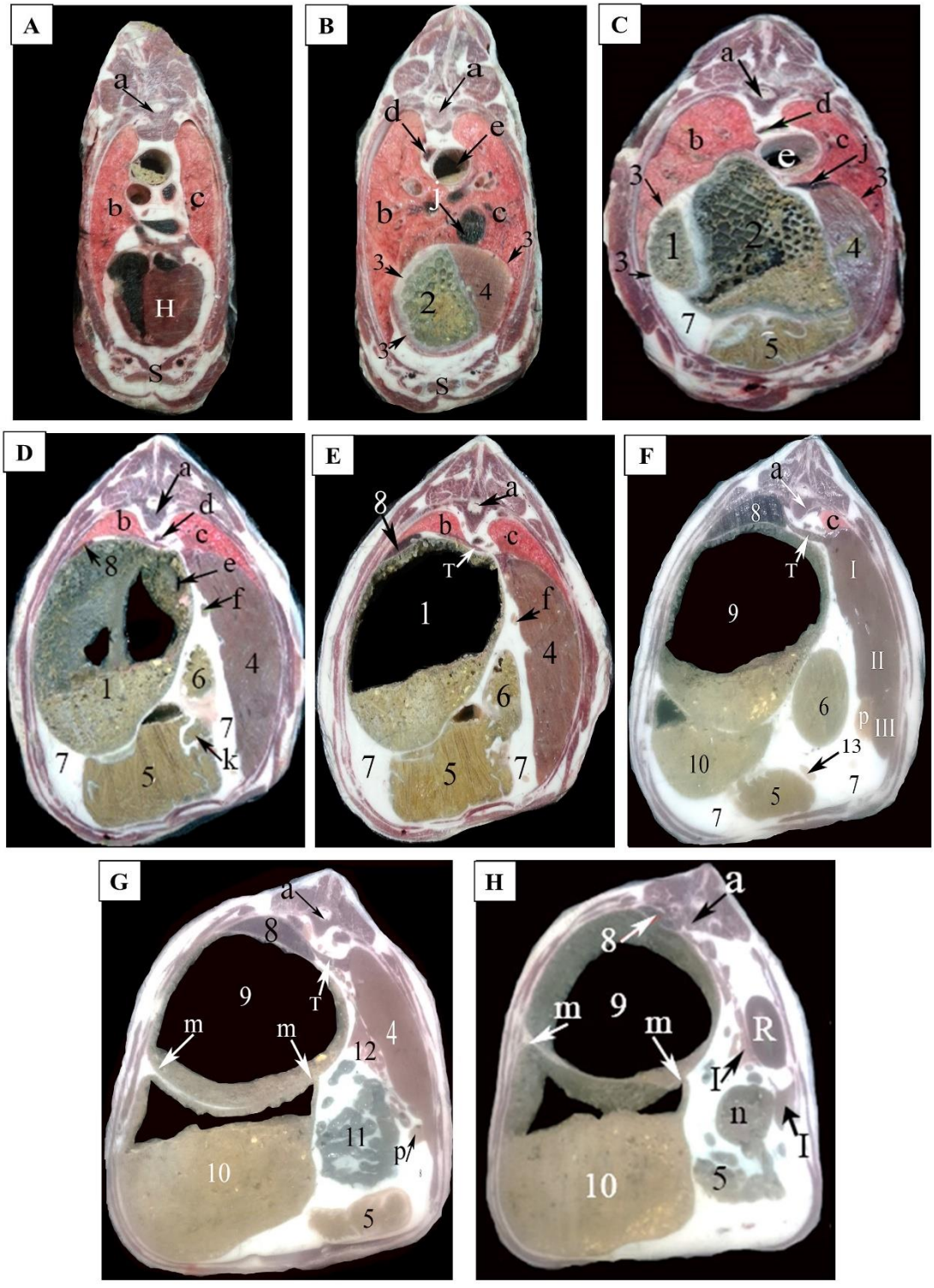

Plate 2: Photomacrographs showing representative frozen cross-anatomical sections, of goats aged between 17-24 months, at the level of fifth (A), sixth (B), seventh (C), eighth (D), ninth (E), tenth (F), eleventh (G) and twelfth (H) thoracic vertebrae.1- Ruminal atrium, 2- Reticulum, 3- Diaphragm, 4- Liver (I- Caudate lobe of liver, II- Right lobe of liver, III- Quadrate lobe of liver), 5- Abomasum, 6- Omasum, 7- Greater omentum, 8- Spleen, 9- Dorsal ruminal sac, 10- Ventral ruminal sac, 11- Intestine (Jejunum), 12- Pancreas, 13- Abomasal lymph node , a- Vertebra, b, c- Left and right lungs, d- Aorta, e- Oesophagus, f- Portal vein, H- Heart, J- Caudal vena cava, k- Omaso-abomasal orifice, m- Right and Left longitudinal pillars, n- Caecum, P- Gallbladder, R- Right kidney, S- Sternum, T- Lumbar part of diaphragm (Root)

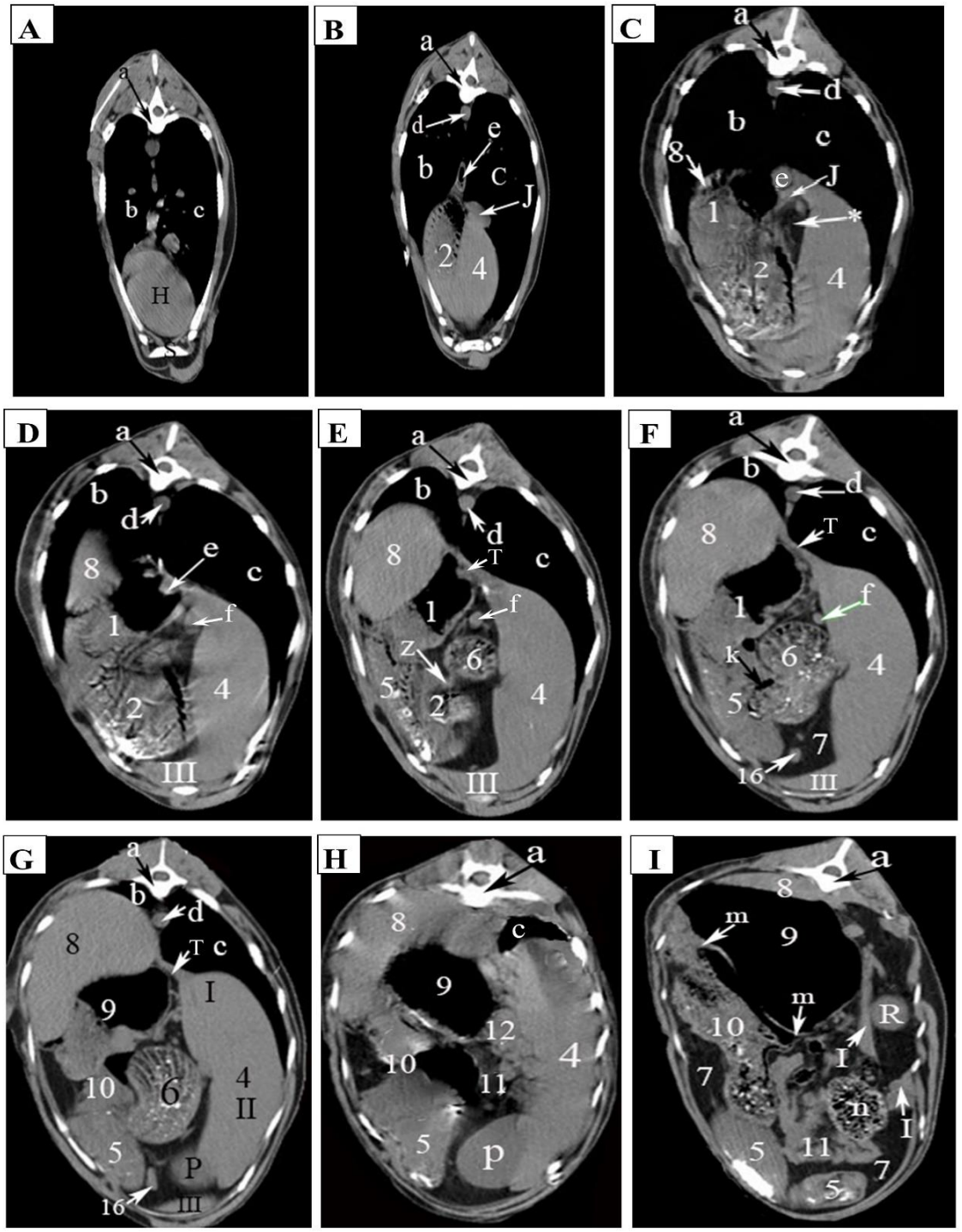

Plate 3: Photomacrographs showing representative CT images, of goats aged between 17-24 months, at the level of fifth (A), sixth (B), seventh (C), eighth (D and E), ninth (F), tenth (G), eleventh (H) and twelfth (I) thoracic vertebrae. 1- Ruminal atrium, 2- Reticulum, 3- Diaphragm, 4- Liver (I- Caudate lobe of liver, II- Right lobe of liver and III- Quadrate lobe of liver), 5- Abomasum, 6- Omasum, 7- Greater omentum, 8- Spleen, 9- Dorsal ruminal sac, 10- Ventral ruminal sac, 11- Intestine (Jejunum), 12- Pancreas, 16- Abomasal lymph node, a- Vertebra, b, c- Left and right lungs, d- Aorta, e- Oesophagus, f- Portal vein, H- Heart, J- Caudal vena cava, k- Omaso-abomasal orifice, m- Right and Left longitudinal pillar, n- Cecum, P- Gallbladder, R- Right kidney, S- Sternum, T- Lumbar part of diaphragm (Root), Z- Reticulo-omasal orifice, and *- Porta hepatis

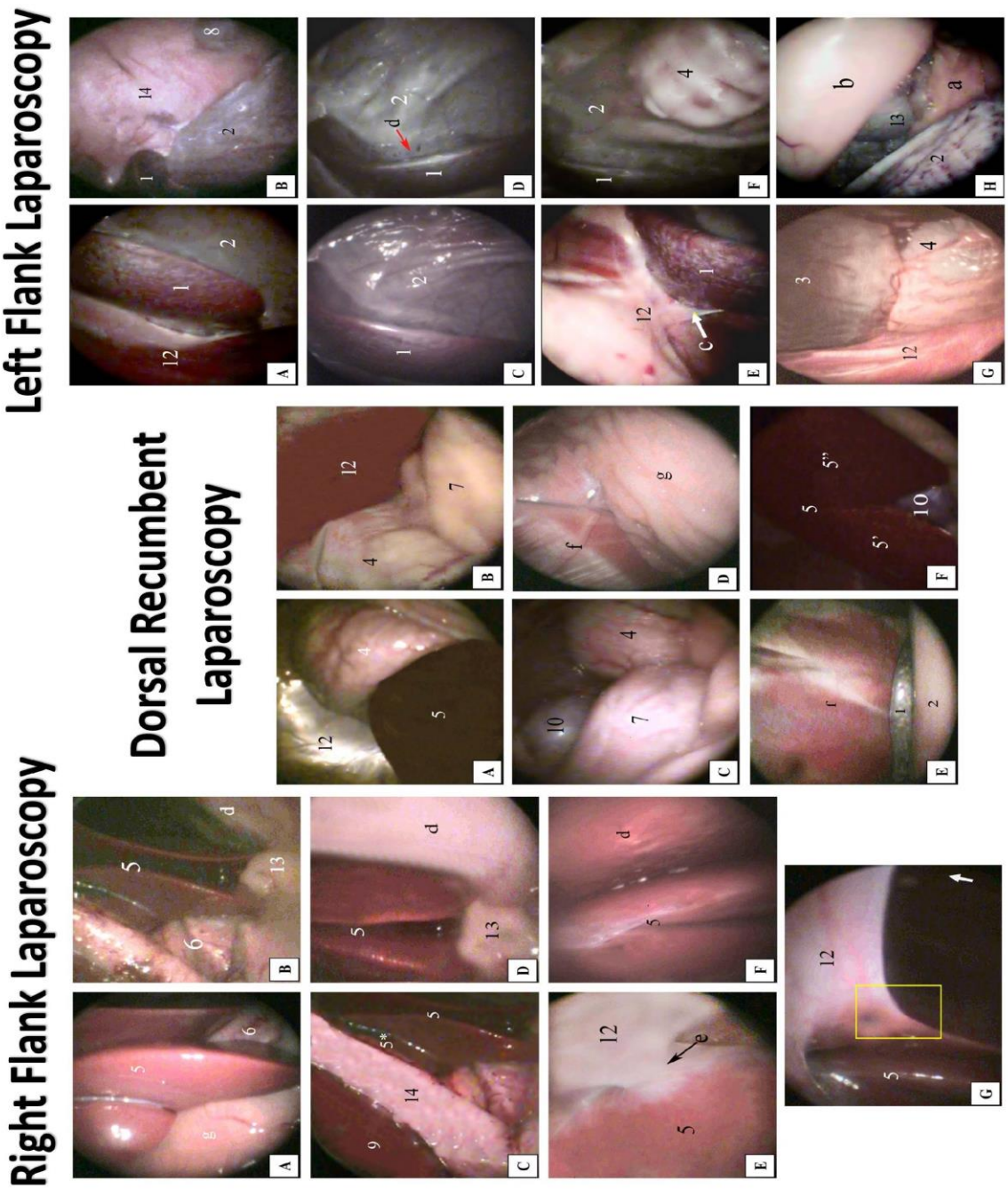

Plate 4: Representative photomacrographs of Right flank laparoscopy, of goats aged between 17-24 months. Note: the telescope was inserted to the right side of the abdomen till reaching the level of the liver. Showing: 5- Liver, 5*- caudate lobe of the liver, 6- Omasum, 9- Right kidney, 12- Diaphragm, 13- Intestine (duodenum), 14- Peri-renal fat, d- Right abdominal wall, g- Greater omentum, e- Coronary ligament, and Yellow rectangle- Right triangular ligament. Dorsal recumbency laparoscopy. Note: the telescope was directed dorsally and to the right side of the abdomen till reach the liver (A), ventral to show reticulum and abomasum (B and C) and caudally till the caudal abdominal region (D). Showing: 1- Spleen, 2-Dorsal ruminal sac, 4- Reticulum, 5- Liver, 5'- Right lobe of the liver, 5"- Quadrate lobe of the liver, 7- Abomasum, 10- Gallbladder, 12- Diaphragm, f: Left abdominal wall, and g: Greater omentum. Left flank laparoscopy. Note: the telescope was directed dorsally till reaching the level of the dorsal ruminal sac and spleen (A, B, C, E, F and H) and directed ventrally to the left side till reach the reticulum (D and E). Showing: 1- Spleen, 2- Dorsal ruminal sac, 3- Ventral ruminal sac, 4- Reticulum, 8- Left kidney, 12- Diaphragm,13- Intestine (colon), 14- Peri-renal fat, a- Intra-abdominal fat, b- Dorsal abdominal wall, c- Phrenico-splenic ligament, and d- Gastro-splenic ligament

Liver: Sequence examination of the frozen sections from the level of the fifth to twelfth thoracic vertebrae (Plate 2) revealed that the liver was entirely located to the right half of the intrathoracic part of abdominal cavity. It was dark red in colour. At the level of sixth thoracic vertebra (Plate 2/B), it had a triangular shape in cross section and was related to the reticulum medially, right abdominal wall laterally and diaphragm dorsally. Also, at the level of seventh thoracic vertebra (Plate 2/C), it was related to diaphragm dorsally, reticulum and abomasum medially right abdominal wall laterally. While, at the level of eighth and ninth thoracic vertebrae (Plates 2/D and 2/E), the liver was related to the right abdominal wall and diaphragm parietally and ruminal atrium, omasum, abomasum, greater omentum, and portal vein viscerally. At the level of the tenth thoracic vertebra (Plate 2/F), the shape of liver changed to elongated oval in outline and it was related medially to the dorsal ruminal sac, greater omentum, omasum and gallbladder, laterally to right abdominal wall and dorsally to the root (lumbar part) of diaphragm. In addition, at the level of eleventh thoracic vertebra (Plate 2/G), the liver was related medially to pancreas, intestine, greater omentum and dorsal ruminal sac, laterally to right abdominal wall and dorsally to the lumbar part of diaphragm. Finally, at the level of the twelfth thoracic vertebra (Plate 2/H), a small part of the caudate lobe of the liver was visible capping the right kidney and its peri-renal fat. There is not much difference between these and previous data in sheep and goat (15,16), and in ruminants in general (17,18).

The portal vein (*V. portae*) appeared as a circular structure on the visceral surface of the liver, at the level of the eighth and ninth thoracic vertebrae, as shown in (Plate 2/D and 2/E). Its location is ventro-lateral to the caudal vana cava (*Vena cava caudalis*). While, the latter vein was detected dorsal to the parietal surface of the liver at the level of the sixth and seventh thoracic vertebrae (Plate 2/B and 2/C). In addition, larger hepatic veins (*Vv. hepaticae*) were distinguishable within the hepatic parenchyma.

Through examining sections at the level of tenth and eleventh thoracic vertebrae, the gallbladder (*Vesica fellea*) was seen as a fluid filled vesicle rested at the ventral part of the visceral surface of liver (Plate 2/F and 2/G). Others observed it at the level of tenth or eleventh rib (16,17). Meanwhile, no relation was detected between the gallbladder and the right abdominal wall, in contrast with information provided by Getty and Nickel and co-workers (18,19).

Spleen: Examined sections at the level from eighth to twelfth thoracic vertebrae, spleen was detected at the left side of the abdomen just ventral to the vertebral column & ribs (Plate 2/D : 2/H). The spleen appeared as a triangular organ that is related to dorsal ruminal sac viscerally, diaphragm and left abdominal wall parietally. Its relations to the vertebral column, dorsal ruminal sac and diaphragm were as previously described in ruminants (17). However, it was shown to be related to the reticulum, in ruminants, (18) and the pancreas, in sheep, (16), but it was not feasible to detect these relations in the present cross or even longitudinal sections (in point 3.1.1.2).

Frozen sections technique described herein, is in accordance with previous anatomical texts and research describing position, size and relation of liver and spleen in ruminants, particularly in goats. However, some findings did not match with the previously reported information such as in the portal vein and gallbladder, which highlight either differences in ruminant subspecies used or the physiological condition, and highlight the need for more revised versions of the currently available information.

Computed tomography (CT)

Detailed examination of soft structures viewed using soft tissue window, the location, relation; homogeneity and shape of each organ under investigation were observed. During this process, each transverse CT image was matched with the conforming frozen cross-section at the same level. The bones (thoracic and lumbar vertebrae, ribs and sternum) were used as landmarks for detecting soft tissue structures. The structures were identified

according to their radiodensity, shape and location. The visible organs in CT scanning with their extension are tabulated in (Table 1).

Abdominal CT in study goats demonstrated most of the typical anatomical findings which appeared in frozen cross-sections. What is more, it shows advantages over regular radiography, since it provides soft tissue structures without superimpositions or artefacts. In addition, its information is diagnostic without the need for contrast materials (20).

Liver: The liver was apparent as homogenous soft tissue density at the right side of the epigastric region from the level of sixth to twelfth thoracic vertebrae (Plates 3/B : 3/I), but, in another goat study its extension was between the fifth thoracic and eleventh lumbar vertebrae (13). Meanwhile, in Holstein-Friesian calves, it extended from the seventh thoracic to the first lumbar vertebrae (21). In another small ruminant species; Jebeer gazelles (22), the liver extended from the eighth thoracic further till the level of second lumbar vertebra (22). This highlights an obvious extension differences between ruminant species.

It was elongated in shape and prolonged from the diaphragm dorsally to the sternum and ventral abdominal wall ventrally. At the levels from eighth to tenth thoracic vertebrae (Plate 3/D: 3/G) but not at the fifth (Plate 3/A), the quadrate lobe was revealed as a small tapered lobe on the ventral abdominal wall. This lobe could be clearly recognized from the right medial hepatic lobe by the gallbladder at the level of tenth thoracic vertebra (Plate 3/G). The right and left lobes were distinguishable by their side location on the CT image. The liver was observed between right abdominal wall, diaphragm, rumen, reticulum, omasum, abomasum and sternum. These relations and the three distinguishable liver lobes were previously reported in goats (13,23).

The hepatic portal (*Porta hepatis*) was seen as a hypodense area medial to the liver at the level of seventh thoracic vertebrae (Plate 3/C). Also, the portal vein was visible medial to the liver as a hypodense band at the level of eighth and ninth thoracic vertebrae (Plate 3/D: 3/F). While, the caudal vena cava was found as a hypodense irregular band dorsal to the visceral surface of liver at the level of sixth and seventh thoracic vertebrae (Plate 3/B and 3/C). Meanwhile, the hepatic veins could not be identified. All this was in agreement with previous reports in goats (13).

The gallbladder was visible as pear-shaped structure at the level of tenth and eleventh thoracic vertebrae (Plate 3/G and 3/H). It was seen to the right side of the ventral part of the visceral surface of the liver with hypodensity (noticeably less density compared to hepatic density); as it was filled with bile. At the level of the tenth thoracic vertebra (Plate 3/G), it was seen between the quadrate and right hepatic lobes. These findings were in a line with those previously mentioned in goats (23) and in Holstein-Friesian calves (21). However, in Jebeer gazelles, the gallbladder was at the level of thirteenth thoracic and first lumbar vertebrae (22). This difference is mainly due to the difference in the caudal extension of the liver between both species.

Spleen: The spleen was shown at the left cranial abdominal region from the level of seventh to the level of twelfth thoracic vertebrae (Plate 3/C: 3/I). However, in another study on goats, it appeared from the eighth thoracic to the second lumbar vertebrae (13). While, in Jebeer gazelles the spleen extended between the ninth thoracic and second lumbar vertebrae (22). Whether these variations in extension are merely due to the state of fullness of the rumen or due to anatomical differences it is not clear in any of the studies and requires further investigation.

The spleen was present between the diaphragm and left abdominal wall dorsally and the dorsal ruminal sac ventrally. It appeared as a large organ with rounded angles and hypodense homogenous soft tissue density; started oval then triangular and finally crescentic in shape depending on the section number. Its homogenous soft tissue density, similar to liver's density, was previously confirmed in goats (13,23) and bovine (21).

Computed tomography, was highly comparable to findings reported in frozen cross sections with the added advantage of ability to perform frequent scanning and follow-up, in addition it is completely non-invasive and no

need to sacrifice the animal under investigation. There were slight variations in CT scans compared to frozen cross sections. For example, this study showed variation in the size and extension of the spleen noted between CT and frozen cross-sections. This could be due to the fact that study goats were live and only sedated, and not bled. Also, slight movement and/or respiration during CT scanning could have participated in such variations, but this needs further investigation.

Laparoscopic study

Laparoscopic study was done on both standing and dorsal recumbent (reverse Trendelenburg) positions. Access through the right flank region showed the liver and its relation with other organs. The left flank approach exposed the spleen and its relations, while, the dorsal recumbency position identified both liver and spleen. The liver and spleen were feasibly characterized by their normal anatomical shape, position and relation.

Liver: Thorough laparoscopic examination of goats showed that, the parietal surface of the liver was viewed in the right epigastric region in standing position (Plate 4). This finding is in agreement with previous reports in goats, horses and ruminants (10,24–27). The liver was also detected in dorsal recumbency approach towards the right side of the cranial abdominal region. This was previously reported in caprine (9), in horses (27–29) and in adult cattle (26).

It had a bright red to brown red colour with smooth surface and sharp edges, as previously shown in small animals (14). But in llama, another small ruminant, the ventral border was fimbriated (30). The hepatic lobation was clearly visible; mainly the left lobe, right lobe, quadrate and caudate lobes. The latter was related to the right kidney dorsally, but it was practically impossible to show the renal impression or hepatorenal ligament due to extensive amount of peri-renal fat (Plate 4/C), so we were unable to observe these structures.

In dorsal recumbent goat, the quadrate lobe was separated from the right hepatic lobe by the gallbladder (Plate 4/F), also this was in agreement with previous findings (9). The left hepatic lobe made a lateral relation with the reticulum in the right side of dorsal recumbent goats, similar finding was observed in bovine (24). Meanwhile, in dogs and cats (31) and in horses (25,27) the left lobe was only visible through the left lateral/paralumbar laparoscopy.

In right flank region, the liver was medially related to the cranial part of duodenum, greater omentum and omasum, laterally to right abdominal wall (Plate 4). The right triangular ligament was viewed in right flank laparoscopy and located between the right lobe of the liver and diaphragm (Plate 4/G), in a line with previous reports in llama (30) and in equine (32). The coronary ligament could be revealed also between the parietal surface of the liver and tendinous part of diaphragm (Plate 4/E), also reported in equines (32) but no details were found in ruminants in the literature. Meanwhile, the left triangular ligament was difficult to examine in the studied goats, in contrary to equines where it could be observed through left flank laparoscopy (32).

In dorsal recumbency laparoscopy, the gallbladder had a pear-shape with a bluish colour and was (embedded between the right and quadrate hepatic lobes (Plate 4/F), these matched previous results in goats (9,14). It was related medially to the reticulum and abomasums (Plate 4/C). It was difficult to identify in standing goats due to omental covering, plus we did not use tissue forceps in our laparoscopic approach, this also was reported previously in large ruminants (24,25).

Spleen: The spleen had a smooth surface with a bluish red colour with white dotted areas giving it a mottling appearance, as previously reported in goats (14), in llama (30), in horse and cattle (25,26,29,32). This characteristic anatomical appearance and position help surgeons identify the spleen to plan surgical interventions, such as splenectomy.

It was present in the left side of the intrathoracic part of abdominal cavity in standing and dorsal recumbent goats between the dorsal ruminal sac viscerally and the abdominal wall parietally (Plates 4/E and 4/A: 4/F), which was also confirmed by previous reports in goats (9), in horse and cattle (10,25–27,29). However, another study on horses

reported that it could be detected through both right and left sides of abdomen (32).

The spleen attached to the dorsal ruminal sac by gastro-splenic ligament medially along the visceral surface of the spleen (Plate 4/D) and to the tendinous part of diaphragm by the phrenico-splenic ligament (Plate 4/E). While, in goats, previous studies gave no observation to the gastro-splenic ligament, only the ventral extremity of the spleen was seen related to the ventral ruminal sac (9). In equines, this ligament was visible between the stomach and visceral surface of spleen (32). There was no direct relation between the spleen and the left kidney (Plate 4/B), other that the spleen was cranial to the left kidney, this was also previously described in goats (14).

Laparoscopy is ideal for real-time monitoring of internal organs as liver and spleen, and for obtaining biopsies or guiding internal injections. We showed for the first time the coronary ligament of the liver through right flank laparoscopy. However, laparoscopy is slightly invasive, requires local and/or general anaesthesia and shows a risk for postoperative complications which can be very costly in veterinary practice.

Conclusions

This study compared CT scanning with frozen cross sections in addition to abdominal laparoscopy, focusing mainly on the liver and spleen of goats. We provided comprehensive serial cross-sectional and CT images, in addition to laparoscopic images from different approaches with the final goal of updating our current knowledge about the anatomical shape, position and relations of liver and spleen in goats. Data and images provided in this study will be of immense value for specialists in anatomy, surgery, radiology and veterinary education.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary data

The supplementary data file is available upon reasonable request by e-mailing the corresponding author. The file contains detailed methodology used herein in addition to supplementary plates and tables.

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Original Research Article

A STUDY ON BOVINE BABESIOSIS AND TREATMENT WITH REFERENCE TO HEMATOBIOCHEMICAL AND MOLECULAR DIAGNOSIS

Mohamed A. Hashem[1], Ahmed N.F. Neamat-Allah[1]*, Mohamed A.Gheith[2]

[1]Department of Clinical Pathology, Faculty of Veterinary Medicine, Zagazig University,
[2]Veterinarians at Veterinary Clinic, Dakahlia Governorate, Egypt

*Corresponding author, E-mail: anattia@vet.zu.edu.eg; drnemovete@yahoo.com

Abstract: This study was carried out during the period from May to December 2015 on thirty crossbred female cows in Sherbeen city, Dakahlia Governorate and two to four years of age. Twenty cows suffered from fever, anorexia, increase in respiratory and heart rates, anemia, pale to icteric mucous membranes and red urine. Babesiosis was diagnosed clinically and confirmed by detection of intra-erythrocytic stages of the Babesia in Giemsa stained blood film, polymerase chain reaction amplification (PCR) and sequencing of 18S rRNA gene. Hemoparasites were detected in thirteen blood samples by microscopic examination, whereas PCR were positive in twenty. The hematological findings revealed a marked decrease in the erythrocyte count, hematocrit %, hemoglobin concentration and mean corpuscular hemoglobin concentration with a significant increase in mean corpuscular hemoglobin and mean corpuscular volume values in Babesia-infected cows when compared with healthy control. On the other hand there was a significant leucopenia and thrombocytopenia along with a significant eosinophilia. The biochemical findings of infected cows revealed a significant increase in activities of serum alanine aminotransferase, aspartate aminotransferases, alkaline phosphatase and lactate dehydrogenase. In contrast a significant decrease in serum levels of total proteins, albumin, globulins, sodium and potassium. While serum bilirubin (total, direct and indirect), urea and creatinine levels were significantly increased. After administration of single I/M imidocarb dipropionate 12% (1.7 mg/kg BW) to Babesia infected cows, there was an improvement in hematological and biochemical parameters. It concluded that molecular detection of *B. bigemina* more sensitive than blood smear. Treatment infected cows with imidocarb improves the clinical signs, hematological and biochemical parameters that indicate recovery of infected cows.

Key words: babesiosis; cow; erythrogram; imidocarb; PCR; 18S rRNA; Egypt

Introduction

Babesiosis is a tick borne hemoprotozoosis worldwide affecting several species of mammals and caused by multiplication of apicomplexans in the Babesia genus intra-erythrocytes. The large number of species described more than one hundred is an evidence of the evolutionary success of this parasite (1). The most economical tick borne disease of livestock is bovine babesiosis. It spreads in Africa, Australia, Central and South America.

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Many species of Babesia can cause bovine babesiosis but the most important bovine babesias are B. *bigemina*, B. *bovis* and B. *divergens* (2). The disease is characterized clinically by sudden onset of acute fever, weakness, rumen stasis, anorexia, anemia and an increase in heart and respiratory rates, pale or icteric mucous membranes, in addition to hemoglobinuria (3).

Economic importance for babesiosis are increased due to abortions, reduction of milk or meat production and even mortality, so increase the demand of encounter the tick vector and vertebrate host by acaricide and therapeutics approach respectively (2). Routine classical diagnosis of acute cases of babesiosis is based on the examination of blood smears that is considered the gold standard, while, PCR and gene sequencing allow the diagnosis of faint parasitemia that couldn't be identified by the classical methods (4).

Imidocarb dipropionate is a urea derivative used in veterinary practice as an antiprotozoal agent for the treatment of babesiosis and other hemoprotozoosis (5). Due to the huge losses and increasing trend of babesiosis in cattle, the present study was implemented to diagnose the infected cattle with Babesiosis in the relation to some hematological and biochemical changes occurs during and after drug therapy.

Material and methods

Animals

The present study was implemented during the period from May to December 2015 on thirty crossbred female cows in Sherbeen city, Dakahlia Governorate, aged from two to four years. Ten cows were clinically healthy and confirmed by laboratory means, used as a normal control and twenty cows showed clinical symptoms of babesiosis (fever, pale or icteric mucous membranes and hemoglobinuria) and confirmed by detection of intra-erythrocytic stages of the hemoparasite in stained blood film from ear vein. The diseased cows were treated with Imizol®(Schering-Plough Animal Health, Germany). Each ml contains imidocarb 85mg as 12% imidocarb dipropionate by a single I/M injection with 1.7 mg / kg BW (6).

Blood Samples

The blood samples (n=30) were gathered from the jugular vein of all animals (control and infected cows) during the feverish period and 7 days post treatment. The samples were separated into two divisions. The 1st was put in chemically free tubes containing anticoagulant (dipotassium salt of EDTA) for hematological and molecular studies, while the 2nd division was collected in centrifuge tubes without anticoagulant for separation of serum. After clotting, the blood samples were centrifuged at 3000 rpm for 15 minutes and the clear serum was carefully aspirated into chemically free and clean tubes and stored at –20°C until used later for biochemical parameters analysis.

Molecular diagnosis of Babesiosis by PCR

Whole blood sample (200µl) was used for extraction of genomic DNA by G-spin™ Total DNA Extraction Kit (iNtRON Biotechnology, Inc., South Korea) following the manufacturer instructions.

The complete nucleotide sequences of 18S rRNA of all piroplasms were downloaded from GenBank and aligned by MAFFT alignment in Geneious software. Consequently, one primer set was designed from a conserved area using Integrated DNA technology online website (https://eu.idtdna.com/PrimerQuest/Home/Index). The primer set amplify 733 bp from the 18S rRNA gene of piroplasms in the examined blood samples (n=30). The sequences of the primers are forward Piro-18-F2: 5′-'ACT GTC AGA GGT GAA ATT CTT AG-3'' and reverse Piro-18-R2:' 5′- AAT AAT TCA CCG GAT CAC TCG- 3''. The amplification condition was as follow, denaturation initiated at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 63.1°C for 30 sec, extension at 72°C for 30 sec, and final extension at 72°C for 5 min (4) ,5 µl of the PCR product was electrophoresed along with 100bp DNA molecular weight 1% agarose gel containing ethidium bromide (at the rate of 0.5µg/ml) at constant 80V for 30 minutes in 1X TAE buffer. To confirm presence of B.

bigmina, DNA sequencing of the amplified product of 18S rRNA region (733 bp) was performed in one representative sample. The GenBank accession number obtained was AAA6558. It was performed in an automatic sequencer at Macrogen Corp. (Seoul, South Korea), after purification of amplicons using a PCR purification kit Solgent Co Ltd (South Korea). The sequences were trimmed then analyzed using Geneious bioinformatics software (Biomatters). Consequently, the trimmed sequences were identified in GenBank by search in basic local alignment search tool (BLAST).

Hematological studies

Hematological examination included estimation of RBCs count, Hb concentration, PCV, MCV, MCHC, MCH, platelets, total and differential leukocytic counts by using full automatic digital cell counter (Sysmex XS-800i, Germany).

Biochemical studies

The obtained serum from centrifugation of coagulated blood was used for estimation of alanine and aspartate aminotransferases (ALT and AST) according to Reitman and Frankel (7). Alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) according to Wu (8). Total proteins, albumin, globulins and albumin/globulins ratio (A/G ratio) were measured as previously reported (9-12), respectively. A total and direct bilirubin level was evaluated according to Tietz (13) while indirect bilirubin was calculated by subtraction of direct bilirubin from total bilirubin (12). Evaluation of urea, creatinine, sodium and potassium were performed according to previous reports respectively (14-16).

Statistical analysis

Data obtained from this investigation was statistically analyzed using the one way analysis of variance (ANOVA) (17). Means at the same row have different letters are significantly different.

Results and discussion

Prior to the treatment, the clinical examination revealed that twenty out of thirty cows with a case history of anorexia and loss of condition revealed pyrexia above (41°C), weakness, depression, rumen stasis, increase in respiratory and heart rates, conjunctival and vaginal mucous membranes were ranged from pale in mild cases to dark yellowish or icteric discoloration (Figure 1A) in more progressive cases, in addition to hemoglobinuria (Figure 1B). The microscopic examination of Giemsa stained blood smear divulge double pear shaped (pyriform) of *Babesia* inside RBCs (intra-erythrocytes) in thirteen clinically infected cows (Figure 2). The newly designed primer set succeeded in amplification of 733 bp of 18S rRNA region in all piroplasms. The nucleotide sequence analysis confirmed the presence of *B. bigmina* in the twenty diseased cows. There is no correlation between the number of RBCs containing pyriform Babesia and severity of the clinical symptoms, where in seven cases Giemsa stained blood smears give a false negative results that could attributed to the site of blood collection and the thickness of blood film. But, PCR succeeded to discover existence of protozoa even with minute levels of infection as reported by Constable et al. (18).

The sudden onset of pyrexia in this study could be referred by a response to the impact of the toxic substances on thermoregulatory that generated during the metabolism of Babesia (18). On the other hand, hemolysis of RBCs leads to anemia and anemic hypoxia resulting in increase in respiratory and heart rates which might be a compensatory mechanism of the body for proper oxygenation of the tissues (18). Hemoglobinuria present in infected animals could be also referred to intense hemolysis related to presence of *Babesia* spp. inside RBCs resulting in hemoglobinemia and therefor hemoglobinuria (19).In current study the observed clinical findings were in accordance with results of previously recorded signs in babisiosis (20,21).

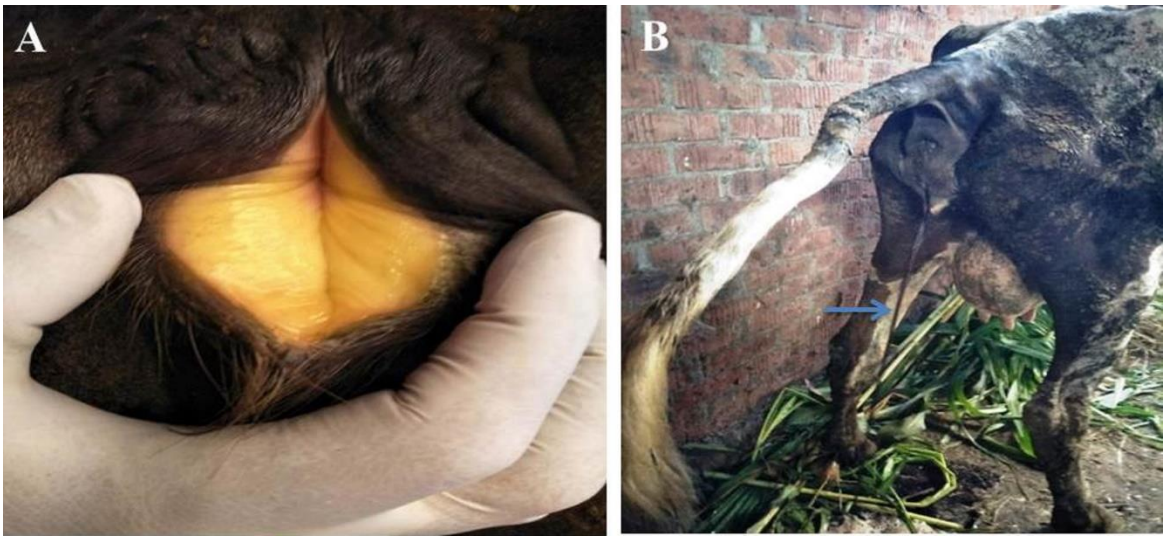

Figure 1: Clinical signs of babesiosis in naturally infected cow showing icteric vaginal mucous membrane (A) and dark red to brown urine (B)

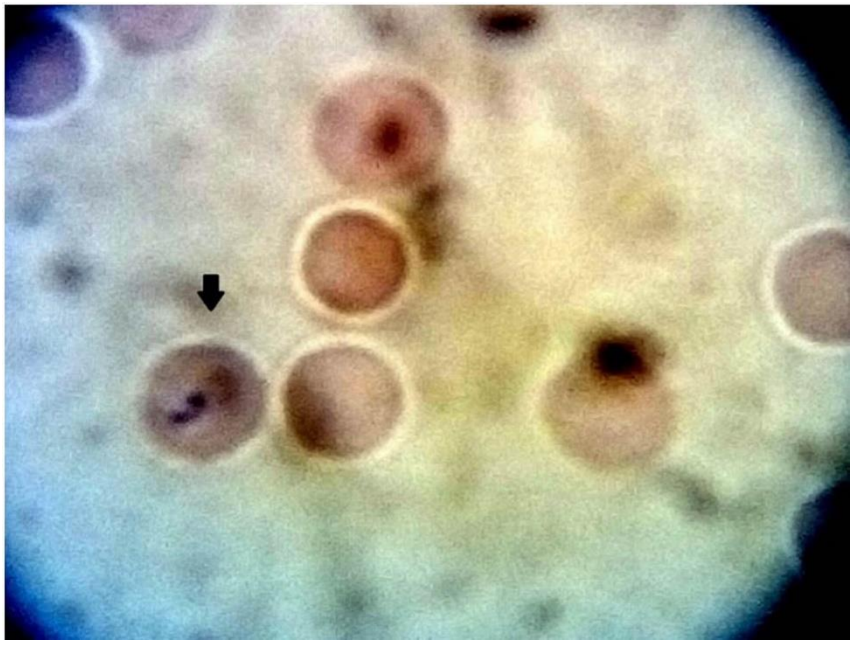

Figure 2: Giemsa-stained blood smear of naturally infected cow showing intra-erythrocytic Pyriform (Pear-shape) of *Babesia bigemina* in pairs (120 X)

Treatment of Babesia-infected cows with imidocarb dipropionate in this study lead to improvement in the clinical signs in the treated cows with subsiding of high temperature, heart and respiratory rates in addition to the color of urine changed from dark red/brown to slight yellow (normal color) that suggested the success of treatment. Imidocarb dipropionate became the product of choice in treatment and control of babesiosis, in addition to its therapeutic usefulness, it also witness to be effectiveness as preventive when used twice of therapeutic dosage (3). Among its advantage it is the solely babesiacide that continually clears the host of parasites, and cows treated with imidocarb may finish up with a sterile solid immunity (22).

Concerning the hematological findings in this study (Table 1), a significant falloff in the mean of RBCs count, HCT%, Hb concentration and MCHC with an increase in MCV were observed in cows infected with *B. bigemina*

when compared with healthy control animals that indicated a macrocytic hypochromic anemia. These results may be attributed to mechanical impairment caused by binary cleavage of trophozoite intra-erythrocytes (23), production of anti-erythrocyte antibodies directed against structural erythrocytes membrane either infected or the uninfected (24), and due to the increase in erythrophagocytosis by activated macrophages (25). The same results were obtained previously (6,26,27). The macrocytic hypochromic anemia may be due to increased erythropoiesis and release of a significant number of reticulocytes from the bone marrow which occurred as a response to the hemolytic process (28). MCH values revealed a significant increase in Babesia infected non treated cows when compared with control animals that confirm hemolytic condition (29).

Table 1: Erythrogram and leucogram (means ± SE) of control non-infected cows, Babesia infected cows before and 7 days after treatment with imidocarb dipropionate 12% by a single I/M injection dose 1.7 mg/ kg BW

Parameters	Cows			P-value
	Control	Before treatment	Seven days after treatment	
Hemoglobin (g/dl)	9.91± 0.27^{a}	5.48± 0.23^{c}	7.45± 0.26^{b}	0.01
RBCs (x10^{6}/µl)	6.85± 0.21^{a}	3.28± 0.14^{c}	5.00± 0.17^{b}	0.00
Hematocrit (%)	30.03±0.68 ^{a}	17.92± 0.74^{c}	22.87± 0.8^{b}	0.00
MCV(fl)	43.84± 0.89^{b}	54.63± 1.68^{a}	45.74± 1.06^{b}	0.01
MCH (pg)	14.47± 0.36^{b}	16.70± 0.4^{a}	14.90± 0.29^{b}	0.03
MCHC (%)	33.00± 0.25^{a}	30.58± 0.44^{b}	32.58± 0.43^{a}	0.01
WBCs (x10^{3}/µl)	9.86± 0.32^{a}	5.48± 0.41^{c}	8.5± 0.3^{b}	0.00
Lymphocytes (x10^{3}/µl)	5.43± 0.27^{a}	2.35± 0.25^{c}	4.55± 0.23^{b}	0.00
Monocytes (x10^{3}/µl)	0.43± 0.02	0.47± 0.02	0.44± 0.02	0.74
Neutrophils (x10^{3}/µl)	3.72± 0.09^{a}	2.38± 0.14^{c}	3.22± 0.09^{b}	0.00
Eosinophils (x10^{3}/µl)	0.12± 0.006^{b}	0.15± 0.007^{a}	0.14±0.009^{a,b}	0.04
Basophils (x10^{3}/µl)	0.14± 0.008	0.12± 0.006	0.12± 0.007	0.83
Platelets (x10^{3}/µl)	529± 27.08^{a}	273± 4.82^{c}	422± 21.38^{b}	0.01

******Highly significant difference at $p \leq 0.01$**; ***significant difference at $p \leq 0.05$, N S: not significant
RBCs: total erythrocytic count, MCV: mean corpuscular volume,MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular hemoglobin concentration, WBCs: white blood cells
Different superscript letters at the same row were significantly different.

Regarding leucogram in this study (Table 1), a significant falloff in WBCs, lymphocyte and neutrophil counts along with a significant increase in eosinophil count was observed in infected cows before treatment compared with control cows. The monocytes and basophils counts were non-significantly changed. One mechanism involved in leucopenia in Babesia infected cows before treatment includes the ability of platelets to bind with activated endothelial cells to interact with leucocytes and induce "secondary capture". The subsequent neutrophil-endothelial interaction could contribute to the decrease in WBCs count (30). Moreover, the mechanisms of neutropenia is yet to be refined whether it's an effect of direct damage to the hematopoietic precursor cells, splenic sequestration, raised neutrophil adherence, or a series of all (31) as well as neutropenia is common during acute infections (32). Lymphopenia observed in Babesia-infected cows before treatment may be attributed to stress (12). Another reason for neutropenia, lymphopenia and subsequently leukopenia may be attributed to sequestration of leucocytes in the spleen (33). While the significant increase in eosinophils due to infected cows mostly with parasitic tick infestation on skin cause allergic reactions (12). Thrombocytopenia was seen in Babesia infected cows before treatment could be attributed to several mechanisms such as platelet sequestration inside the spleen, destructed platelet as immune mediated disease and progress of disseminated intravascular coagulation (26,27). Following administration

of imidocarb dipropionate to Babesia-infected cows, an improvement in the hematological parameters was recorded which represented by an increase in the mean levels of RBCs count, HCT%, Hb concentration toward the normal values and this may be attributed to the elimination of the *Babesia* spp. from blood of the treated animals (34).

Regarding the biochemical investigations in the present work (Table 2), this disclosed that there was a significant increase in activities of serum ALT and AST of cows infected with *B. bigemina* when compared with control non-infected cows. Also, hepatic cell degeneration resulted from enormous hemolysis may occur in synchronization with hypoxia may lead to increase in serum AST and ALT activities (35). Erythrocytes also contain AST; therefore, the hemolysis occurred during the disease considered as an important cause of increase serum AST activity (32). Also, there was a significant excess in serum activity ALP of cows infected with *B. bigemina* when compared with healthy non-infected cows, ALP derives from the bile ducts and does not leak from the hepatocyte with increased membrane permeability, so cholestasis probably caused by hepatomegaly could be the reason of the marked increase in ALP activity (32). Also a significant increase of LDH that is most abundant in erythrocyte and hepatic cells can function as a marker for erythrocyte hemolysis and hepatocellular injury (12, 32).

Table 2: Serum liver function parameters (means ± SE) of control non-infected cows, Babesia infected cows before and 7 days after treatment with imidocarb dipropionate 12% single I/M injection 1.7 mg/ kg BW

	Cows			
Parameters	**Control**	**Before treatment**	**Seven days after treatment**	**P-value**
ALT (U/L)	31.3± 3.77^{c}	75.2± 3.59^{a}	46.85± 2.55^{b}	0.00
AST (U/L)	65.5± 3.55^{c}	136.5± 5.33^{a}	87.15± 5.18^{b}	0.00
ALP (U/L)	108.3± 5.43^{c}	287.7± 8.34^{a}	184.1± 9.23^{b}	0.00
LDH (U/L)	53.1+± 1.02^{c}	69.7± 2.13^{a}	58.91±0.98^{b}	0.00
T. Proteins (g/dL)	6.78± 0.08^{a}	5.27± 0.09^{c}	5.88± 0.1^{b}	0.00
Albumin (g/dL)	3.54± 0.13^{a}	2.8± 0.08^{c}	3.14± 0.04^{b}	0.01
Globulins (g/dL)	3.24± 0.1^{a}	2.47± 0.1^{b}	2.74± 0.1^{b}	0.00
A/G ratio	1.11±0.04	1.16±0.07	1.21±0.06	0.62
T. Bilirubin (mg/dL)	0.32± 0.02^{c}	1.66± 0.11^{a}	0.72± 0.04^{b}	0.00
D. Bilirubin (mg/dL)	0.18± 0.01^{b}	0.29± 0.02^{a}	0.2± 0.005^{b}	0.00
Ind. Bilirubin (mg/dL)	0.14± 0.01^{c}	1.37± 0.09^{a}	0.52± 0.03^{b}	0.00

******Highly significant difference at $p \leq 0.01$, N S: not significant**,**
AST**:** aspartate aminotransferase, ALP: alkaline phosphatase, ALT**:** alanine aminotransferase ,LDH: lactate dehydrogenase and A/G ratio: albumin/ globulins ratio.
Different superscript letters at the same row were significantly different.

Concerning serum proteinogram, a significant decrease in the serum levels of total proteins, albumin and globulins were recorded in cows infected by *B. bigemina* and these could be due to decrease protein production as a result of deprivation of diet protein resulting from anorexia and fever accompanied infection. Also, disturbed hepatic functions and loss of proteins in urine can play a role (36). The present data disclosed a significant rise in levels of serum bilirubin (total, direct and indirect) in cows infected with *B. bigemina* when compared with control cows which may be attributed to the massive hemolysis occurring throughout the infection period resulting in hypoxia that cause degeneration of the hepatic cell that leads to increase level of serum bilirubin (34 ,37).

Regarding kidney function tests, a significant rise in levels of serum urea and creatinine with a significant decrease in both sodium and potassium values in Babesia-infected cows in compare with the control cows. The observed elevation of urea and creatinine levels (Table 3) in Babesia-infected cows could be resulted from kidney dysfunction. This impairment in renal function may be due to necrotizing,

separation of epithelial cells of renal tubules in proximal convoluted tubules and hemoglobin casts (38). The increase in levels of serum creatinine and urea could be also referred to systemic hypotension that occurs in Babesia infection lead to kidney vasoconstriction that have be the most principal cause of kidney hypoxia and more inculpate than hemoglobinuria in damaging of renal tissue, also hemolytic anemia may participate to deficient oxygenation lead to degenerative changes in the cytoplasm of the renal tubules lining epithelial cells in association with perivascular mononuclear leucocytic cellular infiltrations mainly lymphocytes and macrophages (35).

Table 3: Serum kidney function parameters (means ± SE) of control non-infected cows, Babesia infected cows before and 7 days after treatment with imidocarb dipropionate 12% single I/M injection 1.7 mg/ kg BW

Parameters	Cows			P-value
	Control	Before treatment	Seven days after treatment	
Urea (mg/dL)	17.7± 1 [b]	42.1± 2.37[a]	18.5± 0.35 [b]	0.00
Creatinine (mg/dL)	1.1± 0.03 [b]	1.98± 0.13 [a]	1.3± 0.06 [b]	0.01
Sodium (mmol/L)	141.5± 0.7 [a]	137.05±0.76 [b]	140.7± 0.48 [a]	0.00
Potassium (mmol/L)	4.2± 0.04 [a]	3.9± 0.03 [c]	4.09± 0.03 [b]	0.00

**Highly significant difference at $p \leq 0.01$

Different superscript letters at the same row were significantly different.

The hyponatremia observed in this study in cows infected with Babesia may be produced by reduction of renal perfusion and hypotension , that have been percept throughout the course of babesiosis as described by Matijatko et al. (39). In this status, reduction of glomerular filtration invigorates the renin angiotensin aldosterone system, lead to reservation of sodium and water. Furthermore, activation of antidiuretic hormone liberate in hypotension cases causes reduced water excretion. The dilution of sodium despite of the retention leads to hyponatremia (40).

Hypokalemia noticed in this study in cows infected with *B. bigemina* when compared with healthy non-infected cows could be due to forfeiture of potassium ions by the alimentary tract or kidneys, or from potassium translocation into intracellular fluids (41). Also deg-enerative and necrotic alterations in the renal proximal tubules of may contribute in the development of hypokalemia (42).

After administration of imidocarb dipropionate to Babesia-infected cows, there was an improvement in biochemical parameters that indicates recovery from the possible liver and kidney damage. The present results were in agreement with Sevinc et al. (43), while they were in disagreement with results of Kumar et al. (44). The disagreement may be due to different animal species and different blood parasite subspecies.

Conclusion

It could be concluded that molecular detection of *B. bigemina* is more sensitive than blood smear. The hematological and biochemical parameters are adversely affected in *B. bigemina* naturally infected cows, which could be improved by single dose of imidocarb.

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Conflict of interest

The authors declare that they have no competing interests.

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Original Research Article

EFFECT OF NATURAL AND SYNTHETIC FOOD COLORANTS ON SPERMATOGENESIS AND THE EXPRESSION OF ITS CONTROLLING GENES

Metwally Montaser[1,2], Rasha A.E. Abiya[3,4] ,Mohamed Afifi[5,6]*, Salina Saddick[7], Ayed S. Allogmani[8], Omar A. Almaghrabi[6]

[1]Department of Zoology, Faculty of Science, Al-Azhar University, 11884 Cairo, Egypt. [2]Department of Biotechnology, Faculty of Science, Taif University, 21974 Taif, P.O. Box 888. Saudi Arabia; [3]Department of Zoology, Women's College for Arts, Science & Education, Ain Shams University, Cairo, Egypt; [4]Department of Biology, Faculty of Applied Science, Umm Al-Qura University, Makka Saudi Arabia; [5]Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt; [6]Department of Biological Sciences, Faculty of Science, University of Jeddah, Jeddah, Kingdom of Saudi Arabia; [7]Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia; [8]Department of Biological Sciences, Faculty of Science, University of Jeddah, Khulais branch, Jeddah, Kingdom of Saudi Arabia

*Corresponding author, E-mail: mama200100@gmail.com

Abstract: The use of food additives is controversial. However, data regarding their effects on fertility genes are still very sparse. The present study was designed to assess the effects of two coloring agents, carmoisine (synthetic) and curcumin (natural) on the expression of some genes with fertility impact. Sixty three male Sprague-Dawley albino rats were orally administered carmoisine and curcumin in three doses, acceptable daily intake (ADI), 5x- and 10x- ADI for 15, 30, and 45 days. Sperm analysis and testicular expression level of biomarkers Testin, Glial cell derived neurotrophic factor (GDNF), tyrosine kinase receptor (c-KIT), follicle stimulating hormone receptor (FSHR), A kinase anchor protein 3 (PRKA3), spermatogenesis associated 7 (Spata7), Stage-specific embryonic antigen-1 (SSEA1) genes were measured and supported with histopathological studies on rat testes tissues. The recorded results revealed significant down regulation of the tested genes in rats supplemented with carmoisine in time and dose dependent manner. However, these declines were also observed after treatment with medium and high doses of curcumin. Sperm counts were significantly decreased after carmiosine treatment in a dose dependent manner, it was 74.6±6.36, 74.00±6.63, 49.00±0.28 and 147.00±3.2 for ADI, 5xADI, 10xADI and control group respectively, without any changes after curcumin treatment. Also, histopathological studies indicated deleterious effect with medium and high doses of carmiosine. In Conclusion, carmoisine induced hazardous effects on fertility at different levels when consumed in concentrations higher than the acceptable daily-authorized level (50 mg/kg b.wt). However, curcumin as a natural food color is saver than carmoisine up to certain levels.

Key words: carmoisine; curcumin; spermatogenesis genes; rat testis

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Introduction

Coloring agents, either natural or synthetic, have been used in food industry from many centuries to make the food more acceptable. The misuse of the coloring agents especially the synthetic one causes a serious damage to human body (1). Historically natural compounds have been used 400 BC, while synthetic one have been used in late 1800S and early 1900S. One of the most usually used natural compound is curcumin and one of the most usually synthetic compound was carmoisine (2).

Curcumin is the active ingredient of turmeric (the substance derived from the root extract of *Curcuma Longa* plant), is a powerful coloring agent used in many countries including China, India, Saudi Arabia and others for many years. It has a powerful anti-inflammatory, antioxidant and many reports confirm its anticancer activities due to its phenolic compounds (3). Curcumin has anticarcinogenic effect against many cancer types including liver, breast, colon, pancreatic and ovarian cancer producing its effect through induction of programmed cell death and cell-cycle arrest. Curcumin posses potent antioxidant properties, it potentiated the antioxidant system through activation of enzymatic and non-enzymatic antioxidants, and inhibition of pro-oxidants production (4). Curcumin has been used to improve male fertility, the *in vitro* application of corcumin in a concentration of 50 μM/ml of culture medium enhanced the spermatozoa activity and potentiated the spermatozoa antioxidants through reduction of the intracellular superoxide anion production (5). Curcumin mitigated the *in vitro* sperm injury induced by H_2O_2 through induction of sperm mitochondrial cytochrome B (Cyt B) and NADH dehydrogenase 5 (NADH), resulting in improvement of sperm motility of leucocytospermic patients (6). Curcumin enhanced male fertility through induction of 3β-HSD and 17β-HSD activities, testosterone concentration, sperm motility and morphology with improvement of oxidative stress status in testis of male rats exposed to pesticides (7). Carmoisine (E 122) is an azo dye allowed by European Union (EU) as coloring agent for food and permitted by FAO and WHO in 1983 with ADI 0-4 mg/kg B.wt/day. Uncontrolled use of carmoisine leads to many health problems for humans (8). Carmoisine may produce the deleterious effects through induction of lipid peroxidation and suppression of antioxidant enzymes activity (9). Moreover, it induces a genotoxicity through induction of DNA damage (10). Overuse of carmoisine has led to many reproductive problems in mice, which manifested by decrease in sperm count, motility, viability and increase of sperm abnormalities (11). There are many conflicted mechanisms for the effect of carmoisine on reproductive function including the induction of imbalance in sperm and testis antioxidant system through generation of free radicals and inhibition of antioxidants (12).

This study aimed to monitor the action of one natural food coloring agent (curcumin) and one synthetic (carmoisine) on rat testicular tissue and sperm through investigating the testicular tissue histology, sperm characters and the expression of Testin, GDNF, c-KIT, FSHR, PRKA3, Spata7 and SSEA1 genes controlling spermatogenesis.

Material and methods

Chemicals

Carmoisine ($C_{20}H_{12}N_2O_7S_2Na_2$) and curcumin were ordered from Lobachemie PVL Ltd Company (Mumbai, India). These chemicals were prepared in three different concentrations, low, medium and high by their dissolving in distilled water in doses equivalent to acceptable daily intake (ADI), 5 folds of ADI (5xADI) and 10 folds of ADI (10xADI), respectively per each.

Animals and experimental design

All procedures of the current experiment have been approved by the Ethical Committee of the Faculty of Science, King Abdalaziz University, under the number of FS 16582.

A total of 63 young male Sprague-Dawley albino rats weighting about 160–180 g were used in the present study. Rats were obtained from Animal House, King Abdel Aziz University, Jeddah, KSA and kept under

observation for about 7 days before treatment to exclude any intercurrent infection. They were maintained in stainless steel cages at normal atmospheric temperature of 27 ± 5˚C as well as under good ventilation and were kept on standard diet. Rats were randomly divided in to 7 groups, control group (c) received distilled water; groups 2-4 received carmoisine in doses of acceptable daily intake (ADI, 50 mg/kg b.wt.), 5 folds of ADI (5xADI) and 10 folds of ADI (10xADI), respectively (12) and groups 5-7 were treated with a dose equivalent to ADI of curcumin (15.75 mg/kg b.wt), 5 folds of ADI (5xADI) and 10 folds of ADI (10xADI), respectively (8). Three rats were taken randomly from each group every 15 days for a period of 45 days, sacrificed, their testes were removed, the left one was kept in neutral buffer formalin for histological studies and the right one was kept in RNAlater (catalogue number R0901. Sigma-Aldrich Co. St. Louis, Missouri,USA) for molecular assays.

Histopathological studies

Specimens from the testes were collected and fixed in 10% buffered neutral formalin solution, dehydrated in gradual ethanol (70-100%), cleared in xylene, and embedded in paraffin. Five-micron thickness paraffin sections were prepared and then routinely stained with Hematoxylin and Eosin (HE) dyes and then examined microscopically (13).

Sperm analysis

Conventional method of sperm count was done on sample derived from the cauda epididymis (14). Sperms were collected by mincing the epididymis with anatomical scissors in 1 ml saline. Ten microliters of sperm suspension were placed on a slide for counting using hemocytometer and expressed as million/ml of suspension.

Molecular Assays

Expression level of fertility genes, Testin, GDNF, FSHR, PRKA3, Spata-7, SSEA1, c-KIT, and Beta-2-microglobulin (B2m) were semi-quantitated. The mRNA expression levels were examined by reveres transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from 40 mg testes using Biozol (Biolabs, USA). Synthesis of cDNA with total RNA was performed using reverse transcriptase (Thermo Scientific, Waltham, Massachusetts, US). Subsequently, PCR-amplification was performed using specific primers for each gene, GDNF, F: 5'-CAC CAG ATA AAC AAG CGG CG-3' R: 5'-TGG AGC CAG GGT CAG ATA CA-3'; TESTIN, F: 5'-AAT GGG GCA TGA AGG GCT AC-3' R: 5'-TCA CTA CCC CTG TCC CCT TT-3'; c-KIT, F: 5'-GAT GCT CAA ACC AAG TGC CC-3' R: 5'-ACA GGA AGG CTC CGT TGA GT-3'; FSHR, F: 5'-GTA ACC TCG CCT TCG CTG AT-3'. R: 5'-TCC AGC CCA ATA CCA TGA CG-3' ; PRKA3, F: 5'-GGC TTA CCC AGG AGA CCA AC-3'. R: 5'-CAC TAG GTT TGT GAG GCG GT-3'; Spata-7, F: 5'-GAG GAA GGA GTT GGC ACG AT-3'. R: 5'-CTC TGG CGA AGG GAT GAG TG-3'; SSEA1,F- 5'-ATT TGC CTC TGT CCT GTC CTG-3'. R- 5'-GAT CCT GGG CTC CGA AAC TG-3' and B-2-macroglobin gene (internal control for the semi-quant-PCR) F: 5'-GTC TCA GTT CCA CCC ACC TC-3' R: 5'-GAC GGT TTT GGG CTC CTT CA-3'.

Primers were designed by using Primer3 software (http://bioinfo.ut.ee/primer3/) as per the published *Rattus norvegicus* gene sequence in the National Center for Biotechnology Information (NCBI) database. The optimized PCR conditions were, heating for 1 min. at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. The final extension step was 5 min at 72 °C. PCR products were separated on 1.5% agarose gels visualized under UV light and analyzed using Alfa Ease FC software.

Statistics

Data were represented as means ± SE. Statistical analysis was evaluated by one-way analysis of variance (ANOVA). Once a significant F test was obtained, LSD comparisons were performed to assess the significance ($P < 0.05$) of differences among various treatment groups. Statistical Processor System Support "SPSS" for Windows software, Release 16.0 (SPSS, Chicago, IL) was used.

Results and discussion

Histopathology findings

Testes of control rats showed uniform seminiferous tubules with complete spermatogenesis and interstitial connective tissue. Sometimes, the lumina of the little number of seminiferous tubules was empty. Tubular epithelium was intact and contained Sertoli cells resting on the basement membrane, together with spermatocytes and spermatogonia. Round and elongated spermatids were embedded in or associated with the Sertoli cells at different stages of the spermatogenic cycle (Figure 1A and B). Meanwhile, testicular tissue of rats received carmoisine showed degenerative changes, atrophy and necrosis in the majority of the seminiferous tubules. The changes were increased gradually with increasing the dose and periods. The testes of acceptable daily intake carmoisine were mostly normal particularly at the 15 and 30 days post treatment (PT) meanwhile, on the 45th day revealed focal mild testicular degeneration of single or several layers of vacuolated spermatocytes besides congested interstitial blood vessels **(**Figure 1C and D**)**. Few desquamated spermatocytes were seen in the lumen of some seminiferous tubules (Figure 1E). These lesions became prominent in the higher doses (5 and 10 folds) and represented by shrunken, disorganized seminiferous tubules with irregular, buckled basement membrane and incomplete spermatogenesis. Other tubules showed coagulative necrosis, round cells infiltrations and depletion of germinal epithelium with hyalinization of the luminal contents (Figure 1F), particularly in 30 and 45 days groups. Moreover, the seminiferous tubules were almost devoid of spermatids and spermatozoa. Vacuolar degeneration of spermatogonia and Sertoli cells was evident. Degenerated germinal epithelial cells were sloughed in the lumina of most seminiferous tubules. Regarding to the interstitium, there were congestion of the interstitial blood vessels and edema that was represented by faint eosinophilic material. The current results are in line with previous results by Visweswaran and Krishnamoorthy (15), and Ghonimi and Elbaz (16) who stated that carmoisine produced free radicals, which in turn cause damage to the cellular compartment system of rat testis. Conversely, the rats received curcumin showed normal testicular tissue with improvement of spermatogenesis. The latter was represented by presence of elongated spermatids and huge numbers of spermatozoa in all the lumina of the seminiferous tubules. These findings were visualized in all doses and periods; but they were more prominent in the higher doses and longest period (Figure 2). Curcumin was reported to have not only enhancing but an ameliorative action as well (17)

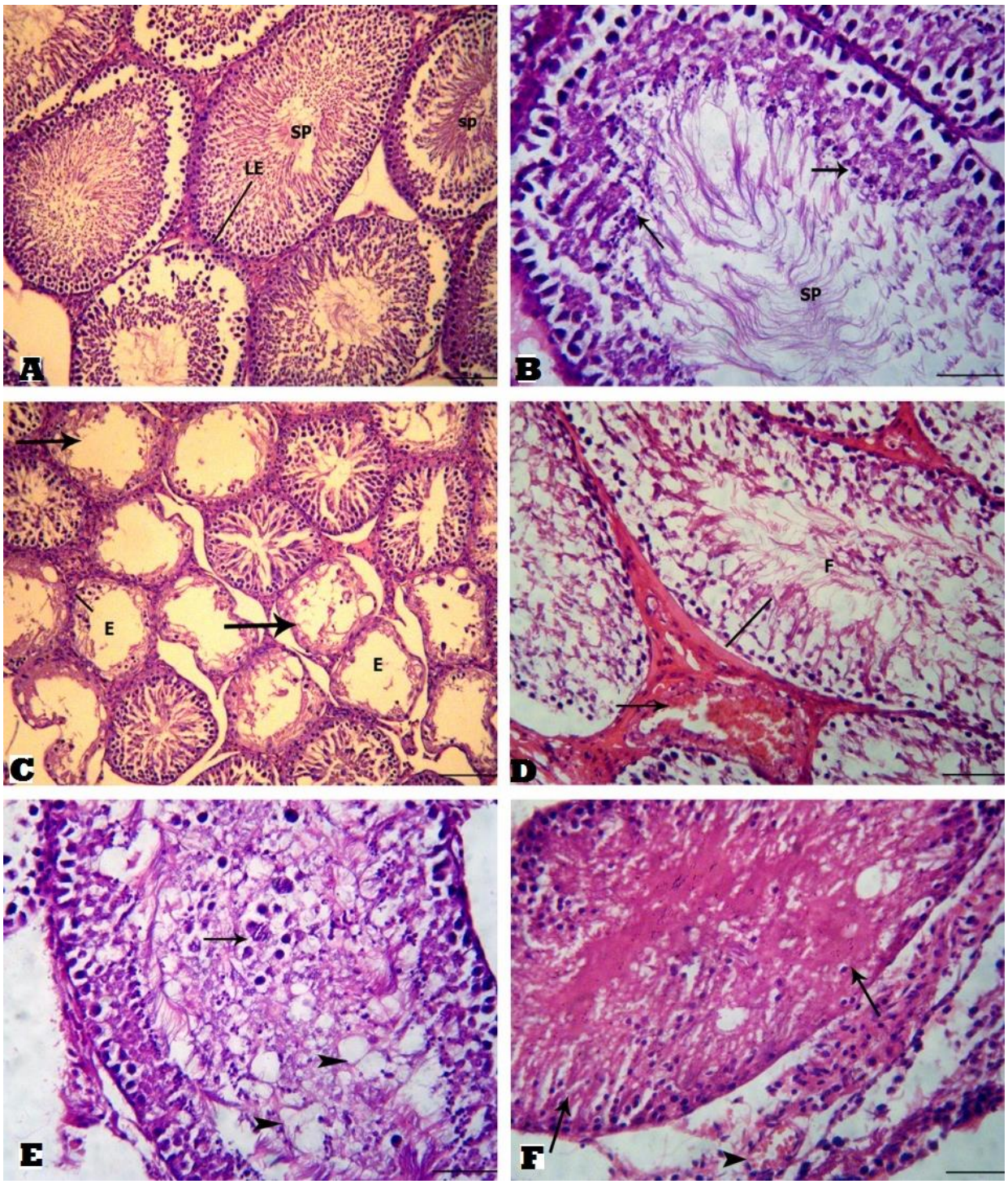

Figure 1: Histopathological effects of carmoisine on rat testes as studied with H &E staining. It showed normal lining epithelium and spermatogenesis in testes of control group (A and B). Carmoisine-treated rat testes were with focal mild testicular degeneration of vacuolated spermatocytes (C and D) and congested interstitial blood vessel (D). Desquamated spermatocytes in the lumen of seminiferous tubule were recorded (E and F). (H&E x 100for A and C and 400 for B, D, E and F)

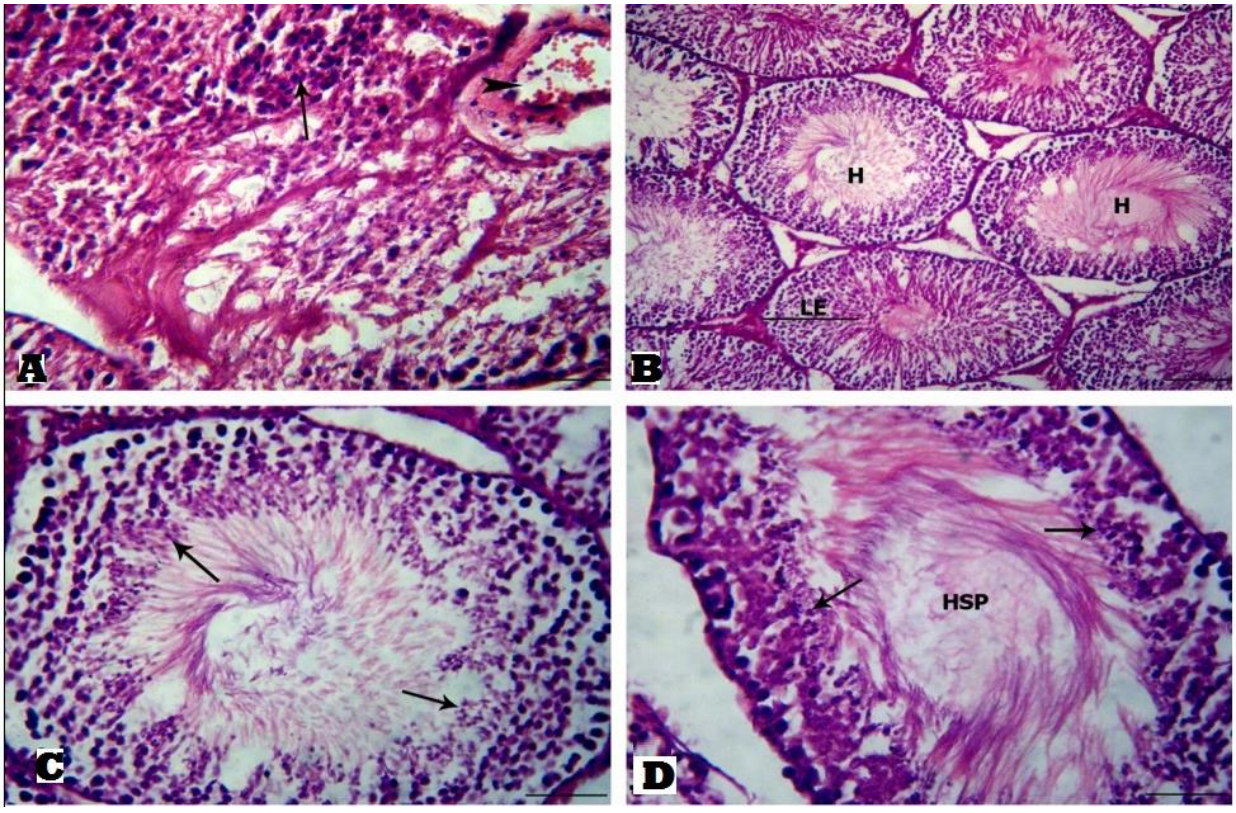

Figure 2: Histopathological effects of curcumin on rat testes as studied with H&E staining. Curcumin treated rat testes showed normal testicular tissue, improvement of spermatogenesis and presence of elongated spermatids and huge numbers of spermatozoa in all the lumina of the seminiferous tubules. (H&Ex 100 for B and 400 for A, C and D

Sperm analysis

Sperm count showed a gradual decrease in all tested groups over the course of carmoisine treatment (Figure 3). After 15 days of treatment, there was significant ($P < 0.05$) decline in sperm count that increased in a proportional rate by increasing the dose (ADI, 5xADI and 10xADI) as compared to control and tested groups (74.6±6.36, 74.00±6.63, 49.00±0.28 and 147.00±3.2, respectively). At the same time, significant decreases in sperm counts ($P < 0.05$) were recorded in the 30th day as compared to control. However, these effects were non-significant ($P > 0.05$) between the two interval times (15 and 30 days). At the end of the experiments (45th day), the lowest sperm count was recorded after ADI. However, medium and high doses, being significantly ($P < 0.05$) lower than that of the corresponding control (67.33±4.63, 62.16±3.87, 45.83±6.09 and 150.33±0.33, respectively). Sperm count was decreased in our study, and sperm characteristics were also affected in previous studies (18). Curcumin treatment showed non-significant ($P > 0.05$) effects on sperm count particularly at 15th and 30th days of treatment (Figure 3). Meanwhile, at the 45th day, significant ($P < 0.05$) decrease in sperm count was resulted in using high dose (10xADI) as compared to control (129.33±10.39 and 150.33±0.33, respectively). Also, slight decreases in sperm count were recorded at 15th day into 132.3±16.8, 133.6±18.3 and 137.3±10.1 due to ADI, medium and high doses, respectively. These beneficial effects remained visible and statistically relevant throughout dose interval during 30 days treatment groups (Figure 3).

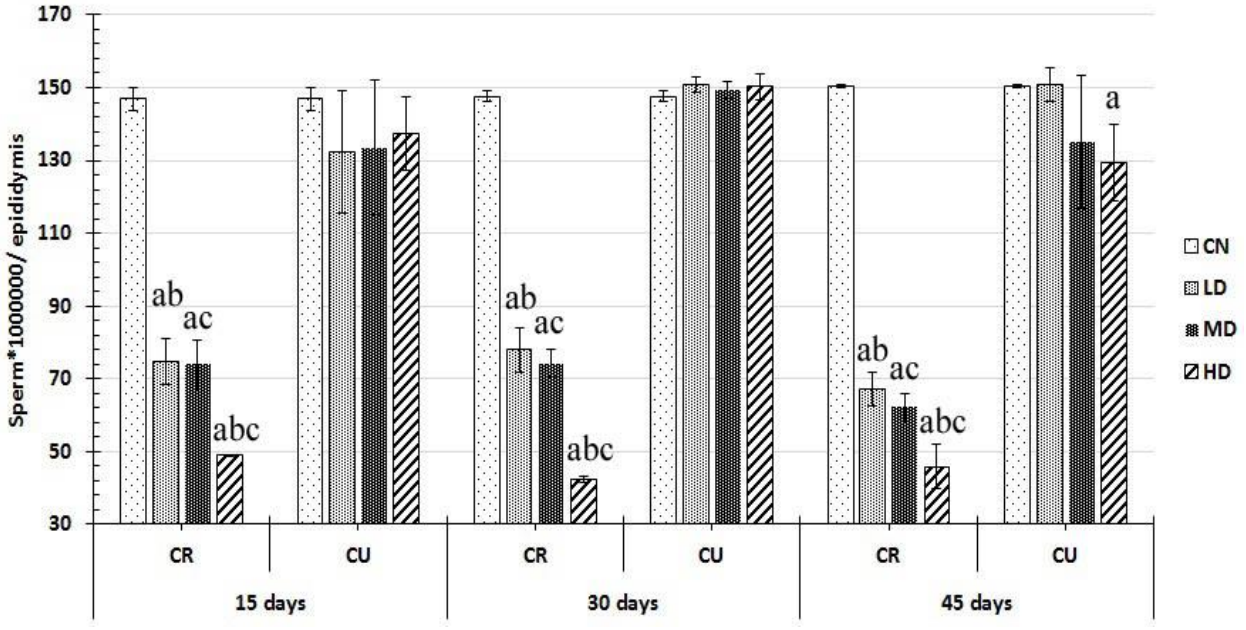

Figure 3: Histogram represents epididymis sperm counts in control and experimental rats. The letter (a) denotes significant (P <0.05) difference as compared to the control; however column with the same letter are in significant relation. Carmoisine (CR), curcumin (CU), control (CN), low dose (LD), medium dose (MD) and high dose (HD)

Level of spermatogenesis gene expression

Testin, GDNF, FSHR, PRKA3, Spata-7, SSEA1 and c-KIT genes expression levels (Figures 4 and 5) were fluctuated around that of the control, in a relative rate along time and dose interval. After 15 days of carmoisine treatment, levels of mRNA expressed Testin gene were slightly increased with increasing the dose ADI, 5x ADI and 10x ADI as compared to the control (0.363± 0.021, 0.524± 0.079, 0.380± 0.041and 0.316± 0.059, respectively), this increase was significant (P <0.05) in 5xADI group. Moreover, the expression levels resulted in curcumin treatment were increased to 0.473± 0.044, 0.386± 0.017and 0.433±0.020 from the control 0.316± 0.059 (low, medium and high doses, respectively). Only ADI dose induced significant (P <0.05) increase as compared to the control. At the 30 and 45 days groups, all tested doses of curmiosine and curcumin induced significant declined mRNA expression level of Testin gene than control except the low ADI dose of curcumin elevated the level from 1.400± 0.095 and 1.430±0.065 of the control into 1.590±0.017 and 1.496±0.029 after 30 and 45 days of treatment, respectively (Figure 5). Testin is a marker in monitoring germ cell-Sertoli cell interactions throughout spermatogenesis as the steady state testin mRNA was correlated with extensive renewal of cell-cell junctions during development (19), on the spot of this previous record, we could explain the current decrease in sperms released out of seminiferous tubules.

DGNF gene level was significantly increased from 0.409±0.005 of the control to 0.454±0.028 with low dose of carmoisine after 15 days treatment. However, there was significant (P <0.05) decrease due to treatment with medium and high dose (0.378±0.015 and 0.392±0.006, respectively). By increasing time and dose of carmoisine treatment, level of tested gene was slightly increased at 15th day. However, significant (P <0.05) decrease of the gene expression level was recorded after 45 days of treatment from 1.140±0.100 of the control to 0.413±0.016, 0.423±0.016 and 0.651±0.029 for ADI, 5xADI and 10xADI, respectively. Acceptable daily intake dose of curcumin and its 5 folds, significantly (P <0.05)

decreased the expression pattern of GDNF gene after 15 days treatment. Meanwhile, 10xADI dose resulted in significant increase from 0.409±0.005 of the control to 0.579±0.001. However, after 30 days of treatment, there were significant increase in the level of GDNF gene especially in groups received low and medium doses of curcumin. Significant decrease was recorded after 45 days treatment in time dependent manner from 1.140±0.100 of control to 0.711±0.079, 0.485±0.052 and 0.438±0.066, respectively (Figure 5). The decrease in expression of GDNF could indicate to negative differentiation of stem spermatogonia (the foundational cells of spermatogenesis) as it was defined to be a marker of stage-specific replication and differentiation of stem spermatogonia (20).

Both carmoisine and curcumin treatments induced significant down regulation of FSHR gene. These effects were time and dose dependent. After 15 days, the expression level of FSHR gene was significantly declined from 0.340±0.047 of the control to 0.286±0.018, 0.380±0.015 and 0.336±0.013 due to carmoisine treatment (ADI, 5xADI and 10xADI doses, respectively). In addition, there were significant decreases in the expression levels of FSHR gene in dose dependent manner at the 30th and 45th day of carmiosine treatment. Figure (5) illustrated down-regulation of FSHR gene resulted from curcumin different doses and treatment duration. This decline was significant between groups of the same duration of treatment. At 15 days group, the level of FSHR gene decreased from 0.340±0.047 of control to 0.303±0.016, 0.226±0.016 and 0.350±0.020 duo to ADI, 5xADI and 10xADI doses of curcumin treatment, respectively. Similarly, significant ($P < 0.05$) decreases were recorded after 30 and 45 days of curcumin treatment with all tested doses (Figure 5). The poor expression in the receptor of FSHR leads to low response to the hormone and consequently poor yield of sperms (21), which support the current results of epididymal sperm count due to carmoisine and curcumin treatments.

The expression level of PRKA3 gene was similar to that of FSHR gene, at the 15th day of carmoisine treatment, there was non-significant ($P > 0.05$) decrease recorded with ADI, 5xADI and 10xADI doses. However, significant decreases were obtained as result of ADI, 5xADI and 10xADI treatment at 30th day as compared to control 0.616±0.103, 0.573±0.017 and 0.563±0.012, respectively. Meanwhile, at the 45 day group low, medium and high doses significantly ($P < 0.05$) decreased the expression level (1.263±0.052, 0.460±0.020 and 0.483±0.017, respectively) as compared to control (1.640±0.075) and between groups of the same interval time. Similarly, curcumin treatment suppressed the level of PRKA3 gene expression with all tested groups in dose and time dependent manner (Figure5). At 15 day group, the level of PRKA3 was decreased from 1.460±0.034 of control to 1.330±0.072, 0.440±0.032 and 0.516±0.013 with ADI, medium and high doses, respectively. After 30 days of treatment, PRKA3 gene expression was significantly decreased to 0.586±0.014 and 0.373±0.049 in medium and high doses, respectively as compared to the control. However, ADI dose induced significant elevation of gene expression into 1.503±0.008. PRKA3 was reported to be related to sperm activity and capacitation (22), consequently, the current decrease in expression results could indicate negative effects of food colorant on rat fertility.

Both carmoisine and curcumin treatments showed gradual decline in Spata-7 gene expression in a dose dependent manner at 30 and 45 days groups. The largest effects were obtained after carmoisine treatment with medium and high dose at the 30th and 45th day, the level of spata-7 gene was significantly decreased from1.713±0.112 of control to 0.723±0.02, 0.726±0.024, 0.740±0.011 and 0.753±0.006, respectively. Similarly, curcumin treatment for 30 and 45 days induced significant decrease of Spata-7 gene expression. This decrease was significant ($P < 0.05$) between groups of the same time of treatment. However, at 15 days of curcumin treatment, ADI dose caused significant elevation in level of Spata-7 gene expression (Figure 5). Several spatas, and RNA splicing variants, have been identified, most of which

act in diverse areas of testicular biology. For one example, SPATA7 is thought to act in testicular germ cell tumors in a human embryonic carcinoma cell line (23). The hazardous effect of carmoisine on rat fertility is supported with the current results and a previous study that used Spata7 as testis genotoxicity and fertility marker (24).

Stage-specific embryonic antigen (SSEA-1) gene was expressed in heterogeneous pattern along time interval of carmoisine treatment. Over expression was detected after 15 days of treatment with medium and high doses (0.756±0.123 and 0.756±0.066, respectively). Meanwhile, this effect was reversed after 30 days of treatment causing significant down regulation of gene level to 0.423±0.053 and 0.366±0.026 with medium and high doses, respectively from that of control 0.773±0.071. Also at 45 day group, the expression level decreased significantly from 0.961±0.033 of the control to 0.833±0.029 and 0.706±0.014 after ADI and high doses, respectively. All these increments were significant ($P < 0.05$) in comparing between groups of the same duration. After 15 days of curcumin treatment, SSEA1 gene expression was significantly elevated from 0.686±0.087 (control) to 1.383±0.091, 0.743±0.028 and 0.843±0.033 after ADI, medium and high doses, respectively. Similarly, at 30 and 45 days group, curcumin induced overexpression of SSEA1 gene level with the all tested doses except medium and high doses at 45 days showed significant decrease from to 0.693±0.029 and 0.763±0.014, respectively (Figure 5). SSEA1 expression was used as marker for both germ cells, interstitial cells and for prepubertal undifferentiated spermatogonia (25). Carmoisine treatment for 15 days induced significant increase in level of C-KIT (tyrosine kinase receptor) gene expression from control (0.610±0.036) to 1.586±0.043, and 0.753± 0.069 with medium and high doses, respectively. However, these effects were reversed by increasing the treatment duration. After 30 days of treatment, the level of C-KIT gene expression was decreased from control (1.580±0.075) gradually with increasing doses to 0.983±0.023, 0.836±0.041and 0.676±0.029, respectively. Also significant decreases were appeared after treatment for 45 days to 1.593±0.098, 0.560±0.011 and 0.583±0.012 after ADI, 5xADI and 10xADI doses, respectively. These decreases were found to be significant between groups (Figure5). Curcumin treatment induced gradual significant ($P < 0.05$) decrease in level of C-KIT gene expressions. Level of C-KIT was decreased to 0.273±0.029 and 0.433±0.044 at 15th day of curcumin treatment (medium and high doses, respectively). Meanwhile, significant decreased ($P < 0.05$) in C-KIT gene expression levels were found in 30 and 45 days groups with tested doses. The decrease was significant between groups with the same interval time (Figure 5). Fluctuations in expression of c-KIT reflects the action of food colorants on spermatogonial proliferation and maturation of round spermatids in mice (26) as it was previously used as a marker for pre-meiotic human spermatogenesis stages and Leydig cells (27). c-KIT was also supposed to possess a regulatory function in normal testicular tissue by possibly providing the microenvironment necessary for spermatoge-nesis (28).

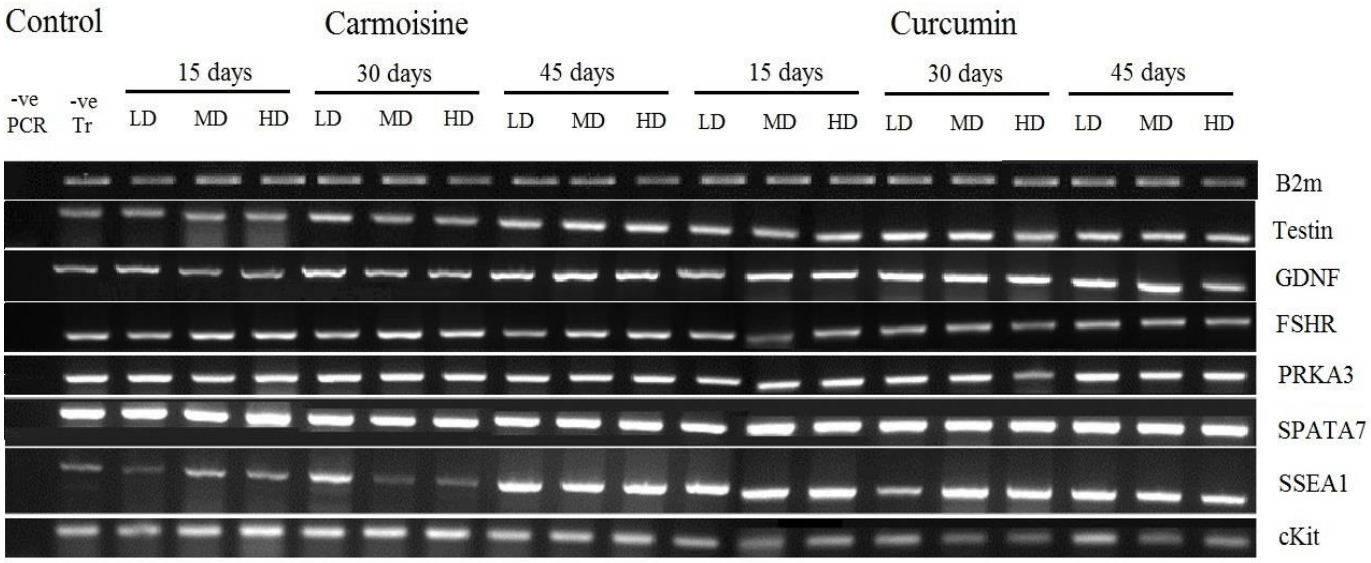

Figure 4: A panel represents mRNA expression patterns of control and rats given the food colorants carmoisine and curcumin. Glial cell derived neurotrophic factor (GDNF); tyrosine kinase receptor (c-KIT); follicle stimulating hormone receptor (FSHR); A kinase anchor protein 3 (PRKA3); spermatogenesis associated 7 (Spata7); Stage-specific embryonic antigen-1 (SSEA1). The house keeping gene b-2-microglobin (B2M) was used as internal control for gene expression. Negative controls for PCR reaction (-ve PCR) and for treatments (-ve Tr) were used

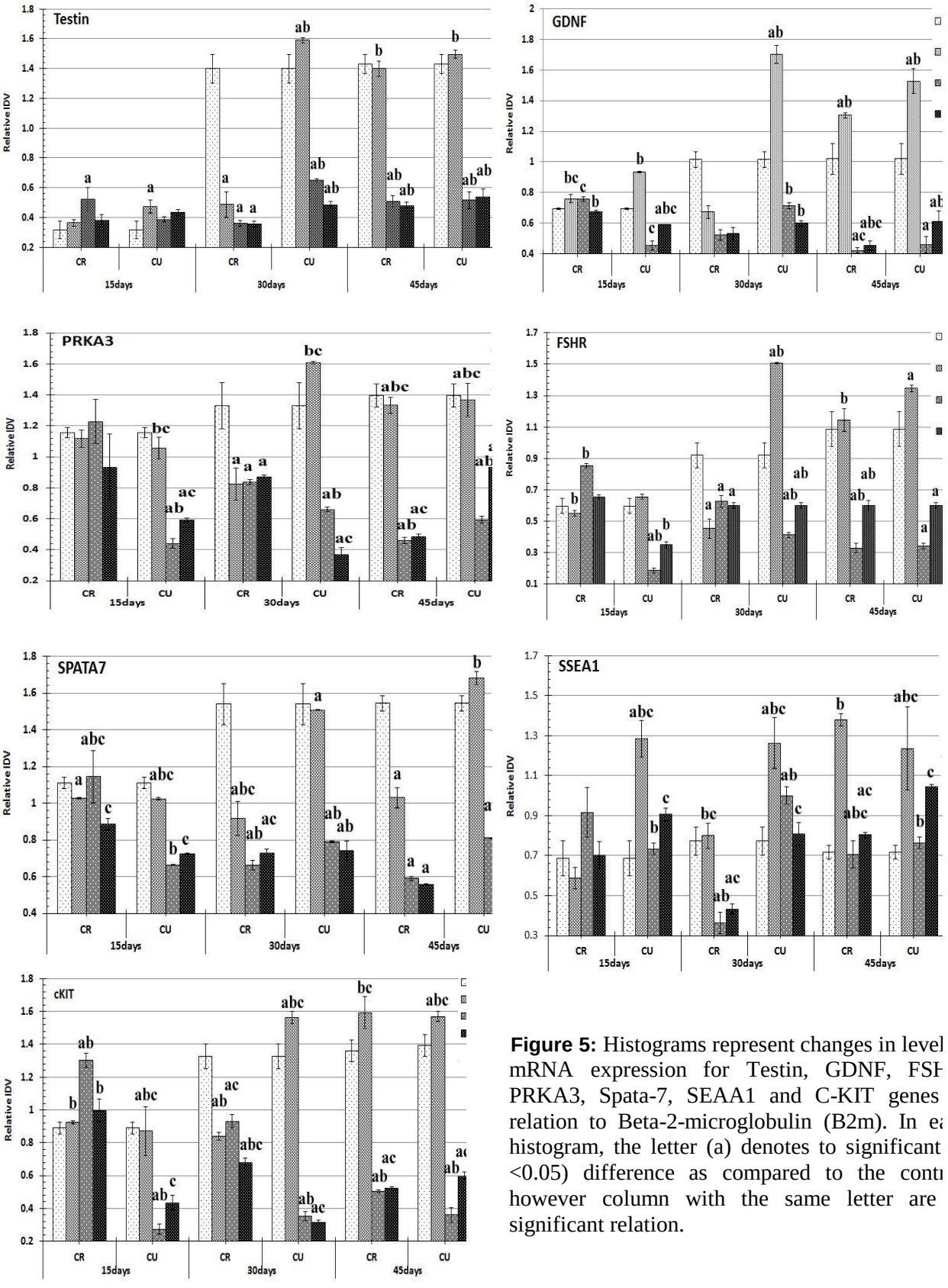

Figure 5: Histograms represent changes in level mRNA expression for Testin, GDNF, FSH PRKA3, Spata-7, SEAA1 and C-KIT genes relation to Beta-2-microglobulin (B2m). In ea histogram, the letter (a) denotes to significant <0.05) difference as compared to the conti however column with the same letter are significant relation.

Conclusion

From the current study, we can conclude that carmoisine has hazardous effects on fertility at different levels when consumed in concentrations higher than the acceptable daily-authorized level. However, curcumin as a natural food color is milder than carmoisine to certain levels.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Original Research Article

INFLUENCE OF STOCKING DENSITY ON GROWTH PERFORMANCE TRAITS, BLOOD CHEMISTRY AND THE EXPRESSION OF *HSP*70 AND *IGF*-I GENES IN NEW ZEALAND WHITE RABBITS

Khairy M. El-Bayoumi, Tamer M. Abdel-Hamid*, Mahmoud S. El-Tarabany, Wafaa R.I.A. Sherief

Animal Wealth Development Department, Faculty of Veterinary Medicine, Zagazig University, Sharkia, Egypt, 44511

*Corresponding author, E-mail: drtamoabha18111980@gmail.com

Abstract: This research was done to mainfest the effect of stocking density on growth performance traits, blood chemistry and the expression of heat shock protein (HSP) 70 and insulin-like growth factor I (*IGF*-I) genes in growing New Zealand White rabbits. A total of 75 rabbits at weaning (35 days of age) were randomly assigned into three stocking densities of 12, 20 and 28 rabbits/m^2 from weaning until 13 weeks of age. Rabbits housed at 28 rabbits/m^2 had the lowest feed intake, live body weight and body weight gain, but they had the highest feed to gain ratio when compared with the lower densities. Moreover, rabbits housed at 28 rabbits/m^2 showed the highest serum creatinine, glucose and cortisol levels compared with rabbits housed at 20 and 12 rabbits/m^2. High stocking density up-regulated the expression of *HSP*70 gene when compared with the lower densities. Meanwhile, *IGF*-I mRNA expression was significantly reduced in the rabbits housed at 28 rabbits/m^2. In conclusion, high stocking densities (28 rabbit/m^2) had negative impacts on growth performance traits and the stress-related parameters (serum glucose, cortisol and creatinine). Moreover, the expression of *HSP*70 gene was increased, with a remarkable reduction in *IGF*-I gene expression in the high stocking group.

Key words: rabbits; growth performance; *HSP*70; *IGF*-I; biochemical parameters

Introduction

Domestic rabbits (oryctolaguscuniculus) are considered a non-conventional livestock species that appear to be a cheap and a common source of high quality animal protein in developing countries. This may be attributed to the established prolificacy, rapid growth rate, high feed conversion ratio, early maturity, small housing area and high genetic selection potential of rabbits (1). The rabbit's meat is of high quality protein with lower calories, fat and cholesterol levels when compared with other sources of meat (2).

One of the most important ways to increase kit survivability and growth performance in the rabbit farms is to create a conductive environmental condition, among which is the appropriate stocking rate (3). The optimal cage space should allow each growing rabbit to stretch along one side of the cage and sit up straight at all age intervals (4). Stocking density in rabbits affects growth and litter size at weaning. Feed intake and daily gain of growing rabbits were higher in less crowded housing (5).

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The European Food and Safety Authority (6) recommended a minimum space of 625 cm^2/rabbit; not more than 40 kg/m^2 at the end of fattening in order to avoid disturbance of rabbit behavior. Growth performance of growing rabbits housed at density lower than 16 rabbits/m^2 was not affected (7,8).

Heat shock proteins (HSP) are considered as molecular chaperone proteins, which have the ability to interact with other proteins reversibly and to promote the folding, the formation and the transmembrane transport of these proteins (9). When an animal is exposed to harmful environmental condition (stress), the metabolism tends to synthesize more HSP, which help the cell to recognize the damaged protein for consequent repair. In addition, HSP has an essential role in the conservation of cellular life as they inhibit apoptosis (10).

Insulin-like growth factor-I is manufactured by almost all tissues and has a vital role in cell growth, differentiation and transformation (11). I*GF*-I gene expression in liver is affected by physiological (12) and nutritional status (13). To our knowledge, no previous scientific researchs that investigated the influence of stocking density on *HSP*70 and *IGF*-I genes` expressions in rabbits. Thus, the present work was aimed to manifest the effect of stocking density on growth performance, some biochemical parameters and expression of *HSP*70 and *IGF*-I genes of New Zealand White (NZW) rabbits.

Material and methods

The present study was carried out at a rabbit farm of Animal Wealth Development Department, Zagazig University, Sharkia-Egypt. This study was carried out during the period from the first of April to the end of May 2017.

Animals, housing and management

Seventy five male NZW rabbits weaned at 35 days of age with an average weight of 545±3.4 g were randomly assigned to three stocking densities (12, 20 and 28 rabbits/m^2). The allowed cage space was adjusted to provide the required stocking density with equal group size (five rabbits per cage) and each stocking density was replicated 5 times. Rabbits were ear-tagged, and housed in wire cages equipped with feeder and automatic nipple drinker. About 14 hours photoperiod was maintained, the housing temperature was 26±2°C and the relative humidity was 68±4 % throughout the experimental period. All rabbits were fed ad libitum a commercial pelleted diet, which consisted of 18% crude protein, 2.5% crude fat, 2651 kcal/kg digestible energy and 12.65% crude fiber from weaning until 13 weeks of age. The experimental rabbits were exposed to the same environmental condition and managed according to the rabbit's management standards.

Growth performance

Biweekly, the live body weight (LBW) was recorded individually for each rabbit at 5, 7, 9, 11 and 13 weeks of age, as well as the feed consumption. The feed conversion ratio and the body weight gain (BWG) were calculated at different week intervals from 5 to 13 weeks of age. The experimental unit was individual cage to calculate the average feed consumption and feed conversion ratio.

Blood sampling

At the end of the experimental period (13 weeks of age), five rabbits from each group were randomly taken (one rabbit from each replicate) and blood samples were collected in plain tubes to obtain serum. Separation of serum was done by centrifugation at 3000 rpm for 15 min, then kept at -20 °C. The total serum protein, albumin, triglycerides, cholesterol, glucose and creatinine were estimated using a commercial diagnostic kit (Elitech Clinical System SAS- Zone Industrielle- 61500 SEES, France); globulin level was estimated by subtracting the obtained albumin level from the obtained total protein level (14). The concentration of serum cortisol was detected (Cortisol ELISA Kit, Cayman Chemical).

Gene expression

RNeasy Mini kit (Qiagen, Germany) was used for RNA extraction following the manufacturer's protocol, 25 mg of thigh muscle were taken from the slaughtered rabbits and homogenized. Quantitect Reverse Transcript-

ion kit (Qiagen, Heidel-berg, Germany) was used for reverse transcription of the first-strand cDNA from total RNA following the manufacturer's protocol. The Real-Time PCR was performed on a Rotor-Gene Q cycler (Qiagen, Germany) using Quantitect SYBR Green PCR kits (Qiagen, Heidel-berg, Germany). The forward and reverse primers (15,16) for each gene are summarized in Table (1). The Q-PCR mixture contained of 12.5 ml 2x SYBR Green PCR Master Mix, 1µl of each primer (10 Pmol/ml), 2 µl cDNA and 8.5 µl RNase-free water in a total volume of 25 µl. The cycle counts and amplification conditions were as follows: initial activation at 95 C˚ for 15 min and then 40 cycles of denaturation at 94 C˚ for 15 sec, annealing at 60 C˚ for 15 sec and elongation at 72 C˚ for 15 sec.

Table 1: Oligonucleotide primer sequences used for semi-quantitative reverse transcription polymerase chain reaction

Gene	Primer	Sequence (5′-3′)	Reference
***IGF*-I**	forward	AGGAGGCTGGAGATGTACTG	(15)
	Reverse	AAATGTACTTCCTTCTGAGTCT	
***HSP*70**	forward	GAGGTCACCTTCGACATCGA	(16)
	Reverse	CTTGCCCGTGCTCTTGTC	
GAPDH	forward	ATTGCCCTCAATGACCACTTTG	(15)
	Reverse	TCTTACTCCTTGGAGGCCATGT	

IGF-I = Insulin-like growth factor I; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase; HSP = Heat shock protein

Fold changes in the target genes` expressions were calculated by comparing $2^{-\Delta\Delta CT}$ (CT: cycle threshold) method (17) with the GAPDH housekeeping gene. The difference between mean ΔCT of the treatment group and the control group is ΔΔCT, where the difference between the mean ct gene of the interest and the internal control gene in each sample is ΔCT then statistical analysis was applied.

Statistical analysis

One-way analysis of variance (ANOVA) procedure of the Statistical Package for Social Sciences version 21.0 (SPSS for Windows 21.0, Inc, Chicago, IL, USA) was used for analysis of the obtained data. The multiple comparisons of means were done by using the Duncan's Multiple Range Tests (DMRT). Results were documented as means ± standard errors (SE), the value of $P < 0.05$ was used to indicate statistical significance. The statistical model that used as the following:

$$Yij = \mu + Si + eij$$

Where: Yij, The animal observation; µ, Overall population mean; S_i, Fixed effect of i^{th} stocking density and e_{ij}, Residual error.

Results

Growth performance traits

Rabbits housed at 28 rabbits/m^2 had the lowest LBW at 9^{th}, 11^{th} and 13^{th} week of age and the lowest BWG at 7-9, 11-13 and 5-13 week intervals compared with those housed at either 12 or 20 rabbits/m^2 (Table 2). The feed consumption of rabbits housed at 28 rabbits/m^2 was significantly lower at all age intervals when compared with the rabbits housed at 12 rabbits/m^2 (Table 2). In general, rabbits housed at 28 rabbits/m^2 had the highest feed conversion ratios at all age intervals studied compared with rabbits housed at 12 rabbits/m^2 and 20 rabbits/m^2 except for 5-7 weeks interval of age, but only significant results was obtained at 7-9 and 11-13 weeks interval of age.

Table 2: Growth performance traits of New Zealand White rabbits as affected by cage stocking density

Variable	Stocking density (rabbits/m^2)			*P*-value
	12	20	28	
Live body weight (g)				
5th week	714.00±23.75	651.00±22.54	656.37±12.33	0.082
7th week	1062.40±29.54	1036.20±29.83	1009.57±24.39	0.455
9th week	1475.07±29.41^a	1463.40±30.70^a	1365.37±29.17^b	0.022
11th week	1832.47±29.01^a	1753.40±31.20^{ab}	1658.46±31.93^b	0.001
13th week	2210.87±32.51^a	2014.68±36.29^b	1866.46±30.83^c	0.003
Body weight gain (g/d)				
5-7 weeks	24.24±0.86	27.51±2.02	25.21±1.33	0.399
7-9 weeks	28.81±1.38^a	30.51±1.09^a	25.43±0.93^b	0.002
9-11 weeks	24.84±1.71	20.71±0.95	20.93±1.54	0.182
11-13 weeks	27.03 ±1.24^a	18.52±1.08^b	14.86±0.72^c	0.001
5-13 weeks	26.73±0.69^a	25.14±0.70^a	21.61±0.44^b	0.001
Feed consumption (g/d)				
5-7 weeks	64.73 ±0.65^a	63.84 ±0.63^{ab}	62.43 ±0.31^b	0.036
7-9 weeks	86.26 ±0.915^a	73.72 ±0.91^b	72.79 ±0.51^b	0.001
9-11 weeks	90.09 ±1.23^a	75.15 ±0.98^b	73.83 ±1.06^b	0.001
11-13 weeks	89.33 ±0.91^a	89.43 ±0.59^a	81.67 ±0.37^b	0.001
5-13 weeks	82.60 ±0.44^a	75.54 ±0.54^b	72.68 ±0.38^c	0.001
Feed conversion ratio				
5-7 weeks	2.69±0.12	2.70±0.24	2.81±0.21	0.904
7-9 weeks	3.03±0.17^a	2.49±0.09^b	3.00±0.12^a	0.004
9-11 weeks	3.74±0.24	3.90±0.26	3.99±0.20	0.787
11-13 weeks	3.40±0.15^b	5.27±0.34^a	5.67±0.16^a	0.001
5-13 weeks	3.12±0.08	3.18±0.11	3.44±0.09	0.053

Means within the same row having different superscript letters are significantly different at (P<0.05)

Blood biochemical parameters

Rabbits housed at 12 rabbits/m^2 recorded the highest significant total serum protein, globulin and cholesterol concentrations compared with those housed at 20 and 28 rabbits/m^2 (Table 3). However, rabbits housed at 28 rabbits/m^2 recorded the highest serum creatinine, glucose and cortisol levels compared with rabbits housed at 20 and 12 rabbits/m^2. The serum albumin level was increased in rabbits housed at 28 rabbits/m^2 compared with their counterparts housed at 20 rabbits/m^2. The effect of stocking density on serum triglyceride level was non-significant.

Gene expression

The expression of *HSP*70 mRNA was significantly higher in rabbits housed at 28 rabbits/m^2 compared with those kept at 20 and 12 rabbits/m^2 (Figure 1). The *IGF*-I gene showed a reduction of the transcript level for rabbits housed at 28 rabbits/m^2 than those kept at lower densities (Figure 2).

Table 3: Effect of stocking density on blood biochemical parameters of New Zealand white rabbits at 13 weeks of age

	Stocking density (rabbits/m^2)			
Variable	**12**	**20**	**28**	***P*-value**
Total protein (g/dl)	5.94±0.03^a	4.82±0.01^c	5.60±0.05^b	0.001
Albumin (g/dl)	3.70±0.05^a	3.07±0.07^b	3.79±0.06^a	0.001
Globulin (g/dl)	2.24±0.04^a	1.74±0.07^b	1.81±0.08^b	0.003
Creatinine (mg/dl)	0.41±0.01^b	0.40±0.012^b	0.45±0.01^a	0.013
Cholesterol (mg/dl)	126.67±0.67^a	100.33±0.39^b	76.71±0.89^c	0.001
Triglycerides (mg/dl)	147.02±25.99	149.22±1.35	173.63±0.37	0.433
Glucose (mg/dl)	76.63±0.28^c	79.45±0.34^b	81.83 ±0.81^a	0.001
Cortisol (µg/dl)	5.59±0.54^b	6.01±0.13^b	8.35±0.24^a	0.001

Means within the same row having different superscript letters are significantly different at (P <0.05)

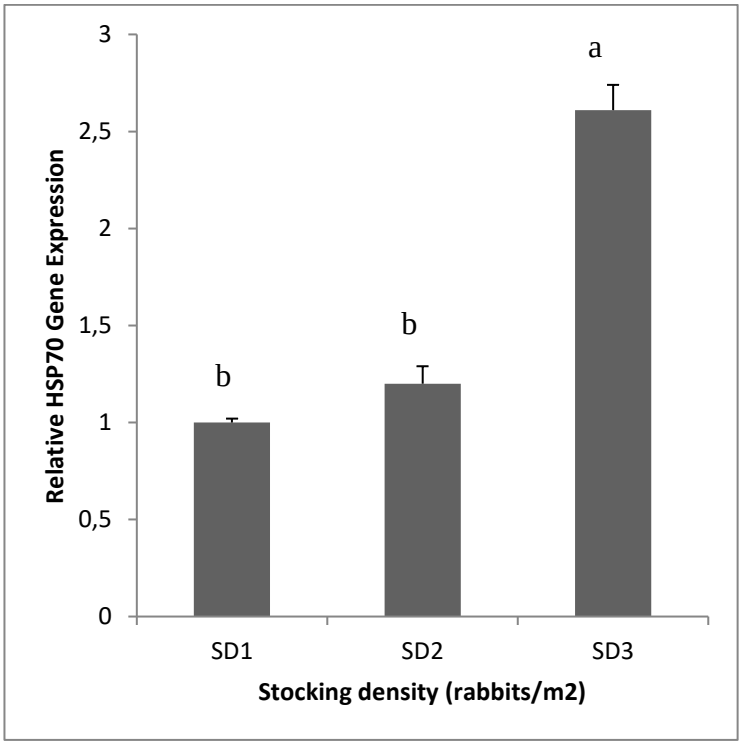

Figure 1: Effect of stocking density on heat shock protein (HSP) 70 mRNA expression in New Zealand White rabbits. SD1, 12 rabbits/m^2; SD2, 20 rabbits/m^2; SD3, 28 rabbits/m^2.Values were means±SEM. The bars represented the treatment with different superscripts (a, b) were significantly different at p <0.05

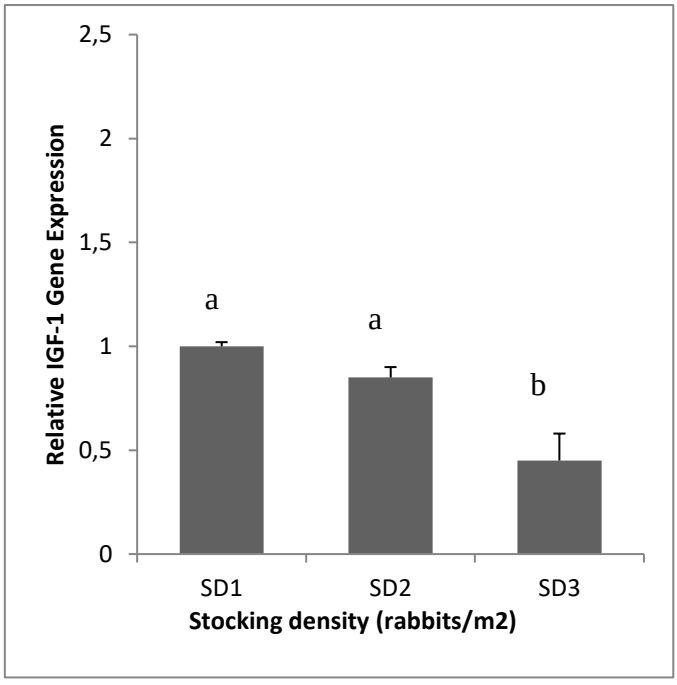

Figure 2: Effect of stocking density on IGF-I mRNA expression in New Zealand White rabbits. SD1, 12 rabbits/m^2; SD2, 20 rabbits/m^2; SD3, 28 rabbits/m^2. Values were means ± SEM. The bars represented the treatment with different superscripts (a, b) were significantly different at p <0.05

Discussion

The present results revealed that higher stocking density depicted a negative effect on growth performance traits of growing rabbits which were harmonious with those reported in previous studies (7,18-21). In another study by Mbanya et al. (22), lower results of final body weights and weight gains have been recorded in rabbits housed at 10 rabbits/m^2 than those housed at 5 rabbits/m^2. Among four stocking densities (6, 12, 18 and 28 rabbits/m^2), the last one had the greatest impairment effect on daily gain and feed intake (FI) than the others (23). But controversy results were repoted in various studies (8,24,25) in which the stocking density had no effect on growth performance of rabbits. Increasing the number of rabbits from 1 to 3 or 5 rabbits/cage (70x40x60 cm) led to a reduction in daily weight gain (4), also, groups having 5 rabbits/cage showed higher feed conversion ratio . The higher feed intake for pellet feed recorded in rabbits housed at lower density (4 rabbits/m^2) could be attributable to greater physical activity which increase the energy requirement (26). The lighter body weights that were found in the rabbits housed at higher density might be attributable to the lower feed intake, the lower feed utilization and the stresses that the rabbit exposed which are reflected in the higher glucose and cortisol levels in the serum of these rabbits.

In partial agreement with the present results, Ebru Onbasilar and Ílyas Onbasilar (4) detected that serum cholesterol and triglyceride levels in growing rabbits were not influenced by cage density. Contradicted with the present results, Kalaba (21) found that the levels of albumin in blood plasma of rabbits were not changed by stocking density (4, 8, 12 and 16 rabbits/m^2); but in parallel with the current study, he observed that plasma total protein, globulin and cholesterol levels were significantly reduced in rabbits kept at the highest density (16 rabbits/m^2) than those housed at a lower cage density, whereas plasma glucose and cortisol levels were apparently higher in rabbits housed at the same cage density. The total protein, globulin and creatinine concentrations were higher (p≤0.05) in rabbit housed at 1 and 2 rabbits/cage (50x60x30 cm) than those housed in 3 and 4 rabbits/cage, while cholesterol was decreased by increasing the cage density (3).

Controversy results were reported by Abd El-Monem et al. (27) who noted that there was no difference among rabbits housed at four stocking densities (2, 3, 4 and 5 rabbits/cage) in plasma total protein, albumin, globulin and creatinine levels. The cage density of 8.6, 12 and 17.2 rabbits/m^2 had a non-significant effect on blood parameters (total protein, albumin, cholesterol and glucose) in 13-week-old rabbits (28). On the contrary, there were no significance differences between single and group housed female rabbits in corticosterone level (29).

The changes in *HSP* gene expression levels are considered an essential biomarker for detection of stress and ideal environmental condition (30). HSPs are essential for permitting cells to cope with acute stressor, especially those affecting protein synthesis (31). Moreover, previous studies have reported that synthesis of HSPs (Hsp27, Hsp70, and Hsp90) has increased under heat stress and these proteins play an important defensive role against stress-induced tissue harmful metabolites so maintaining integrity of organs in broilers (32,33). The *HSP*70 mRNA expression was significantly increased in rabbits kept at 20 and 28 rabbits/m^2 compared with those kept at 12 rabbits/m^2, which could be explained by the higher temperature at which the rabbits were kept in conjunction with high stocking density which increase this heat stress and these results were in agreement with Yu and Bao (34) and Gu and coauthors (35) who found that exposure of broilers to high temperature led to an increase in expression of *HSP* compared with those kept under normal temperature. *Hsp*27, *Hsp*70, and *Hsp*90 mRNA expressions in bursa and spleen of black-boned chickens kept at high temperature were significantly higher (P <0.01) than those kept under normal temperature (36). Similarly, Ramsay et al. (37) and Dhabhar (38) reported an increase in the expression of *HSP*70 after exposure to crowding in European Sea bass, Dicentrarchus Labrax and Atlantic cod and Gadusmorhua. expression of *HSP* gene in rainbow trout was significantly affected by stocking density (39, 40). *HSP*70 gene expression was increased by increasing stocking density from 0.0578 to 0.077 and 0.116 m^2/bird and from 0.100 to 0.063 m^2/bird (41,42). On the contrast, expression of *HSP*70 gene in the liver and head kidney of Senegalese sole was declined by increasing stocking density from 7 to 30 kg/m^2 (43).

Previous authors reported that *IGF*-I has an essential role in the growth of broiler as broilers exhibited slow growth had lower levels of *IGF*-I (44). High blood concentrations of *IGF*-I were associated with higher protein synthesis levels and lower protein breakdown levels and, thus larger skeletal muscle mass (45). In addition to this, higher expression of *IGF*-I gene in the liver and higher level of *IGF*-I in blood have been detected in broilers choosen for higher growth rate compared with those selected for slower growth rate (46).

The present results revealed a reduction in growth performance of rabbits that were kept at high stocking density and relatively high temperature (28-33°C) which could stimulate a physiological process related to exposure to stress that resulted in reduction of IGF-I mRNA expression and serum IGF-I concentration. IGF-I showed reduced transcript level for rabbits housed at higher stocking density and these present results were in agreement with Brockmark et al. (47) who detected that Atlantic salmon reared at low density had higher levels of IGF-I than those kept at higher density. Similarly, Salas-Leiton et al. (43) found that total IGF-I mRNA levels reduced significantly at higher density (30 kg/m^2) in liver but not in the kidney in Senegalese sole. Black-boned chickens reared at high temperature (37±2°C) had low serum concentration of IGF-I (P < 0.01) than those reared at normal temperature (24±2°C) (36).

Conclusion

In conclusion, high stocking densities (28 rabbit/m^2) had a negative impact on growth performance traits and the stress-related parameters (serum glucose, cortisol and creatinine). Moreover, the expression of *HSP*70 gene has increased, while the expression of *IGF*-I gene has decreased in rabbit housed at high stocking density.

Conflict of interest

The authors declare no conflict of interest

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Original Research Article

HUMAN HEALTH RISK ASSESSMENT OF HEAVY METALS AND TRACE ELEMENTS RESIDUES IN POULTRY MEAT RETAILED IN SHARKIA GOVERNORATE, EGYPT

Rasha M. El Bayomi, Wageh S. Darwish*, Shimaa S.M. Elshahat, Abdelsalm E. Hafez

Department of Food Control, Faculty of Veterinary Medicine, Zagazig University, El-Zeraa str. 114, 44519-Zagazig, Egypt

*Corresponding author, E-mail: wagehdarwish@gmail.com; wagehdarwish@yahoo.ca

Abstract: The objective of this study was to estimate the residual concentrations of copper (Cu), zinc (Zn), cadmium (Cd), lead (Pb) and mercury (Hg) in muscles and livers of chicken (broilers and layers), turkeys and quails marketed in Egypt. Metal-metal correlations were further analyzed. Moreover, metal's dietary intake and health risk assessment among Egyptian consumers were calculated. Livers had higher metal residues compared with the muscles in all species examined. The ranges (µg/g ww) of the elemental concentrations in the livers of the examined bird species were 0.03-0.46 (Cu), 1.77-10.33 (Zn), 0.05-1.09 (Pb), 0.02-0.15 (Cd) and 0.13-0.99 (Hg). Such ranges (µg/g ww) in the muscle samples were 0.007-0.60 (Cu), 0.69-4.64 (Zn), 0.01-0.55 (Pb), 0.02-0.13 (Cd) and 0.11-0.94 (Hg). Correlation analysis among metals revealed both tissue-dependent and inter-species differences for the accumulation patterns of metals. The potential risk assessment of all investigated metals in poultry meats revealed no significance risk on Egyptians. However, intake of repetitive small concentrations of metals may lead to severe toxicological implications.

Key words: poultry; heavy metals; risk assessment

Introduction

Poultry meat is an excellent source of high quality animal protein that is required for feeding convalescents, adults, young children and infants (1). Increasing the popularity of poultry meat is mainly due to its ideal chemical composition including essential polyunsaturated fatty acids, essential amino acids, as well as easy digestibility, availability, acceptance and low price compared with other meat (2).

Contamination of meat with heavy metals is a serious hazard because of their toxicity, bioaccumulation and biomagnification in the food (3). The main sources of poultry exposure to heavy metals are feed, drinking water and litter contamination (4,5). These metals find their way to the human food mainly via the consumption of contaminated meat (6).

Toxic metals with severe health hazards include lead (Pb), cadmium (Cd) and mercury (Hg) (7). Human exposure to heavy metals may lead to several acute or chronic adverse effects (8). The main toxic effects of lead include hypertension, reproductive dysfunction, gastrointestinal track damage, nephropathy, and damage of central and peripheral nervous systems (9). The toxicity of cadmium is linked to its interactions with zinc and other essential elements leading to sever renal dysfunction and

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respiratory symptoms (10). Mercury is a neurotoxin that causes neuro-behavioral alterations, renal damage and gastrointestinal toxicity (11). Zinc (Zn) and copper (Cu) are essential trace elements that play several physiological and biological roles in maintenance of animal performance and health, however, high intake of these elements can cause health problems (12).

As humans are on the top of the ecosystem, it is essential to evaluate their dietary exposure to such toxicants (13). Moreover, the human health risk assessment due to consumption of poultry meat contaminated with heavy metals is a major task of food hygiene. Therefore, this study aimed to estimate the concentrations of metals (Cu, Zn, Cd, Pb and Hg) in fresh poultry meat samples (muscle and liver) collected from Sharkia Governorate, Egypt and to evaluate the risks associated with consumption of such meat. Furthermore, metal-metal correlations were analyzed to investigate their inter-species differences in metal accumulation pattern.

Material and methods

Chemicals and reagents

The standards of the tested metals and other reagents including nitric and perchloric acids were of analytical grade and purchased from Merck, Darmstadt, Germany.

Collection and preparation of samples

Eighty samples from breast muscles and liver of layers, broilers, turkeys and quails (10 samples, each) retailed for human consumption were randomly collected from Zagazig city, Sharkia Governorate, Egypt. After being sacrificed in poultry meat shops, 10 grams from each sample were identified and wrapped separately in polyethylene bags, then stored at –20°C until metal extraction and measurements. The analysis of heavy metals (Cu, Zn, Cd, Pb and Hg) was estimated by Atomic Absorption Spectrometry (AAS) (Per Kin Elmer model (spectra-AA10, USA) in the Central Laboratory, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Analytical procedures

One gram of each tissue sample was digested overnight in 5 mL volume of a mixture 3 volumes of 65% nitric acid and 2 volume of 70% perchloric acid (14, 15). The tubes were moist heated for 3 h at 70°C followed by cooling and filtration. The filtrates were kept at 4°C until measurement of the metals. Levels of Hg were measured using hydride generation/cold vapor atomic absorption spectroscopy, while in case of Cu, Zn, Pb and Cd, a graphite furnace was used (Perkin Elmer® PinAAcle™ 900T atomic absorption spectrophotometer (Shelton, CT, USA).

Quality assurance and quality control

The standard reference (DORM–3, fish protein) (National Research Council, Canada) was used for validation of the analytical method. The recovery rates (%) for Cu, Cd, Pb, Zn and Hg were 98%, 98%, 97%, 96% and 95, respectively. The instrumental limits of detection (LOD) were 0.02, 0.005, 0.01, 0.02 and 0.02 μg/g for Cu, Cd, Pb, Zn and Hg, respectively. The concentrations of Cu, Cd, Pb, Zn and Hg were expressed as μg/g wet weight (ww). Residual metal concentrations in the examined samples were compared with the maximum permissible limits of metals set by FAO/WHO (16).

Metal's dietary intakes and health risk assessment

The potential health risks of Egyptian adults due to consumption of metal-contaminated poultry meat were assessed based on calculation of the estimated daily intake (EDI), hazard quotient (HQ), and hazard index (HI) of the metals examined. EDI was calculated using the equation described by the United States Environment Protection Agency (USEPA) (17) as follows

$$EDI = (C \times FIR)/BW$$

Where, BW is the body weight of the Egyptians adults, which was set as 70 kg. C is the concentration of the metal in the tested samples (μg/g ww). FIR is the rate of the ingestion of poultry meat (muscle) in Egypt,

which was estimated to be 39.53 g/day (18) and the ingestion rate of liver tissue was set as 0.1 g/day (19). EDI values were expressed as (µg/kg BW/day) for the heavy metals. EDI values were then compared with metal's tolerable daily intakes (TDIs) (FAO/WHO) (20).

The assessment of heavy metal-related non-cancer risks followed the guide lines recommended by USEPA (21) using the following equation:

$$THQ = \frac{ED \times C \times FIR \times EF}{RFD \times BW \times AT} \times 10^{-3}$$

Where THQ is the target hazard quotient; C is the heavy metal concentration in poultry meat (µg/g); FIR is the food ingestion rate (g/day); ED is the exposure duration (70 years, average lifetime); EF is exposure frequency (365 days/year); RFD is the oral reference doses for Cu, Cd, Pb, Zn and Hg (0.04, 0.001, 0.004, 0.3 and 0.0016 mg/kg BW/day) (22). AT is the average exposure time (365 days/ year × exposure years, assumed as 70 years); BW is the average adult body weight (70 kg).

Hazard index (HI) was generated to evaluate the risk of the combined metals using the following equation: HI = ΣHQi, Where i represent each metal. When the value of HQ and/or HI exceeds 1.0, there is a concern for potential health effect (23).

Statistical analysis

Heavy metals concentrations were recorded as means ± standard errors (SE). Results were analyzed using one-way analysis of variance (ANOVA) (SPSS for Windows 21.0). Duncan's test was used for statistical analysis. Comparing metals in muscles and liver of the same bird was done by T-test. The value of $P<0.05$ was used to indicate statistical significant differences. Correlation analyses among the measured metals were calculated based on Spearman's coefficient using JMP program (SAS Institute, Cary, NC, USA).

Results and discussion

Levels of the tested heavy metals

Residual concentrations of copper (µg/g ww) were recorded in Table (1). Samples collected from turkeys had the highest Cu residues (0.13 ± 0.12) compared with other bird species, particularly in the muscle. The recorded concentrations in the present study were far below the values recorded in muscles of broilers and quails in Egypt (6, 24). However, copper residues were below the detection limit on livers of broilers in Philippines (25). All examined samples in the present study had copper residues within the permissible limits set by FAO/WHO and Egyptian authorities (16,26)

Table 1: Heavy metal residual concentrations in the examined meat samples from different poultry species (n = 10 each)

Item		Cu		Zn		Pb		Cd		Hg	
		Mean ± SE (Range)	%	Mean ± SE (Range)	%	Mean ± SE (Range)	%	Mean ± SE (Range)	%	Mean ± SE (Range)	%
Layers	Muscle	0.08 ± 0.02^{ab} (0.06-0.14)	0	2.59 ± 0.10^{b} (2.26-2.86)	0	0.10 ± 0.05^{a} (0.01-0.24)	40	0.06 ± 0.01^{ab} (0.02-0.10)	80	0.45 ± 0.14^{a} (0.11-0.83)	80
	Liver	$0.18 \pm 0.03^{a*}$ (0.11-0.25)	0	$5.53 \pm 0.45^{a*}$ (4.81-7.32)	0	0.21 ± 0.05^{a} (0.05-0.34)	0	0.06 ± 0.01^{a} (0.02-0.09)	0	0.41 ± 0.07^{b} (0.20-0.60)	20
Broilers	Muscle	0.04 ± 0.02^{b} (0.007-0.11)	0	2.46 ± 0.30^{bc} (1.26-2.88)	0	0.31 ± 0.04^{a} (0.17-0.4)	100	0.09 ± 0.01^{a} (0.05-0.13)	80	0.62 ± 0.11^{a} (0.23-0.94)	100
	Liver	$0.21 \pm 0.03^{a*}$ (0.10-0.29)	0	$4.76 \pm 0.44^{a*}$ (3.69-5.86)	0	0.34 ± 0.06^{a} (0.19-0.48)	0	0.10 ± 0.01^{a} (0.04-0.15)	0	0.65 ± 0.12^{a} (0.23-0.99)	80
Turkeys	Muscle	0.13 ± 0.12^{a} (0.04-0.60)	0	3.70 ± 0.33^{a} (2.94-4.64)	0	0.18 ± 0.10^{a} (0.01-0.55)	80	0.07 ± 0.01^{ab} (0.03-0.1)	60	0.44 ± 0.11^{a} (0.17-0.79)	80
	Liver	0.26 ± 0.08^{a} (0.19-0.46)	0	5.56 ± 1.21^{a} (3.51-10.33)	0	$0.45 \pm 0.19^{a*}$ (0.16-1.09)	20	0.10 ± 0.01^{a} (0.06-0.14)	0	0.47 ± 0.13^{ab} (0.13-0.88)	40
Quails	Muscle	0.10 ± 0.05^{ab} (0.02-0.28)	0	1.13 ± 0.35^{c} (0.69-2.54)	0	0.18 ± 0.08^{a} (0.01-0.46)	40	0.04 ± 0.01^{b} (0.03-0.08)	60	0.52 ± 0.05^{a} (0.43-0.71)	80
	Liver	0.15 ± 0.05^{a} (0.03-0.27)	0	2.40 ± 0.38^{b} (1.77-3.82)	0	0.34 ± 0.05^{a} (0.19-0.45)	0	0.08 ± 0.02^{a} (0.04-0.12)	0	0.70 ± 0.15^{a} (0.14-0.97)	40

Means within the same sample (muscle or liver) carrying different subscripted letter are significantly different (p <0.05) based on Duncan's multiple comparisons. *indicates significant difference among muscle and liver of the same bird species. % indicates percentage of samples exceeding maximum permissible limits (MPL) for the tested heavy metals set by Egypt organization for Standardization (EOS 2010)(26) and FAO/WHO (2000) (16). MPL for Cu, Cd, Pb, Zn and Hg (µg/kg ww) in muscles were set to be 15.00; 0.05, 0.1, 50, 0.2, respectively; in liver, they were set to be 15.00, 1.00, 0.5, 50, 0.5, respectively.

In the present study, residual concentrations of zinc were the highest among the examined elements as shown in Table (1). This may be attributed to the addition of zinc to the poultry feed formula for improvement of the physiological body functions (27). Similar to copper, turkeys had the highest residual concentrations of zinc in the muscles among the examined avian species (3.70±0.33 µg/g ww). The detected levels of zinc in the current investigation were comparable to 1.29 and 1.28 (µg/g ww) in the muscles of quails reared in either battery or deep litter systems, respectively in Egypt (4) and lower than those recorded in broilers examined in; Egypt (24), India (28, 29) and Zambia (30) and in free-range chicken from Ghana (31) and Nigeria (32). Zinc levels were within the accepted permissible limits of zinc in meat (16).

Residual concentrations of cadmium were recorded in Table (1). The ranges (µg/g ww) of Cd residual concentrations in the livers and muscles of the examined bird species were 0.02-0.15 and 0.02-0.13, respectively. Levels of Cd in livers and muscles of the examined species were comparable to that recorded in broilers (33) and quails (6) examined in Egypt and and in broilers from India (29). However, higher Cd concentrations were recorded in the livers of free-range chicken collected from Poland (34), Zambia (30), Ghana (31), Egypt (35) and Nigeria (32) and in turkeys collected from Spain (36). Levels of cadmium recorded in muscles were 80%, 80%, 60% and 60% in broilers, layers, turkeys and quails, respectively (Table 1) that exceeded maximum permissible limits of cadmium (16,26) This is may be attributed to the high levels of cadmium in poultry ration, drinking water and/or environment (6,37).

Lead is an environmental toxin that is linked to several health adverse effects (15). The obtained concentrations of lead in the present study were shown in Table (1). The results indicated that turkeys had the highest lead residues among the examined avian species. The average Pb residual concentrations (µg/g ww) in the muscles and livers of turkeys were

0.18±0.10 and 0.45±0.19, respectively. The recorded concentrations in the present study were in agreement with the recorded concentrations in poultry meat samples collected from Poland (34), India (29), Egypt (38). However, lower concentrations of Pb were reported in turkeys from Spain (36), quails from Egypt (6) and broilers from Philippines and Zambia (25,30). Lead had exceeded the established MPL (16,26) in all examined bird species with different percentages as indicated in Table (1). This might be due to the contamination of birds' feed and water with lead that is commonly used in the manufacturing of water pipes (39) and might be contained in bone and fish meals (40).

Mercury occurs naturally in the environment at low levels and characterized by its high bioaccumulation and biomagnification nature (41), it can find its way to food chain via industrial wastes (42), water, feed, dust and soil (31,43). The mean concentrations of Hg in the current study were declared in Table (1). The recorded mercury concentrations in the livers of broilers (0.65±0.12 µg/g ww) were lower than that recorded in broilers in India (28) and in free-range chicken from Ghana (31) and higher than those recorded in poultry meat in Philippines, Zambia and Nigeria (25, 30, 32). In the present study, 80%, 100%, 80% and 80% of the examined samples of layers, broilers, turkeys and quails, respectively were higher than the recommended maximum permissible limits of Hg (16,26).

In general, liver had higher accumulation pattern for metals compared with muscle in the examined avian species, this is may be attributed to the fact that liver is the organ of metabolism and detoxification of xenobiotics in humans and animals (6,15)

Correlation analysis among metals in poultry meat

In order to investigate the inter-metal`s tendency of accumulation, Spearman`s coefficient factors were calculated in both liver and muscle tissues of the different birds as indicated in Tables (2 and 3). Interestingly, the achieved results revealed both interspecies and tissue-dependent variations in the tendency of accumulation of the tested metals. For instances, significant negative correlations were observed between the essential trace elements as copper and zinc from one side and the toxic ones from the other side as clear in case of Cd-Cu ($r = -0.21$, $p = 0.74$ in layers and $r = -0.67$, $p = 0.22$ in layers); Cd-Zn ($r = -0.87$, $p = 0.05$) and Hg-Zn ($r = -0.60$, $p = 0.28$). This may be attributed to the physiological trials of the body for detoxification of such toxicants. Essential elements such as copper and zinc were reported to induce metal binding proteins and antioxidant enzymes (6). These findings agreed with the inter-metal correlations detected in the livers of cattle and sheep (15), chicken (31) and quails (6).

Table 2: Correlation analysis among the examined metals in the muscle tissues of different poultry species

		Layers		**Broilers**		**Turkey**		**Quails**	
		R	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Cd	**Cu**	-0.15	0.81	**0.81**	**0.01**	-0.80	0.11	0.11	0.87
Pb	**Cu**	**0.9**	**0.04**	0.45	0.45	0.45	0.45	-0.11	0.87
Pb	**Cd**	0.15	0.81	0.05	0.93	-0.05	0.93	**-1.00**	**<.0001**
Zn	**Cu**	-0.51	0.39	-0.45	0.45	0.11	0.86	**0.71**	**0.01**
Zn	**Cd**	-0.56	0.32	-0.62	0.27	0.11	0.87	-0.11	0.87
Zn	**Pb**	**-0.77**	**0.05**	-0.11	0.87	-0.31	0.62	0.11	0.87
Hg	**Cu**	0.31	0.62	**-0.89**	**0.04**	**-0.67**	**0.02**	-0.20	0.75
Hg	**Cd**	0.41	0.49	-0.62	0.27	0.15	0.81	-0.40	0.51
Hg	**Pb**	0.44	0.51	**-0.7**	**0.05**	**-0.71**	**0.05**	0.41	0.51
Hg	**Zn**	-0.90	0.04	**0.61**	**0.04**	0	1.00	0.50	0.39

r: spearman`s correlation factor; *p*: probability and Values in bold show significant correlation between each two metals.

Table 3: Correlation analysis among the examined metals in the livers of different poultry species

		Layers		Broilers		Turkeys		Quails	
		r	*P*	*r*	*p*	*r*	*p*	*r*	*p*
Cd	**Cu**	-0.21	0.74	-0.67	0.22	0.58	0.31	-0.21	0.74
Pb	**Cu**	-0.20	0.75	-0.56	0.32	**0.65**	**0.02**	0.31	0.62
Pb	**Cd**	-0.41	0.49	**0.68**	**0.02**	-0.14	0.82	-0.15	0.81
Zn	**Cu**	-0.31	0.62	-0.60	0.28	**-0.86**	**0.05**	0.31	0.62
Zn	**Cd**	**-0.87**	**0.05**	**0.87**	**0.05**	-0.29	0.63	**0.67**	**0.02**
Zn	**Pb**	0.51	0.39	0.56	0.32	-0.55	0.34	0.31	0.62
Hg	**Cu**	-0.11	0.87	0.15	0.80	-0.59	0.29	0.1	0.87
Hg	**Cd**	0.67	0.22	-0.13	0.83	-0.34	0.57	0	1.0
Hg	**Pb**	-0.40	0.50	-0.29	0.64	-0.08	0.89	0.31	0.61
Hg	**Zn**	-0.60	0.28	0.36	0.55	**0.87**	**0.05**	-0.36	0.55

r: spearman`s correlation factor; *p*: probability and Values in bold show significant correlation between each two metals.

Health risk assessment

Daily intake of heavy metals

Estimation of dietary intake of metals is considered as a good tool for identification of the human exposure frequencies to heavy metals. The estimated daily intakes of Cu, Zn, Cd, Pb and Hg were assessed based on the average concentrations of the accumulated metals as indicated in Table (4). The estimated metal intake from consumption of muscles and livers of poultry were lower than the tolerable daily intake of Cu (500), Cd (1.00), Pb (3.57), Zn (300-1000), and Hg (0.71) (20). This may indicate that such metals had minimum potential health risks from consumption of poultry meat in the study area. Similarly, Darwish *et al.* (6) reported that the EDIs of Pb and Cd from consumption of the edible tissues of the quails were below TDI.

Table 4: Estimated daily intake (EDI) (µg/ kg BW) of different tested metals tested

Examined samples		Cu	Zn	Pb	Cd	Hg
Layers	**Muscle**	0.045	1.463	0.057	0.034	0.254
	Liver	0.0003	0.008	0.0003	0.0001	0.001
Broilers	**muscle**	0.023	1.389	0.175	0.051	0.350
	liver	0.0003	0.007	0.001	0.0001	0.001
Turkeys	**muscle**	0.073	2.089	0.102	0.040	0.249
	liver	0.0004	0.008	0.001	0.0001	0.001
Quails	**muscle**	0.057	0.638	0.102	0.023	0.294
	liver	0.0002	0.003	0.001	0.0001	0.001
TDIs		**500**	**300 - 1000**	**3.57**	**1**	**0.71**

EDI: estimated daily intake and TDI: tolerable daily intake.

Target hazard quotient and hazard index

Heavy metals were highly accumulated in the livers compared with the muscle. However, the potential risks from consumption of the livers were minor when compared with the muscle owing to their extremely low ingestion rates (5). The non-carcinogenic risks from consumption of poultry meat were assessed based on the THQs and HI as indicated in Table (5). Amongst all the examined metals, Hg in muscle of layers posed the highest potential health risk (0.219), while Cd was the lowest (Table 5). In general, THQ and HI values were

far below one, which indicate minimum potential hazards for Egyptian consumers from poultry meat. However, this finding should be handled carefully due to the extensive consumption of poultry meat to compensate the shortage in the red meat in Egypt, which may lead to higher accumulation for metals. Therefore, continuous screening for metal accumulation in the poultry meat should be conducted. The recorded THQ and HI values corresponded with that reported in Ghana, Nigeria and Egypt (6,31,32).

Table 5: Hazard analysis due to consumption of poultry meat in Egypt

Examined samples		THQ Cu	THQ Zn	THQ Pb	THQ Cd	THQ Hg	HI
Layers	**muscle**	0.001	0.005	0.014	0.034	0.159	0.213
	liver	>0.000	>0.000	0.001	>0.000	>0.000	0.001
Broilers	**muscle**	0.001	0.005	0.043	0.051	0.219	0.319
	liver	>0.000	>0.000	0.0001	>0.000	0.001	0.001
Turkeys	**muscle**	0.002	0.007	0.02	0.040	0.155	0.229
	liver	>0.000	>0.000	0.0002	0.000	>0.000	0.001
Quails	**muscle**	0.001	0.002	0.025	0.023	0.184	0.235
	liver	>0.000	>0.000	>0.000	>0.000	>0.001	0.001
RFD		0.04	0.3	0.004	0.001	0.002	

THQ: target hazard quotient; **RfD**: oral reference dose for each metal; **HI**: hazard index.

Conclusion

The present study revealed accumulation of Cu, Zn, Cd, Pb and Hg in the livers and muscles of layers, broilers, turkeys and quails marketed in Egypt. Metals varied in their accumulation tendency based on tissue and species. The recorded concentrations had exceeded maximum permissible limits for Cd, Pb and Hg. Non-carcinogenic risk assessment of the examined metals revealed minimum risk among Egyptian populations due to consumption of poultry meat.

Conflict of interest

The authors declare no conflict of interest.

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Original Research Article

EXISTENCE OF VANCOMYCIN RESISTANCE AMONG METHICILLIN RESISTANT *S. aureus* RECOVERED FROM ANIMAL AND HUMAN SOURCES IN EGYPT

Norhan K. Abd El-Aziz[1], Marwa I. Abd El-Hamid[1]*, Mahmoud M. Bendary[2], Asmaa A. El-Azazy[3], Ahmed M. Ammar[1]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia, 44511. [2]Department of Microbiology and Immunology, Faculty of Pharmacy, Port Said University, Port Said, 42511. [3]Abouhamad Veterinary Organization, Ministry of Agriculture, Abou-Hamad, Sharkia, 44661, Egypt

*Corresponding author, E-mail: mero_micro2006@yahoo.com; mero_micro@zu.edu.eg

Abstract: The increased resistance of vancomycin among methicillin resistant *Staphylococcus aureus* (MRSA) has produced a major formidable threat in the therapeutic field. The current study analyzed the vancomycin resistance traits among MRSA isolates recovered from 148 samples of animal and human origins in Sharkia Governorate, Egypt. All staphylococci isolates were examined against 8 antimicrobials and vancomycin minimal inhibitory concentrations (MICs) were then determined among phenotypic vancomycin resistant and intermediate *S. aureus*. Furthermore, all vancomycin-resistant *S. aureus* (VRSA) isolates were exposed to PCR analysis of *mec*A and *van* genes. Herein, 45 of 86 *Staphylococcus* spp. were identified as *S. aureus*, while 41 were coagulase negative staphylococci (CoNS). A higher incidence rate of *S. aureus* was observed in meat products (58.06%), but majority of CoNS isolates were isolated from milk samples (54.54%) with no statistical differences ($P < 0.05$) in the distribution of *S. aureus* and CoNS among all samples. Oxacillin and amoxicillin-clavulanic acid recorded the highest resistance percentages among *S. aureus* (93.33 and 88.89%) and CoNS (75.61 and 87.80%), respectively. Multidrug resistance (MDR) was detected in high proportions of *S. aureus* (64.4%) and CoNS (34.1%). Forty-two of 45 *S. aureus* isolates were MRSA, of which 14 were vancomycin resistant with MIC values ranged from 32-1024 µg/mL. PCR detection of *mec*A and *van* genes in the tested isolates revealed that they were all *mec*A gene positive, while 10 out of them had *van* genes. The *van*B gene was found in 5 isolates with higher MICs (64- 256 µg/mL), while *van*A gene was detected in 4 isolates with MICs of 128-512 µg/mL and only one isolate harbored both *van*A and *van*B genes with MIC value of 1024 µg/mL. According to the upsurge of VRSA prevalence rates, more attentions should be oriented for continuous monitoring of antimicrobial usage with the need for effective drugs against VRSA.

Key words: *S. aureus*; Antibiogram; VRSA; MRSA; *mec*A; *van* genes

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Introduction

Staphylococcus aureus (*S. aureus*) has been documented as a significant cause of a wide range of diseases from wound, skin and bone infections to life threatening pneumonia, devastating septicemia and toxic shock syndrome (1,2). In dairy animals, *S. aureus* is one of the most incriminated pathogens causing clinical and subclinical mastitis worldwide (3). Because of the overuse of antibiotics, there has been a direful increase in the incidence of antibiotic resistant strains, which has complicated the treatment process (1). Penicillin was primarily very effective against most staphylococcal infections, but *S. aureus* began producing β-lactamase enzyme in the mid-1940s, which destroys the penicillin β-lactam ring (4). Later, more than 90% of *S. aureus* strains were penicillin resistant. The increase in this resistance drove the discovery of methicillin drugs, which are semi synthetic penicillin virtually resistant against genetic variations of β-lactamase enzyme. Through evolution, rigorous bacteria those cannot be treated by antibiotics are surviving until the isolation of the first bacterial strain of methicillin resistant *S. aureus* (MRSA) in 1961. Since then, MRSA become a dangerous endemic organism worldwide and listed on the top of the serious problems with a negative impact on public health. MRSA is mediated by the presence of a novel penicillin-binding protein (PBP), PBP-2a, which is expressed by an exogenous *mec*A gene (5, 6).

High scores of MRSA were observed in Egypt compared to other African and Mediterranean countries (7). The dramatic increase in MRSA prevalence raises the challenge of selecting suitable therapy for MRSA infections because of limited therapeutic options. Glycopeptides such as vancomycin has become the cornerstone for treating MRSA infections over the last twenty years (1).

Conceivably, the excessive usage of vancomycin results in the alarming widespread of its resistance and the existence of two types of glycopeptide resistant *S. aureus*. The first type, vancomycin intermediate resistant *S. aureus* (VISA), is due to cross linked and thickened cell wall matrix that sequesters and limits glycopeptides penetration (8). The second one, vancomycin-resistant *S. aureus* (VRSA) is associated with the acquirement of *van*A operon from *Enterococcus faecalis* by a horizontal gene transfer (9).

The increased prevalence of VRSA strains has augmented the controversy regarding the existing and the future role of vancomycin in treating staphylococcal infections and has stimulated great efforts to understand the mechanisms of its resistance. Though, there are several reports of VRSA worldwide, few cases were recorded in different Governorates of Egypt (10,11). According to earlier studies conducted in Egypt, VRSA strains were recognized with many of their infections occurring in humans, while data on VRSA infections in animals is too limited. Based on this rationale, continuous researches should be done for phenotypic and genotypic detection of VRSA to find the ideal antibiotic therapy and to control VRSA infections. Therefore, it was of a great interest to follow up the resistance to vancomycin in *S. aureus* recovered from animal and human origins in Sharkia Governorate and to determine the molecular characterization of the genes conferring this resistance.

Material and methods

Samples

A total of 148 samples were collected from animal (n=97) and human (n=51) sources. The samples from animal origin were milk (n= 66) from cows with clinical signs of mastitis from various dairy farms in Egypt and meat products (n=31) including sausage (n=7), minced meat (n=15) and burger (n=9), which were randomly collected from different supermarkets in Zagazig city, Sharkia Governorate, Egypt. Clinical samples from human origin comprising pus (n=12), urine (n=9), cerebrospinal fluid (C.S.F) (n=8), sputum and peritoneal fluid (n=7, each) and blood and pericardial fluid (n=4, each) were collected from patients admitted to Zagazig University hospital, Sharkia Governorate, Egypt. All samples were obtained under aseptic conditions and quickly

transferred to the laboratory for further bacteriological investigations.

Isolation and identification of staphylococci

The samples were subjected to standard microbiological techniques for isolation and identification of staphylococci (12). Briefly, Baird Parker agar (Oxoid, UK) with an egg yolk–tellurite emulsion supplement (Oxoid, UK) was used for preliminary isolation of staphylococci which were then subjected to Gram staining, oxidation/fermentation (O/F) test and catalase test. The developed colonies were sub-cultured onto mannitol salt agar medium (Oxoid, UK) to confirm mannitol fermentation. Presumptive *S. aureus* colonies were then identified basing on β-hemolysis on blood agar, production of golden yellow pigments onto milk agar, and tube coagulase test. Biotyping of coagulase negative staphylococci (CoNS) was done using the API 20 Staph identification kit (BioMerieux, Marcy l'Etoile, France). Molecular confirmation of putative *S. aureus* isolates was applied by PCR amplification of the *nuc* gene (447bp) using the primer sequences and PCR conditions described previously (13). The sequences of the primer pairs were 5'-GCGATTGATGGTGATACGGTI-3' and 5'-AGCCAAGCCTTGACGAACTAAAGC-3'. The amplification was carried out in a total of 37 cycles under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 0.5 min and extension at 72°C for 1.5 min. The reaction was terminated by a final extension step at 72°C for 3.5 min.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for staphylococci isolates was applied adopting the standardized Kirby-Bauer disc-diffusion procedure (14) using the following antimicrobial discs: amoxicillin/clavulanic acid (AMC, 20/10 μg), oxacillin (OX, 1 μg), vancomycin (VA, 30 μg), clindamycin (DA, 10 μg), gentamicin (CN, 10 μg), ciprofloxacin (CIP, 5 μg), trimethoprim/sulfamethaxole (SXT, 23.75/1.25 μg) and rifampin (RA, 5 μg) (Oxoid, UK). The interpretation of the results was done according to Clinical and Laboratory Standards Institute (CLSI) criteria (15). Staphylococci isolates concomitantly resistant to ≥3 antimicrobial classes were considered as multidrug-resistant (MDR).

Minimum inhibitory concentration of vancomycin (Sigma-Aldrich, USA) was detected against *S. aureus* isolates by broth macrodilution method following CLSI guidelines (15). The interpretive criteria were available in the pertinent CLSI document.

PCR-based detections of methicillin and vancomycin-resistance genes

Genomic DNA was extracted from phenotypic methicillin-resistant and vancomycin intermediate and resistant *S. aureus* isolates using the QIAamp DNA Mini kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. Oligonucleotide primers for *mec*A gene (a determinant of methicillin resistance), 5′-GTA GAA ATG ACT GAA CGT CCG ATA A-3′ & 5′-CCA ATT CCA CAT TGT TTC GGT CTA A-3′ and vancomycin resistance genes, 5′-CAT GAA TAG AAT AAA AGT TGC AAT A-3′ & 5′-CCC CTT TAA CGC TAA TAC GAT CAA-3′) for *van*A and 5′-GTG ACA AAC CGG AGG CGA GGA-3′ & 5′-CCG CCA TCC TCC TGC AAA AAA-3′ for *van*B were selected according to previous reports (16,17). PCR amplifications were performed with a PTC-100 ™ programmable thermal cycler (MJ Research Inc., Waltham, USA) in a total reaction volume of 50 μl consisting of 25μl of Dream *Taq* TM Green Master Mix (2X) (Fermentas, Inc. Hanover, USA), 1μl of each primer (20 pmole) (Sigma-Aldrich, Co., St. Louis, USA), 5μl template DNA and the volume was completed to 50 μl by nuclease-free water. The amplification conditions for *mec*A gene were performed as following: 94°C for 10 min, followed by 10 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 75 s and 25 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 75 s (16). Meanwhile, multiplex PCR amplification was carried out for *van*A and *van*B genes using the following thermal cycling conditions: initial denaturation at 94°C for 5 min, 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at

72°C for 1 min) and a final extension step at 72°C for 10 min (17). An aliquot of each amplicon (8 µl) was loaded on 1.5% agarose gel (Sigma-Aldrich, Co., St. Louis, MO, USA) containing 0.5 µg/mL ethidium bromide (Sigma-Aldrich, Co., St. Louis, MO, USA). A 100 bp DNA ladder (Fermentas, Inc. Hanover, USA) was used as a molecular weight marker. The amplified DNAs were electrophoresed at 100 V for 60 min on a mini horizontal electrophoresis unit (Bio-Rad, USA). The gel was then visualized and photographed under an UV transilluminator (Spectroline, Westbury, USA). For each PCR experiment, appropriate positive and negative controls were included.

Statistical analysis

Data were analyzed by Chi-square test using Statistical Package for Social Sciences (SPSS) version 23.0 (IBM Corp., Armonk, NY). *P* values of < 0.05 were statistically significant.

Results

Characterization of Staphylococci species

Staphylococci isolates were identified by conventional bacteriological methods. All the recovered isolates (n=86) grown onto Baird Parker and mannitol salt agar media were Gram positive cocci, non-spore forming, arranged in grape-like clusters, fermentative and catalase test positive; they were identified as staphylococci. Among them, 45 isolates were β-hemolytic on blood agar, produced the characteristic golden yellow pigments on milk agar and tube coagulase test positive, so they were considered as *S. aureus*. Biochemical identification of CoNS (n=41) using API 20 Staph identification kit revealed 5 biotypes; 12 *S. sciuri* (29.27%) from mastitis milk, 12 *S. xylosus* (29.27%) from mastitis milk (n=10) and human (n=2), 10 *S. lentus* (24.39%) from mastitis milk, 4 *S. chromogenes* (9.76%) from mastitis milk (n=3) and meat products (n=1) and 3 *S. simulans* (7.31%) from human (n=2) and mastitis milk (n=1). Further confirmation of *S. aureus* isolates was conducted by PCR amplification of *nuc* gene (Figure 1A).

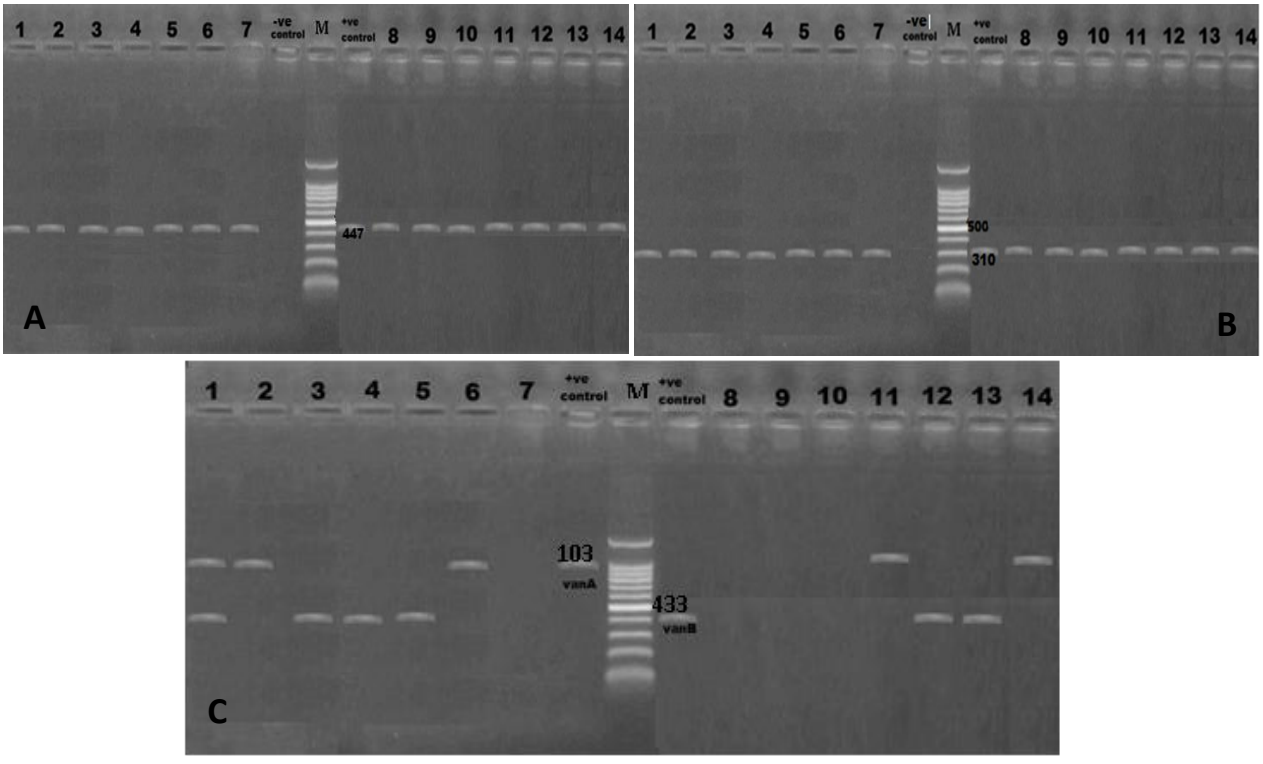

Figure 1: Agarose gel electrophoresis of PCR amplified products of *nuc* (A), *mec*A (B) and *van* (C) genes. Lane M: DNA molecular size marker (100 bp), lanes 1-14: VRSA isolates from minced meat (lanes 1-7), human sources (lanes 8-10), sausage (lanes 11-13) and mastitis milk (lane 14). The size in base pairs (bp) of each PCR product is indicated next to the bands in the +ve control lanes

Incidence of Staphylococcus species in animal and human sources

A total of 86 staphylococcal isolates were recovered from 148 samples (58.10%). Of these, 30.40% (45/148) were *S. aureus* and 27.70% (41/148) were CoNS. It has been observed that 28.87% (28/97) and 33.33% (17/51) of *S. aureus* isolates were recovered from animal and human origins, respectively. Meanwhile, 38.14% (37/97) and 7.84% (4/51) of CoNS isolates were isolated from animal and human samples, respectively. Incidence of *S. aureus* in meat products was 58.06% (18/31) which was higher than milk samples (15.15%, 10/66), but majority of CoNS isolates were isolated from milk samples (36/66, 54.54%). Statistical analysis of our data revealed highly significant differences ($P < 0.05$) in the overall percentage distribution of *S. aureus* and CoNS across mastitis milk, meat products and human samples (Table 1). Incidence of *Staphylococcus* species in diverse samples collected from human and animal origins are illustrated in Table (1).

Table 1: Incidence of *Staphylococcus* species in different samples collected from animal and human origins

Source	Number of analyzed samples (n= 148)	No. of staphylococcal isolates (%)	
		S. aureus	**CoNs**
Mastitis milk	**66**	**10 (.1515)**	**36 (54.54)**
Meat products:	**31**	**18 (.5806)**	**1 (3.23)**
Minced meat	15	9 (60.00)	1 (6.67)
Burger	9	3 (33.33)	0 (0.00)
Sausage	7	6 (85.71)	0 (0.00)
Human:	**51**	**17 (.3333)**	**4 (7.84)**
Pus	12	8 (66.67)	0 (0.00)
Urine	9	1 (11.11)	2 (22.22)
C.S.F	8	2 (25.00)	1 (12.50)
Sputum	7	3 (42.86)	0 (0.00)
Peritoneal fluid	7	3 (42.86)	1 (14.29)
Blood	4	0 (0)	0 (0)
Pericardial fluid	4	0 (0)	0 (0)
Chi-square	-	18.545	42.769
DF	-	2	2
P value*	-	0.000	0.000

CoNS: coagulase negative staphylococci, C.S.F: Cerebrospinal fluid, DF: degree of freedom, *: highly significant.

Antimicrobial susceptibility testing

S. aureus and CoNS were tested against 8 antimicrobial agents (Table 2). *S. aureus* resistance was observed most commonly to oxacillin and amoxicillin-clavulanic acid with percentages of 93.33 and 88.89%, respectively, followed by clindamycin (62.22%). Meanwhile, majority of *S. aureus* showed lower resistance rates against other tested antimicrobials. According to oxacillin/ methicillin susceptibility pattern, majority of the isolates (42/45, 93.33%) were MRSA, while only one isolate (2.22%) was methicillin-sensitive and 2 isolates (4.44%) were methicillin-intermediate. According to vancomycin susceptibility rates (Table 3), 64.44% (29/45) were vancomycin sensitive *S. aureus*, 4.44% (2/45) were VISA, and 31.11% (14/45) were VRSA. Interestingly, all VRSA isolates were MRSA as well as MDR.

Regarding CoNS, resistance was observed most commonly to amoxicillin-clavulanic acid, oxacillin and clindamycin with percentages of 87.80, 75.61 and 58.54%, respectively. Meanwhile, all CoNS exhibited absolute susceptibility (100%) to vancomycin and trimethoprim/sulfamethaxole being the best drugs used for treatment of CoNS infections. Our data showed significant differences in frequency of resistance to several antimicrobials (Table 2) with *P* values < 0.05.

According to the antibiogram results, *S. aureus* and CoNS of human origin (82.35 and 50%) were more multi-drug resistant as compared to those recovered from animal origin (53.57 and 32.43%), respectively

Preliminary screening of vancomycin resistance

The MIC values of vancomycin were determined against all phenotypic vancomycin resistant (n=14) and intermediate (n=2) *S. aureus* isolates with the disc diffusion method. The results showed that 14 *S. aureus* isolates (87.5%) were resistant to vancomycin with MIC values ranged from 32 to 1024 µg/mL, which were classified as VRSA in accordance with the breakpoints published by CLSI. Interestingly, the highly resistance for vancomycin was observed by 7 *S. aureus* from minced meat samples. One of these isolates had MIC value of 1024 µg/mL and the other six isolates had MIC values ranged from 64 to 512µg/mL.

Table 2: Antimicrobial resistance in *S. aureus* and coagulase negative staphylococci isolates from animal and human sources

Antimicrobial agent	Number of resistant *S. aureus* isolates (%)			Total (45)	*P* value	Number of resistant CoNS isolates (%)			Total (41)	*P* value
	Mastitis milk (10)	Meat products (18)	Human (17)			Mastitis milk (36)	Meat products (1)	Human (4)		
AMC	8 (80.00)	18 (100.00)	14 (82.35)	40 (88.89)	0.157	31 (86.11)	1 (100.00)	4 (100.00)	36 (87.80)	0.680
OX	10 (100.00)	17 (94.44)	15 (88.24)	42 (93.33)	0.490	27 (75.00)	1 (100.00)	3 (75.00)	31 (75.61)	0.680
VA	1 (10.00)	10 (55.56)	3 (17.65)	14 (31.11)	0.015	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1.00
DA	3 (30.00)	14 (77.78)	11 (64.71)	28 (62.22)	0.043	20 (55.56)	1 (100.00)	3 (75.00)	24 (58.54)	0.534
CN	1 (10.00)	2 (11.11)	4 (23.53)	7 (15.56)	0.522	0 (0.00)	0 (0.00)	1 (25.00)	1 (2.44)	0.010
CIP	3 (30.00)	0 (0.00)	10 (58.82)	13 (28.89)	0.001	7 (19.44)	1 (100.00)	1 (25.00)	9 (21.95)	0.164
SXT	1 (10.00)	10 (55.56)	3 (17.65)	14 (31.11)	0.015	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1.00
RA	0 (0.00)	4 (22.22)	5 (29.41)	9 (20.00)	0.181	17 (47.22)	1 (100.00)	2 (50.00)	20 (48.78)	0.588

AMC: amoxicillin-clavulanic acid, OX: oxacillin, VA: vancomycin, DA: clindamycin, CN: gentamicin, CIP: ciprofloxacin, SXT: trimethoprim/sulfamethaxole, RA: rifampin, CoNS: coagulase negative staphylococci.

Table 3: Multi-drug resistance profile of recovered staphylococci isolates

No. of antimicrobials to which the isolates were resistant	No of staphylococci isolates (%)					
	S. aureus (45)			CoNS (41)		
	Milk (10)	Meat products (18)	Human (17)	Milk (36)	Meat products (1)	Human (4)
3	0 (0.00)	5 (27.78)	9 (52.94)	9 (25.00)	0 (0.00)	1 (25.00)
4	0 (0.00)	7 (38.89)	2 (11.76)	2 (5.56)	1 (100.00)	0 (0.00)
5	0 (0.00)	2 (11.11)	3 (17.64)	0 (0.00)	0 (0.00)	1 (25.00)
6	1 (10.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)

CoNS: coagulase negative staphylococci

PCR detection of mecA and van genes

All 14 MDR-VRSA isolates (3 from human and 11 from animal origins) were screened by PCR detection of *mec*A and *van* genes. The results indicated that all selected isolates were positive for *mec*A gene (Figure 1B).

Furthermore, PCR amplification of *van* genes confirmed their possession among 10 *S. aureus* isolates only (71.4%); however, the other 4 *S. aureus* isolates (28.8%) were *van* genes negative. Data revealed that *van*B and *van*A genes were found in 5 and 4 isolates (35.7 and 28.6%, respectively) and they were detected simultaneously in one isolate (7.1%). Bands with approximate size of 310 bp were detected for *mec*A gene (Figure 1B), while *van*A and *van*B genes were observed at 1030 and 433 bp, respectively (Figure 1C). Interestingly, *van*A gene was detected in isolates with MICs ranging from 128 to 512 µg/mL, where *van*B gene was observed in isolates with MICs ranging from 64 to 256 µg/mL. In addition, the only isolate gave positive results for both *van*A and *van*B genes exhibited a high MIC value (1024 µg/mL). Characterization of all VRSA isolates elicited from the study is illustrated in Table (4).

Table 4: Description of 14 VRSA isolates elicited from the study

VRSA isolate No.	Source	Results of disc diffusion method			Vancomycin		*mec*A gene	*Van* genes
		S	I	R	MIC (µg/mL)	MBC (µg/mL)		
1	Minced meat	CIP	CN	AMC, OX, VA, DA, SXT, RA	1024	>1024	+	*van*A, *van*B
2	Minced meat	CIP	CN, RA	AMC, OX, VA, DA, SXT	256	512	+	*van*B
3	Human	CIP	RA	AMC, OX, VA, DA, CN, SXT	64	128	+	-
4	Mastitis milk	RA	-	AMC, OX, VA, DA, CN, CIP , SXT	128	256	+	*van*A
5	Sausage	CIP	CN, RA	AMC, OX, VA, DA, SXT	64	64	+	*van*B
6	Minced meat	CIP, RA	-	AMC, OX, VA, DA, CN, SXT	64	128	+	*van*B
7	Human	CN, CIP, RA	DA	AMC, OX, VA, SXT	32	64	+	-
8	Human	CN, CIP	-	AMC, OX, VA, DA, SXT, RA	32	64	+	-
9	Minced meat	CIP, RA	CN	AMC, OX, VA, DA, SXT	128	256	+	*van*A
10	Minced meat	CIP	CN, RA	AMC, OX, VA, DA, SXT	512	1024	+	*van*A
11	Sausage	CIP, RA	CN	AMC, OX, VA, DA, SXT	64	128	+	*van*B
12	Sausage	CIP, RA	CN	AMC, OX, VA, DA, SXT	128	256	+	*van*A
13	Minced meat	CIP	CN, RA	AMC, OX, VA, DA, SXT	256	512	+	*van*B
14	Minced meat	CIP	DA, CN, RA	AMC, OX, VA, SXT	64	128	+	-

S: sensitive, I: intermediate, R: resistant, AMC: amoxicillin-clavulanic acid, OX: oxacillin, VA: vancomycin, DA: clindamycin, CN: gentamicin, CIP: ciprofloxacin, SXT: trimethoprim/sulfamethaxole, RA: rifampin, MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration, +: positive, -: negative

Discussion

Multidrug resistant MRSA is the uppermost cause of community acquired and nosocomial infections worldwide, being associated with high mortality rates. Vancomycin has been suggested as the best drug for treatment of such infections (18). The increase in vancomycin resistance among MDR and MRSA strains has been supposed as a formidable threat in the therapeutic fields (19). Therefore, this study has made an attempt to explore the vancomycin resistance among MRSA isolates originated from human and animal sources (mastitis milk and meat products) for the first time in Egypt.

In the current study, staphylococci isolates were identified phenotypically by conventional microbiological methods as anecdotally reported (20). Further confirmation of all *S. aureus* isolates was conducted based on PCR detection of *nuc* gene as declared previously (13). Identification of the recovered CoNS isolates using API 20 Staph identification kit revealed 5 biotypes which were *S. sciuri*, *S. xylosus*, *S. lentus*, *S. chromogenes* and *S. simulans* with *S. sciuri* and *S. xylosus* being predominant (29.27%, each). They were mostly

from milk samples of cows suffering from mastitis. On the contrary, *S. chromogenes*, *S. haemolyticus*, *S. epidermidis* and *S. simulans* were accounted for 81.3% of all CoNS milk isolates in a recent scientific literature in Belgium (21).

Herein, *S. aureus* was isolated from animal and human origins with percentages of 28.87 and 33.33%, respectively. Incidence of *S. aureus* in meat products was 58.06% which was higher than mastitis milk (15.15%). Abd El-Hamid and Bendary (22) recorded a lower percentage of *S. aureus* in human subjects (26.67%). In previous studies, the isolation percentages of *S. aureus* in mastitis milk samples were 23 and 24%, respectively (23,24) which were higher than the frequency documented in the current study. Meanwhile, another study carried out in Egypt reported a lower incidence rate of *S. aureus* in milk samples from cases of cow mastitis (10.94%) (22). Additionally, a lower incidence rate of *S. aureus* (10%) was previously found in meat products by Ammar and coauthors (25). The differences in incidence rates among studies are interrelated to sampling, geographic area, environmental factors and the detection methods.

Resistance to commonly used drugs is an alarming emergent concern in human and veterinary fields. Considering the sequential changes in the burden of the resistance problem, detecting its determinants is important for managing control efforts (26). In the present study, the results of the antimicrobial susceptibility testing showed that *S. aureus* resistance was observed most commonly to oxacillin exhibiting 93.33% MRSA isolates, followed by amoxicillin-clavulanic acid (88.89%) and clindamycin (62.22%). In contrast, 37% resistance to amoxicillin-clavulanic (27) and 20% resistance to clindamycin (19) have been previously reported. In addition, twenty one (72.41%) of 29 *S. aureus* strains were MRSA as declared by Hasan and joint authors (19). This variation in resistance might be correlated to the type of antimicrobial agent recommended for treatment in various geographical areas.

Regarding CoNS, the antimicrobial resistance was observed most commonly to amoxicillin-clavulanic acid (87.80%), followed by oxacillin (75.61%), while all the recovered isolates showed 100% susceptibility to vancomycin and trimethoprim-sulfamexazole. The previous findings were consistent with a recent previous study (26), where CoNS showed significant increasing temporal trends in oxacillin and amoxicillin-clavulanate resistance. Moreover, a previous work carried out in Pakitsan revealed that their CoNS isolates exhibited high resistance levels to oxacillin (70.3%) and amoxicillin (74.8%), but low resistance rates were observed against ciprofloxacin (35.2%), amoxicillin/clavulanate (32.8%), clindamycin (16.3%) and vancomycin (2.6%), which was in harmony with our results indicating that ciprofloxacin, clindamycin and vancomycin are effective agents for treatment of CoNS infections (28).

Moreover, antibiogram results revealed that MDR pattern was pronounced in majority of *S. aureus* and CoNS isolates of human origin (82.35 and 50%) comparing to those recovered from animal origin (53.57 and 32.43%), respectively, which is consistent with another study in India (29). This may be attributed to that the extensive use of antibiotics in Egypt has rendered the frequently used antibiotics fully ineffective in treatment of staphylococcal infections.

A myriad of studies have focused on vancomycin resistance among MDR-MRSA isolates (18,19) revealing the necessity for effective and new drugs against MRSA. In this study, 31.11% (14/45) of *S. aureus* isolates were categorized as VRSA with MIC values ranged from 32-1024 µg/mL, they were all MDR and MRSA as was indicated by the presence of *mec*A gene. The highly resistance for vancomycin was observed in 7 *S. aureus* from minced meat samples. PCR amplifications exhibited the possession of the tested *van* genes among 10 *S. aureus* isolates only (71.4%); *van*B and *van*A genes were detected in 5 and 4 VRSA isolates with percentages of 35.7 and 28.6%, respectively and they were detected simultaneously in one isolate (7.1%). Previously, a similar level of VRSA was

observed in Egypt as 32% of *S. aureus* isolates (commonly from minced meat) were vancomycin resistant, 87.50% out of them possessed the *van* genes with a high predominance of *van*B (50%) (25). In contrast, all examined *S. aureus* isolates were vancomycin sensitive in another study in Pakistan (30).

Conclusion

Our study has focused on the great emergence of VRSA among isolated MRSA from mastitis milk, meat products and human sources as a first report at least in Egypt. In light of the results, a major fraction of VRSA was detected among recovered MRSA isolates indicating the unawareness and undiscerning usage of the broad-spectrum antibiotics and thereby revealing the need for novel and effective antibiotics against MRSA.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

MORPHOLOGICAL AND HISTOLOGICAL STUDIES ON PARATHYROID GLAND OF ADULT MALE GOAT *(CAPRA HIRCUS)*

Nehal I.A. Goda, Shafika A. El sayed, Rasha R. Beheiry*, Suzan A.A. Ismail

Department of Histology and Cytology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt

*Corresponding author, E-mail: rasharagab2006@yahoo.com

Abstract: The parathyroid glands are essential endocrine glands as they produce hormones that maintain calcium within the normal level in blood through secretion of parathormone. The removal of parathyroid glands leads to fatal levels of hypocalcemia. The number of parathyroid glands is species specific. The current investigation was performed on 20 healthy freshly slaughtered adult male goats. Fifteen specimens were immediately fixed in 10% buffered neutral formalin, then processed for histological and immunohistochemical examination. Other 5 specimens were handled for transmission electron microscope. The present work was conducted to study the morphological and histological characteristics of parathyroid glands of goat. Parathyroid glands comprised of two pairs, each pair formed from external and internal glands. External parathyroid gland was rounded or oval in shape. Its location was varied in the same animal where it may be located cranial to thyroid gland or beside the submandibular salivary gland. Internal parathyroid gland was embedded inside thyroid tissue. It appeared as pale rounded area at the end of cranial part of the right and left thyroid lobe. The histological findings demonstrated that, each parathyroid gland is surrounded by a thin capsule of dense irregular connective tissue. The glandular parenchyma is divided by short thick septa into ill distinct compartments. Each compartment had numerous numbers of chief cells. The active chief cells were polygonal in shape with oval nuclei. Immuno-histochemical findings revealed that they are positively reacted against chromogranin antibody. Electron microscope revealed that the cytoplasm have abundant mitochondria, rough endoplasmic reticulum, evenly distributed golgi apparatus and numerous secretory granules. On the contrary, the inactive chief cells have more vacuolated cytoplasm which contains less cell organelles.

Key words: parathyroid gland; chief cells; ultrastructure; chromogranin

Introduction

Goats play an important role in the food and nutritional security of the rural poor people (1). The economic returns of goat are rising every year in developed countries (2).

Parathyroid glands are the last real organ recognized in humans (3). They are essential for life in most animals and human (4). They assume an extraordinary part in the direction of the calcium metabolism inside the body (5,6).

The glands' parenchyma consists of densely packed cellular structure that are arranged in

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various forms including follicles or anastomosing cords and nests of polygonal cells. They are isolated by single or double layered collagen and reticular fibers with fibrocytes. The parathyroid cells are classified according to the presence of glycogen and lipid droplets into darkly stained active secreting and lightly stained inactive resting cells (7).

The active chief cells are polygonal in shape. They have light, spherical or oval indented nuclei. The inactive chief cells are polygonal outlines and have less cell organelles than the active cells. They have more vacuolated cytoplasm and central nucleus (8). In rat the staining properties of the cytoplasm reflects different functional status of the chief cells (9).

In mouse, chief cells have some morphologic changes as indicated by various phases of the secretory cycle (10). In *Rattus rattus*, the chief cells are oval in shape (11), the active stage is polygonal in shape, the plasma membranes of adjacent active chief cells have complex interdigitations, while, the inactive chief cells are cuboidal and have uncomplicated interdigitations between adjacent cells (12).

Immunohistochemistry revealed that the parathyroid glands contain two major kinds of cells; chief cells and oxyphilic cells. Chief cells are considered as the functional and the most well-known cells (13). The immunocytochemical staining revealed that chief cells contain less secretory granules than other endocrine cells (14). They have densely stained diffuse fine granules containing PTH (parathyroid hormone) and Chromogranin in their cytoplasm (15). The parathyroid cells are stained focally or diffusely to chromogranin antibodies (16).

Chromogranin A is a major protein of the parathyroid glands which is co-stored and co-secreted with parathyroid hormone. Chromogranin A is dispersed essentially in endocrine and neuroendocrine cells (17). Chromogranin A and other subclasses of chromogranin have been found in most endocrine cells with secretory granules in human being (18,19) and animals (20, 21).

The present work aimed to throw more light on the morphology and histological structure of the parathyroid glands of goat and differentiate their different cell types by light and transmission electron microscopes. Also, to investigate localization of chomogranin A immunoreactive cells by immunohistochemical studies.

Material and methods

Animals and Tissue Samples

The current study was conducted on 20 healthy freshly slaughtered adult male goats "*Capra hircus*" taken from Minia El-Kamh abattoir, Sharkia Governorate, Egypt. Fifteen samples were used for the routine histological studies and five samples were used for the electron microscopical studies. The parathyroid glands were dissected from the healthy animals immediately after slaughter. The shape and color of the glands were studied immediately after being dissected.

Tissue preparation

The specimens were immediately fixed in 10% buffered neutral formalin, then dehydrated followed by clearing in Xylol. All specimens were infiltrated with soft melted paraffin in the hot air oven and were embedded in hard paraffin. Using rotary microtome, sections of 5-7 μm thickness were cut. The paraffin sections were stained with Harris's Hematoxylin and Eosin (H & E) stain as a routine staining method to demonstrate the general histological structure. Crossmon's trichrome stain was used for the collagen and muscle fibres (22).

Tissue preparation for immunohistochemistry

Avidin biotin peroxidase method was used. The sections used were mounted on charged slides, then deparaffinized by xylene, and re-hydrated in graded ethanol then washed in phosphate buffer saline (PBS) at pH 7.2 for 5 minutes. To block endogenous peroxidase activity, the sections were immersed in 0.3% hydrogen peroxide in water. The sections were then washed in distilled water several times and then washed in PBS. The sections were then washed in 10% normal rabbit serum (blocking reagent) in a humid chamber for 30 min to reduce non specific binding of immunoglo-

bulins. The sections were incubated with antisera containing the specific primary antibody (chromogranin rabbit monoclonal antibody RM-9112-R7, Thermo scientific, Thermo Fisher Scientific). The sections were then incubated in a humidified chamber at room temperature overnight. Excess reagent was thrown off and the slides were washed in four changes of PBS, 5 min each. Then, the sections were incubated at horse reddish peroxidase polymer for 15 min at room temperature. The slides were rewashed in four changes of PBS, 5 min each. Diaminobenzidine (DAB) was used as chromogen and sections were incubated for 2-4 min at room temperature. Sections were washed in distilled water for 5 min, then were counter stained with Mayer's haematoxylin, dehydrated in ascending grades of alcohol, cleared in xylene and then mounted in Canada Balsam (22).

All the stained sections were examined with a standard light microscope (Olympus BX 21, Objective X 4,10,40,100 and Ocular X 10) and photographed by a digital Dsc-W 800 super steady cyper shot camera (Sony-Japan) at the Department of Histology and Cytology, Zagazig University).

Transmission electron microscopic (TEM) studies

Pieces of tissue about 1 mm^3 were fixed immediately in a buffered glutaraldehyde - formaldehyde fixative (GA/FA) that consists of (1% glutaraldehyde and 10 % formaldehyde in 0.1 M Phosphate buffer at pH 7.4 at 4ºC**)** for 2 h, then fixed in 1% osmium tetroxide. After rinsing in the osmium tetroxide, they were dehydrated in ascending grades of ethanol ending with propylene oxide, and embedded in Epoxy resin. Semi thin sections were cut on a MT2 Sorvall microtome and stained with toluidine blue. Ultrathin sections were cut on a RMC MT6000_ XL ultramicrotome, mounted on copper grids and stained with uranyl acetate and lead citrate. The ready ultrathin sections were examined and photographed at Faculty of Agriculture, Mansoura University by a JOEL electron microscope (JEM 1200 EX II) operating at 80KV (23).

Results

Morphology of parathyroid glands

Parathyroid glands right and left lobes consisted of two pairs; external pair (right and left) and internal pair (right and left). External parathyroid gland was rounded or oval in shape. It had pale reddish color and consisted of right and left lobes. The gland was located anterior to the cranial pole of thyroid gland. External parathyroid gland was embedded inside the remnants of thymic tissue. Internal parathyroid gland consisted of right and left parts. It was embedded inside thyroid tissue. Internal parathyroid gland was very small to be seen in fresh samples where it appeared after making serial segments in the formalinized thyroid lobes. It appeared as pale rounded area at the end of cranial part of the right and left thyroid lobe (Figure 1).

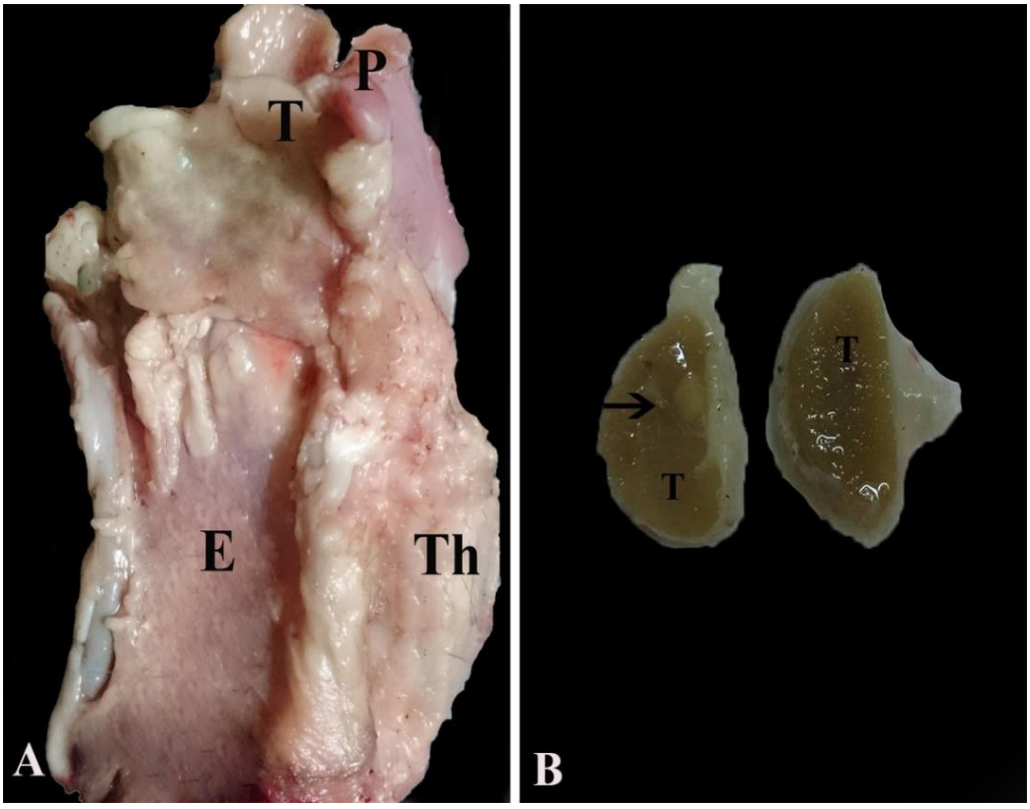

Figure 1: Photomacrograph of goat parathyroid glands. (A) showing "T" remnant of thymus gland, "P" external parathyroid, "Th" thyroid gland and "E" esophagus. (B) showing the less observed internal parathyroid "arrow" embeded within thyroid gland lobe "T" after its separation at the cranial end

Light microscopy

External parathyroid gland was surrounded by thin connective capsule, which consisted of connective tissue fibers and cells. The capsule arises from which irregular short and thick septa dividing the parenchyma into incomplete compartments (Figure 2A). These compartments had densely packed cellular structure of only one cell type of chief cells, which present near the blood capillaries (Figure 2B). In goat parathyroid glands, no oxyphil cells were observed. Chief cells were abundant and evenly distributed inside the lobules. They were separated by interstitial connective tissue including collagen fibers and blood capillaries (Figure 2C). In semi thin section, many vacuoles of different sizes were observed and the chief cells were distributed all over the parenchyma beside the blood capillaries (Figure 2D).

Internal parathyroid gland was embedded inside thyroid tissue (Figure 3A) and separated from them by connective tissue septa containing collagen fibers (Figure 3B).The chief cells were classified into two types according to the staining affinity; darkly stained cells and lightly stained cells. Chief cells were round or oval in shape with pale acidophilic cytoplasm. It had rounded vesicular and centrally located nuclei (Figure 3C). Immunohistochemical observation revealed that the chromogranin immunoreactive cells gave a strong positive reaction with anti-chromogranin, the expression was strongly observed in the cytoplasm of chief cells (Figure 3D).

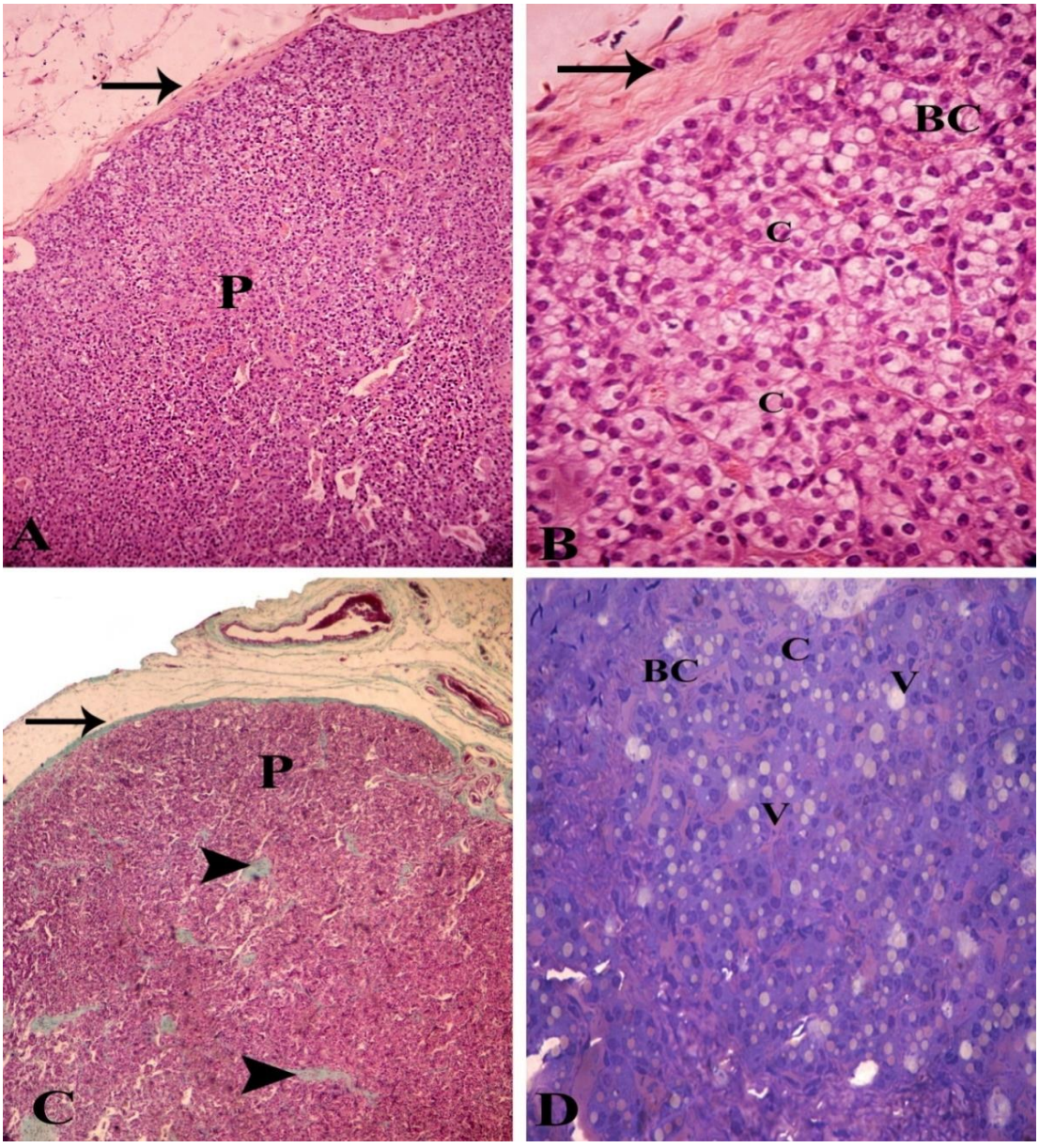

Figure 2: Photomicrograph of the goat's external parathyroid gland showed (A) the glands' connective tissue capsule "arrow"and the parenchyma "P". (B) the glands' capsule "arrow", the parenchyma consists of chief cells "C" which present near to blood capillary "BC". (C) showed the glands capsule had collagen fiber " arrow" the parenchyma "P" consists of chief cells which separated by interstitial connective tissue including collagen fibers (arrow heads) (D) semi thin section of external parathyroid showed many vacuoles "V" of different sizes and the chief cells (C) are distributed all over the parenchyma beside the blood capillaries (BC). (A&B): H&E stain, (C) : Crossmon's trichrome, (D): Toluidine blue x. 100 in (A) x. 50 in (C) and x. 400in (B,D)

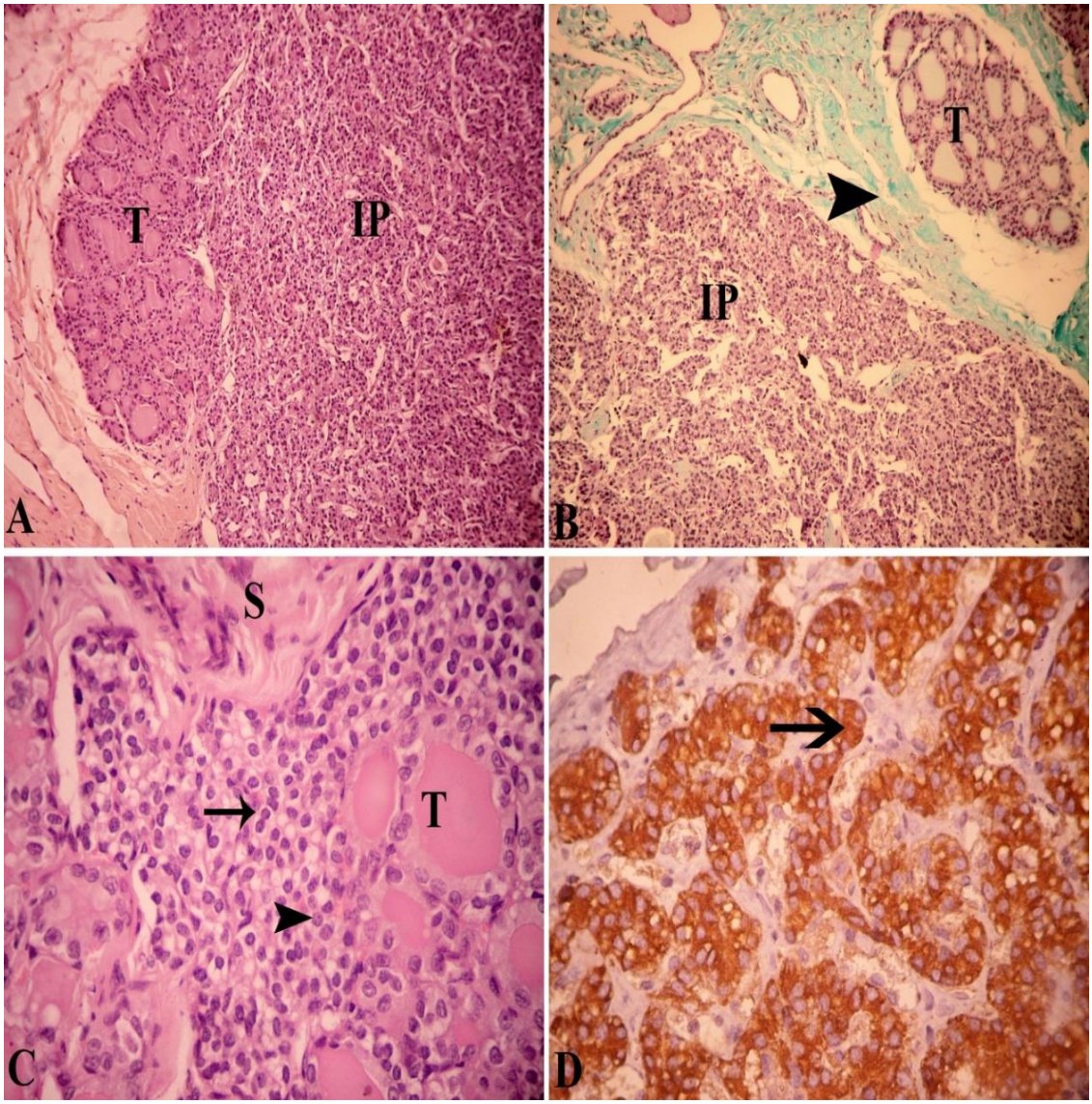

Figure 3: Photomicrograph of the goat's internal parathyroid gland showed (A) the internal parathyroid gland "IP" is embedded inside thyroid tissue "T". (B) the internal parathyroid gland "IP" is embedded inside thyroid tissue "T" and separated by connective tissue septa containing collagen fibers "arrow head". (C) internal parathyroid gland showed the gland is surrounded by connective tissue septa "S" The gland's parenchyma is consisted of light "arrow head" and dark chief cells "arrow". (D) External parathyroid gland showed the immune-reactivity of chromogranin A. The chief cells are positively reacted against chromogranin antibody "black arrow" Stain: (A&C): H&E, (B) : Crossmon'strichrome, (D): IHC staining x. 100 in (A,B) and x. 400in (C,D)

Ultrastructure

The active chief cells were polygonal in shape with oval or round nuclei. Their nuclei were large and light with peripheral heterochromatin spots. The nuclear envelope was indented with many nuclear pores. Their cytoplasm had abundant mitochondria, randomly distributed rough endoplasmic reticulum, evenly distributed golgi apparatus and numerous dense secretory granules. The inactive chief cells had polygonal outlines with more vacuolated cytoplasm which contain less cell organelles and more central nucleus (Figure 4). The inactive chief cells had rounded nucleus and moderate number of mitochondria. They were located beside blood capillary that contain red blood corpuscle (Figure 5).

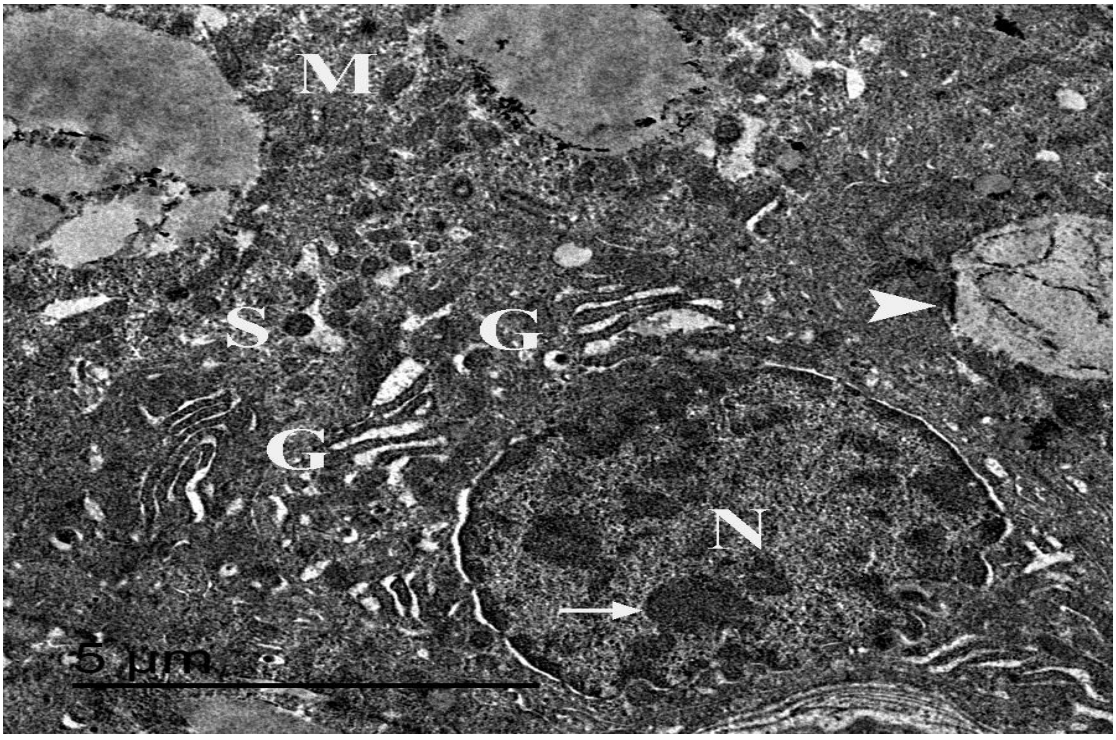

Figure 4: Transmission electron micrograph of goat's parathyroid gland. Ultrathin section showed that the active chief cells have rounded nucleus "N". Their nuclei are large with electron dense heterochromatin spots "arrow". The cytoplasm of these cells have abundant mitochondria "M", evenly distributed golgi apparatus "G" and several secretory granules "S". The inactive chief cells have more vacuolated cytoplasm "arrow head"

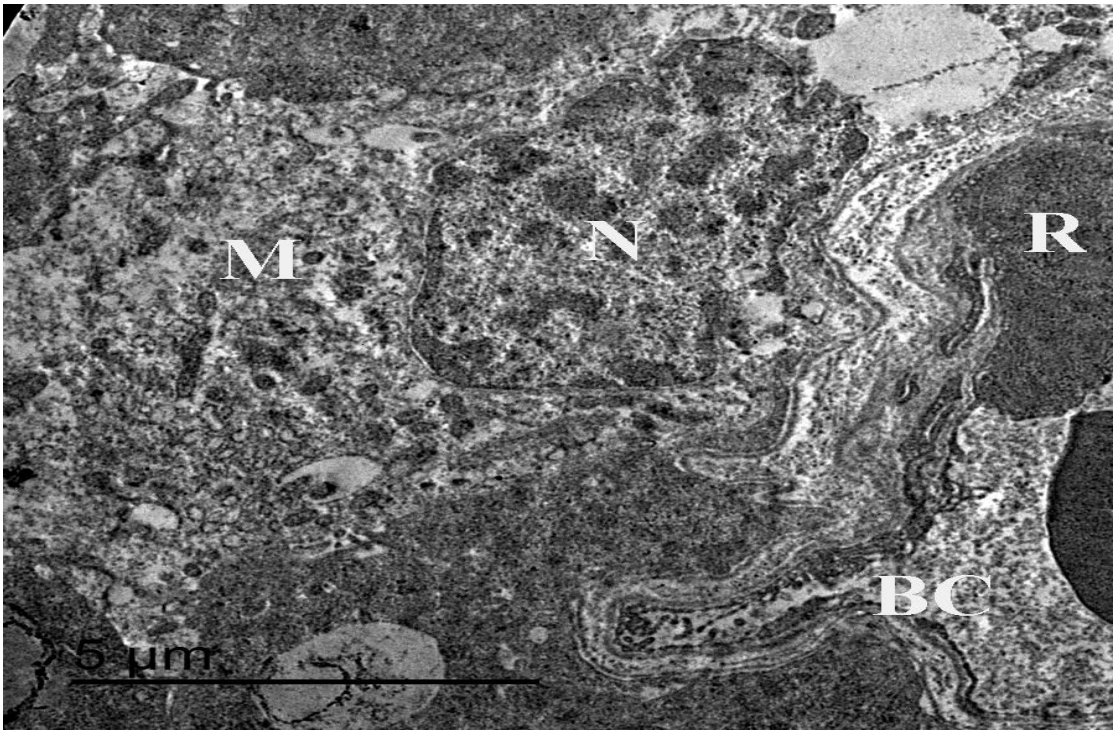

Figure 5: Transmission electron micrograph of goat's parathyroid gland. Ultrathin section showed that the inactive chief cells have rounded nucleus "N". The cytoplasm of these cells has moderate number of mitochondria "M". They are present near blood capillary "BC" containing red blood corpuscle "R"

Discussion

The parathyroid glands vary in number and location in several vertebrates (24). The parathyroid glands are small pale brown flattened oval disk (12). In goat, the parathyroid glands have two embryological origins. The external parathyroid and the internal parathyroid originate from III and IV pharyngeal pouches respectively. It is organized bilaterally. The external parathyroid gland lies ventral to the wing of the atlas. However, the internal parathyroid gland is buried in the medial portion of the cranial half of the thyroid gland (25), our study confirmed the results of above mentioned authors. Besides, in the present work, the location of the external parathyroid gland varied in the same animal for example; it may be located cranial to thyroid gland embedded within remnant of thymic tissue, also it may situated beside submandibular salivary gland.

The present study revealed that the parathyroid glands of goat consisted of external and internal pairs. The external and internal parathyroid glands have been recorded in large ruminants; buffalo (26) and camel (8). External parathyroid gland of camel was located anterior to the cranial pole of the right and left lobe of thyroid gland or 9-16cm dorsal to the dorsal pole of the thyroid gland (8). In our study the internal parathyroid gland was buried inside thyroid lobe. t couldn't be seen in fresh samples. However, it appeared after making serial segments in the formalinized thyroid lobes rather than in camel the internal parathyroid was not circumscribed and cannot be seen by naked eye (5).

The current work indicated that external parathyroid gland was surrounded by thin connective capsule which sent irregular short and thick septa dividing the parenchyma into incomplete compartments. Similar results were reported in buffalo (26). In goat the parathyroid compartments had densely packed cellular structure of only one cell type called chief cell. The same results were confirmed in rats (27). However, the parathyroid gland of horse consists of two types of cells including chief cells and oxyphilic cells (28) which were not detected in goat's parathyroid parenchyma under investigation in current study. Also these cells are absent from the parathyroid glands of the rat and many species of lower animals (11). Its number increases by aging (29). While in human (30) and in camel (8) the oxyphil cells are larger than chief cells. Their cytoplasm is filled with numerous large mitochondria as a need for energy production.

Chief cells were classified into two types; darkly and lightly stained cells. They undergo morphologic changes corresponding to different stages of the secretory cycle (10). They were round to oval in shape with pale acidophilic cytoplasm. Chief cells had round, vesicular and centrally located nuclei. Similar results were recorded in horse by Babu et al. (28) .

Parathyroid hormone (PTH) 3 and chromogranin A (CgA) represent the two major proteins of the parathyroid gland whose secretion is controlled by the concentration of extracellular Ca2+ (31). The most hypotheses for the role of CgA, is that it is a precursor protein for autocrine or paracrine peptides that modulate stimulated endocrine cell secretion (31,32). Using ABC immunohistocheistry; our results confirmed previously mentioned findings through detection of positively reacted chief cells with chromogranin antibody (33).

The active chief cells were polygonal in shape with oval nuclei. Their nuclei were large and light with peripheral heterochromatin spots. The cytoplasm of these cells have abundant mitochondria, randomly distributed rough endoplasmic reticulum, evenly distributed golgi apparatus and numerous dense secretory granules which indicate active protein synthesis. On the contrary, the inactive chief cells have more vacuolated cytoplasm which contains less cell organelles. Similar results were recorded in previous studies in camel (8) and (12). Chen et al. (12) added that these vacuoles may be due to numerous lipid droplets, lysosomes or glycogen particles

Conclusion

Parathyroid glands of adult goat consisted of external and internal pairs. The location of the

external parathyroid gland varied in the same animal. The internal parathyroid gland was buried in the thyroid gland. It had only one cell type called chief cells without oxyphil cells. Two types of chief cells were observed; active and inactive suggesting different stages of activity according to calcium level in the blood. These cells were positively reacted to chromogranin antibody which has an important role in its endocrine function.

Conflict of interest

There is no conflict of interest.

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Original Research Article

BIOCHEMICAL EFFECTS OF GINGER AND/OR GREEN TEA EXTRACTS IN HIGH FAT DIET - INDUCED OBESE RATS

Mohamed A. Hashem[1]*, Nasr A.M. Nasr El-Deen[1], Osman A.E. Ghareeb[2]

[1]Department of Clinical Pathology, [2]Scientific and Medical Research Center 'ZSMERC', Faculty of Veterinary Medicine, Zagazig University, 44511, Sharkia Governorate, Egypt

*Corresponding author, E-mail: mhashem.vet@gmail.com

Abstract: The current study was designed to clarify the sequel of administration of green tea and/ or extract of ginger on body weight, lipid profile, some hormones and some fertility variables in high fat diet (HFD) - induced obese rats. The results revealed that treatment of HFD obese rats with ginger extract decreased the elevated body weight (from 530±5.29 to 295±9.11), lee index (from 0.33±0.008 to 0.27±0.004), serum total cholesterol (from 25±2.57 to 187±2.79), triacylglycerol (TAG) (from 201±2.15 to154±1.82), low density lipoprotein cholesterol (LDL-C) (from 187.8±3.36 to119.6±2.31), very low density lipoprotein cholesterol (VLDL-C) (from 40.21±0.12 to 30.8±0.13), leptin (from 7.74±0.05 to 5.66±0.09), luetinzing hormone (LH) (from 0.30±0.006 to 0.19±0.005) and follicular stimulating hormone (FSH) (from 0.74±0.128 to 0.52±0.012) levels, but significantly elevated the decreased high density lipoprotein cholesterol (HDL-C) (from 21.40±0.50 to 36.40±0.81), serum testosterone (from 0.84±0.02 to 1.32±0.14), sperm count (from 44.20±2.08 to 65.20±0.54), motility (from 64±1.18 to 75.60±0.81) and normality (from 53.80±1.42 to 67±1.51). Similar results were observed with green tea treatment with minor variations. So, the inclusive outcomes may suggest that extracts of ginger and/ or green tea have a significant hypolipidemic effect with body weight reduction in rats fed high fat diet. In addition, the extracts may be qualified for ameliorative the ruined fertility parameters.

Key words: grean tea; ginger; obesity; lipids; sperm count; leptin

Introduction

Obesity is the excess accumulation of fat in the body. It is a condition medicinally termed to storage of superfluous body fat to amplitude that cause a negative health impact, resulting in reduction of life anticipatancy and excess health problems (1). Obesity is mainly caused by combination of excessive intake of high energy food, lack of physical activity and genetic susceptibility (2). Several effects are caused due to obesity including diabetes mellitus (3), hypertension (4), dyslipidemia (5), cardiac alterations (6), metabolic syndrome (7), lung diseases (8), cancer (9) and neurological disorders (10). In last few years, ginger and green tea have become a subject of interest because of their beneficial effect on human health.

Zingiber officinale (Ginger) has been consumed for over 2000 years as a spice. Its root contains polyphenol compounds including derivatives of 6-gingerol, which possess high antioxidant vigor (11). The piquancy of caller

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ginger is due principally to the gingerols that are symmetrical chains of phenols. The most plentiful is 6-gingerol (1), with presence of small quantities of another gingerols with varied chain lengths. The spiciness of dried ginger fundamentally results from shogaols (for example, 6-shogaol (2)), that are exsiccated forms of gingerols. Shogaols are created from the related gingerol during thermic processing. Dissolution averages of 6-gingerol to 6-shogaol were also appeared to be pH dependent and the paramount constancy at pH 4 (12). Many studies revealed several physiological responses of ginger, which may also be salutary for preventing and treat of some medical problems as hyperlipidemia (13), hyperglycemia (14), over weight (15), hepatic diseases (16), renal diseases (17) and gastrointestinal tract **(**GIT) illness (18).

Green tea is a pop drink from the plant of *Camellia sinensis* (Theacease family). Since many thousands years, it is supposed that green tea consumption possess medical effectiveness in the avoidance and treat a lot of diseases and so extend life is often connected to habit of green tea drinking (19). Green tea is manufactured from newly cropped young leaves by prompt steaming to avoid enzymatic fermentation. Steaming procedure inactivate enzymatic action of polyphenolic oxidase, which has the ability for conversion of tea catechins to polymeric derivatives and oligomeric. Though, young leaf handled for completely enzymatic fermentation, in which catechins is modified into arubigins and aflavins, which is a trait of the black tea, while semi-fermentation produces Oolong tea (20). In the recent past years, several studies revealed that green tea promotes health and has been informed for treatment of hyperlipidemia (21), overweight by increasing fat oxidation and energy expenditure (22) and hyperglycemia (23). The purpose of this study is to investigate the possible effects of green tea and ginger extracts on rats obesity induced by a high fat diet (HFD).

Material and methods

Experimental Animals

The present experiment was conducted on 50 apparently healthy male albino rats with average body weight (170-187 g). They were obtained from the Animal House, Zagazig Scientific and Medical Research Center (ZSMRC), Faculty of Medicine, Zagazig University. Rats were kept in clean hygienic house in metal cages and housed in controlled temperature room (19–23 °C), with dark light cycle of 12 hours during all experimental period. The study was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University.

Experimental Diets

Basal and fattening diets with their composition are shown in Table (1). The basal diet was used to meet the rat's nutrient requirements, while the high fat diet (HFD) was formulated to induce obesity in rats as previously mentioned (24). Water and feed were available all the time during the experimental period.

Herbal Plants

Ginger extracts (GE) (Multi-treat Arab Company for Pharmaceutical and Medicinal plants (MEPACO MEDIFOOD)), Egypt. It is present as tablets; one tablet contains 400 mg/kg BW ginger dry extract.

Green tea extracts (Multi –treat Arab Company for Pharmaceutical and Medicinal plants (MEPACO MEDIFOOD)), Egypt. It is present as tablets; one tablet contains 300 mg/kg BW green tea dry extract.

Experimental Design

A 50 mature male Albino rats were divided into 5 equal groups. Group (1) fed on basal diet (control group). Group (2) fed only HFD (60 % fat) for 45 days and served as obese control. Group (3) fed HFD and given one tablet ginger extracts (400 mg/kg BW) in 1 ml distilled water by gavages daily for 45 days. Group (4) fed HFD and given one tablet green tea extracts (300 mg/kg BW) in 1 ml distilled water by gavages daily for 45 days. Group (5) fed HFD and both ginger and green tea extracts in the same aforementioned doses and duration.

Table 1: Composition of the basal and experimental diet fed to healthy mature male albino rats

Ingredient	Basal diet (%)	Experimental diet (%)
Wheat Flour	77	16.07
Meat Meal	6.0	20
Animal Fat	3.5	57.93
Wheat Bran	10	3.0
Lysine	0.6	0.1
Methionine	0.4	0.4
Di-calcium phosphate	1.0	1.0
Sodium chloride	0.5	0.5
Vit. & Min. Mix	1.0	1.0
Calculated Nutrient Composition		
Crude protein	14.01	12.67
Energy (ME; kcal /kg)	3296.6	6239.23
Ether Extract	5.27	60.02
Crude fiber	3.83	3.75
Ash	3.06	5.77
Lysine	1.09	0.81
Methionine	0.61	0.58
Calcium	0.91	2.06
Phosphorus	0.64	1.12

ME: Metabolizable energy.

Determination of body weight, body length and Lee Index

The analyses were done in anaesthetized rats intraperitoneally injected by 0.1 ml of sodium barbiturate (1%) in order to observe the changes in both live body weights and lengths (25). The body weight and body length were measured according to the described methods (26,27). Lee Index: The final body weights and body lengths were used to calculate the Lee index = cube root of body weight (g) divided on nose to anus tall (cm) (26).

Collection of blood samples

By the end of the experiment, blood samples were collected after overnight fasting from retro-orbital venous plexus in a sterile test tube without anticoagulant for separation of serum for biochemical tests.

Biochemical studies

Lipid profile including serum total cholesterol, TAG, and HDL-C were evaluated using colorimetric methods (28-30), respectively; LDL-C and VLDL-C were calculated according to the equation of Friedewald (31) and serum leptin level (32). Serum levels of FSH, LH and testosterone were determined by Enzyme Linked Immunosorbent Assay (ELISA) as previously described **(**33-35), respectively.

Sperm analysis

Sperm motility and count, as well as normality were determined according to the described methods (36, 37), respectively.

Statistical analysis

The obtained data was statistically analyzed using one way ANOVA (SPSS) (38). The significance was set as $P \leq 0.05$. All values were represented as mean± standard error and the highest value was represented by the letter (a).

Results

Rats fed high fat diet (HFD) alone, HFD plus ginger and HFD plus green tea showed a significant rise in final body weight and lee index in comparison to the normal control as illustrated in Table (2). The highest values were recorded in G2. While groups 3 and 4 exposed a statistical falloff in the body weight and lee index, in comparison with G2 and the lowest values were reported in the G3. Moreover, HFD fed rats and treated with both ginger and green tea showed a significant diminution compared with groups (2-4), while in comparison with the

control group, lee index was returned to the normal values.

Lipid profile as illustrated in Table (3) disclosed a significant rise in the serum cholesterol, TAGs, LDL-C, VLDL-C and leptin (Table 4) levels in groups (2-5), in comparison with normal control group. The highest values were recorded in G2. On the other hand, groups (3-5) showed a significant decrease in the aforementioned parameters compared with obese rats (G2) and the lowest values were recorded in the G5. However HDL-C revealed a significant decrease in groups 2-5 compared with normal control. The lowest value was recorded in G2 and the highest value was reported in G5. Obese rats treated with either ginger, green tea or both showed a significant increase in HDL-C compared with obese group.

The serum reproductive hormones (FSH, LH and testosterone) results were shown in Table (4). Serum FSH and LH levels exposed a significant increase in G2 compared with the negative control and a significant decrease in groups 3-5 when compared with the positive control (G2) and return to normal levels in G5.On opposing, serum testosterone level showed a significant decrease in rats fed HFD alone (G2), HFD plus ginger and HFD plus green tea when compared with the normal rats as illustrated in Table (4). Groups (3 and 4) publicized a significant rise in serum testosterone level when compared with G2 and the highest values were reported in G3. Moreover, rats fed HFD diet and treated with both ginger and green tea showed insignificant statistical changes, in comparison with normal control and significant increase compared with groups 2-4.

Regarding semen examination in the present study, Table (5) revealed a significant decrease in semen motility and normality in groups 2-5, in comparison with the negative control group. On the other hand, treatment of obese rats (G3-5) showed a significant increase in the aforementioned parameters compared with obese rats. Sperm count showed similar results except for extracts combination which showed insignificant changes compared with the normal group and significant increase when compared with groups 2-4.

Table 2: The initial body weight, final body weight and Lee index in rats received high fat diet (HFD), ginger and or green tea

Parameters / Groups	Initial body weight (g)	Length (cm)	Final body weight (g)	Lee index
Negative control (G1)	181.00±0.96	24.00±0.03	221.00±6.37^{e}	0.24±0.003^{d}
Obesity (G2)	181.00±0.97	23.84±0.02	530.00±5.29^{a}	0.33+0.008^{a}
Obesity+Ginger (G3)	182.00±1.02	23.92±0.08	295.00±9.11^{c}	0.27±0.004^{c}
Obesity+Green tea (G4)	181.00±0.92	23.92±0.04	400.00±2.7^{b}	0.30±0.005^{b}
Obesity+Ginger+Green tea (G5)	181.00±1.50	24.00±0.06	238.00±4.42^{d}	0.25±0.002^{d}
P value	0.431	0.522	0.000	0.000

Means values ± S.E. within the same column having different letters were significantly different at $P \leq 0.05$.

Table 3: Lipid profile in rats received high fat diet (HFD), ginger and or green tea

Parameters / Groups	Cholesterol mg/dl	Triacylglycerols mg/dl	HDL-C mg/dl	LDL-C mg/dl	VLDL-C mg/dl
Negative control (G1)	152.00±1.77^{d}	127.00±1.09^{d}	42.60±1.02^{a}	87.20±1.98^{c}	25.40±0.10^{c}
Obesity (G2)	251.00±2.57^{a}	201.00±2.15^{a}	21.40±0.50^{d}	187.8±3.86^{a}	40.21±0.12^{a}
Obesity+Ginger (G3)	187.00±2.79^{b}	154.00±1.82^{b}	36.40±0.81^{c}	119.6±2.31^{b}	30.80±0.13^{b}
Obesity+Green tea (G4)	191.00±1.06^{b}	154.00±1.15^{b}	35.20±0.58^{c}	124.4±1.16^{b}	30.80±0.10^{b}
Obesity+Ginger+Green tea (G5)	159.00±1.74^{c}	139.00±0.81^{c}	39.60±0.50^{b}	92.00±2.12^{c}	27.80±0.2^{c}
P value	0.000	0.000	0.000	0.000	0.000

Means values ± S.E. within the same column having different letters are significantly different at $P \leq 0.05$.
HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, VLDL-C: very low density lipoprotein cholesterol.

Table 4: Hormonal changes in rats received high fat diet (HFD), ginger and or green tea

Parameters / Groups	Leptin (ng/ml)	FSH (mIu/ml)	LH (mIu/ml)	Testosterone (ng/ml)
Negative control (G1)	4.48±0.10^{e}	0.36±0.005^{d}	0.15±0.003^{d}	1.65±0.02^{a}
Obesity (G2)	7.74±0.05^{a}	0.74±0.128^{a}	0.30±0.006^{a}	0.84±0.02^{d}
Obesity+Ginger (G3)	5.66±0.09^{c}	0.52±0.012^{c}	0.19±0.005^{c}	1.32±0.14^{b}
Obesity+Green tea (G4)	6.20±0.10^{b}	0.63±0.007^{b}	0.23±0.003^{b}	1.24+0.14^{c}
Obesity+Ginger+Green tea (G5)	4.92±0.21^{d}	0.37±0.008^{d}	0.16±0.005^{d}	1.62±0.18^{a}
P value	0.000	0.000	0.000	0.000

Means values ± S.E. within the same column having different letters are significantly different at $P \leq 0.05$.
FSH: follicular stimulating hormone, LH: luetinzing hormone.

Table 5: Sperm analysis in rats received high fat diet (HFD), ginger and or green tea

Parameters / Groups	Sperm count (million/epididymes)	Sperm motility %	Sperm normality %
Negative control (G1)	74.20±2.98^{a}	82.60±1.43^{a}	75.40±1.12^{a}
Obesity (G2)	44.20±2.08^{c}	64.00±1.18^{e}	53.80±1.42^{d}
Obesity+Ginger (G3)	65.20±0.54^{b}	75.60+0.81^{c}	67.00±1.51^{b}
Obesity+Green tea (G4)	60.40±3.65^{b}	70.40±0.50^{d}	61.60±1.07^{c}
Obesity+Ginger+Green tea (G5)	72.60±1.36^{a}	79.00±0.83^{b}	70.20±0.86^{b}
P value	000	000	000

Means values ± S.E. within the same column having different letters are significantly different at $P \leq 0.05$.

Discussion

Obesity is a complex disease gives rise to the interaction of dietary, a myriad of genetic, environmental factors and lifestyle which make a positive energy balance and lead to augmented mass of body fat (39). A highly significant increase was observed in the body weight and lee index of rats received HFD when compared with the normal control group. Our results nearly parallel to those previously obtained (40). This rise in body weight and Lee index may be attributed to the high caloric diet (contain 60% fat) resulting in fat accumulation in adipose tissue. Not only body mass high energy calorie from overeating; it also promote capacity of stomach which stimulate the gastric mechanoreceptors that adjust signals for eating greedily (41).

Obese rats treated with ginger and/ or green tea extracts showed a significant decline in body weight and lee index compared with obese rats as they stimulate thermogenesis. Also, ginger washes the body by minimize the acidity of stomach and cleaning the digestive tract from food logged in it (42). The obtained results agree with the findings of other researchers (39,40,43). The thermogenic possession of green tea was attributed to its high fulfilled of caffeine and catechin polyphenols (44).

Obese rats treated with ginger and green tea mixture disclosed insignificant changes in lee index compared with the normal control and an improvement in the body weight results compared with high calorie rats and other treated groups. In our opinion, this may be owing to the synergistic effect of both herbal plants.

Regarding to the lipid profile, obese rats revealed a highly significant rise of cholesterol, TAGs, LDL-C and VLDL-C with a highly significant reduction in HDL-C level compared with the negative control. These findings were similar to those of other authors (43,45,46). The obesity is the common source of dyslipidemia as lipid oversupply in a status of obesity results in excess availability of non esterified fatty acid and, in turn, elevated TAGs accumulates in non-adipose tissues, e.g. the liver, muscle and pancreas (5). Ginger and green tea administered to obese rats improved the previous results towards the normal control levels and agree with previous findings (47-49). It accomplished that the hypocholesterolemic effect of ginger or green tea could have probably outcome from the restrain of cellular cholesterol biosynthesis after extract consumption (50). Furthermore, it

was reported that the decrease of cellular cholesterol biosynthesis is closely correlated with increased activity of the LDL receptor, which enhancing the removal of LDL from blood, resulting in a reduction in the serum or plasma cholesterol concentration (51). Administration of combination of ginger and green tea extracts to obese rats improved lipid profile results than individual administration. There is also evidence that polyphenol contents in the tea enhance fecal cholesterol and bile acid excretion (52). Moreover, the hypocholestrolemic effect of ginger may be due to the presence of some chemical constituents in ginger, which inhibit the absorption of dietary fat by inhibiting its hydrolysis, it also stimulate the activity of hepatic enzyme cholesterol-7-alpha hydroxylase, which in turn stimulates the conversion of cholesterol to bile acids, an important pathway of elimination of cholesterol from the body (53).

Studying serum leptin levels in obese animals revealed that there is a highly significant rise compared with the normal rats. Leptin is released as a response to obesity to correct the problem in which leptin is manufactured by white adipose tissue to signal fat accumulates reserves in the body and regulates energy expenditure and food intake (45). However, treatment of rats with obesity showed an improvement in the leptin level in comparison with the obese group. These results nearly analogous to those previously conveyed (47,54). In contrast another study showed no difference in leptin level (55). In our opinion, the cause of decrease in serum leptin was attributed to the beneficial effect of ginger and green tea which represented by decreased body weight and fat store. However, the improvement in rats given the combination of extracts is more than other groups which returned to normal levels. This normalization in serum leptin or lipid profile may be due to the effect that comes from the usage of the green tea and ginger extracts which leads to a lowering effect of free radicals on the cell membrane of cells (49).

Concerning the analysis of reproductive hormones in this study, rats fed with HFD revealed a highly significant diminution in serum testosterone. These results were in harmony with others (56,57). Moreover, obesity that associated with a significant reduction in the levels of testosterone and increased estrogen levels as excess fat indeed causes the male hormone, testosterone, to be transformed into estrogen which decreases testicle stimulation (58). While, ginger or green tea treated rats showed an improvement in testosterone levels compared with obese rats. These results were in groups (2-4) coordination with other findings (59,60). The improvements confirmed the effect of ginger and green tea was very clear in obese animals given the two extracts combination that showed insignificant changes in testosterone compared with the normal control group. However, it has been explained earlier that green tea extract polyphenols has inhibitory effect on leydig cell testosterone production probably through cell signaling pathway, P-450 side chain cleavage and the function of 17β HSD (61).

Rats fed HFD alone revealed a significant upsurge in the serum LH and FSH levels compared with the normal rats. Related findings were previously stated (62). While, ginger or green tea -treated obese rats showed a fall in serum levels of FSH and LH when compared with obese animals. Similar results were previously reported (63). The obtained results in were attributed to the compensatory mechanism as a response to the increased testosterone level by feedback.

A major observation of the current investigation is sperm analysis, feeding rats with HFD revealed a significant decrease in sperm count, motility and normality compared with the normal rats. These results were in line with others (62,64). It has been suggested that hypercholesterolemia causes testicular and reproductive damage by excessive generation of free radicals and oxidative stress, which are cytotoxic to spermatozoa (65-67). While obese rats treated with ginger or green tea showed an improvement in the results of sperm count, motility and normality compared with the obese rats. Ginger and green tea are rich in antioxidant components which display sperm antimutagenic activities due to their radical scavenging and their ability in suppression of the reactive

oxygen species (ROS) activity in a dose-dependent manner and to inhibit apoptosis and so improve semen quality in male rats (68). The improvement in sperm evaluation in obese rats treated with mixture of both plants was marked and consequently, these results confirmed the synergistic effect of both herbal plants.

Conclusion

It could be concluded that feeding high fat diet has deleterious effects on the body weight, lipid profile, leptin, reproductive hormones and fertility. Moreover, administration of ginger and green tea has favorable effects on the studied parameters in obesity with rats induced by high fat diet.

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Conflicts of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

PREVALENCE OF ANTIBIOTIC RESISTANT *V. parahaemolyticus* AND *V. cholerae* IN FISH AND HUMANS WITH SPECIAL REFERENCE TO VIRULOTYPING AND GENOTYPING OF *V. parahaemolyticus*

Heba A. Ahmed*, Rowaida S. Abdelazim, Rasha M.A. Gharieb, Rasha M.M. Abou Elez, Maysa A.I. Awadallah

Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, 44511, Sharkia Governorate, Egypt

*Corresponding author, E-mail: heba_ahmed@zu.edu.eg

Abstract: This study aimed to investigate prevalence, virulence determinants, antibiogram and genotyping of *Vibrio* isolates from retail shrimp and tilapia fish as well as stool samples from gastroenteritis patients in Sharkia Governorate, Egypt. *Vibrio* spp were molecularly confirmed in 25.5% and 3% of fish and human stool samples, respectively. *V. parahaemolyticus* was isolated from 8.9%, 5% and 3% of shrimp, tilapia and stool samples, respectively. However, 0.7% of shrimp and 1.7% of tilapia were found to harbor *V. cholera*. *trh* and *tdh* virulence related genes were assessed in 34 *V. parahaemolyticus* isolates (25 from shrimp, 6 from tilapia and 3 from human stool). The *tdh* gene alone was recorded in 4 (16%) isolates from shrimp and 2 (66.7%) isolates from human stool. However, *trh* gene was detected alone in one (4%) isolate from shrimp. Moreover, both genes were detected simultaneously in one shrimp (4%) and one human stool (33.3%). Tilapia fish isolates were negative for both virulence genes. The resistance of the examined isolates were 100% (each of nalidixic acid and erythromycin), 81.6% (sulphamethoxazol), 73.7% (chloramphenicol), However, susceptibilities to gentamicin (81.6%), ciprofloxacin (73.7%) and 71.1% for each ampicillin/sulbactam and amikacin were observed. Multiple drug resistance was recorded in *V. parahaemolyticus* and *V. cholerae* isolates. Out of 38 isolates, 6 (15.8%) were resistant to all 14 antibiotics with MAR index of 1. Twenty of the isolates (52.6%) were resistant to 5-13 drugs with MAR index higher than 0.286. ERIC-PCR fingerprinting revealed five distinct profiles namely E1-E5 and the discriminatory index of the reaction was 0.5107, indicating low discrimination of the technique. In conclusion, this study revealed the contamination of tilapia and shrimp in fish markets with potentially virulent *V. parahaemolyticus* strains in the study area. Moreover, the presence of human and fish isolates in the cluster indicated the potential of the environmental isolates to cause human infection.

Key words: *Vibrio* spp.; prevalence; antimicrobial resistance; genotyping; ERIC-PCR

Introduction

Fish is highly nutritious food and has various health benefits; however, it also threatens human health because it is an important source of foodborne diseases (1). *Vibrio* spp. naturally inhabit both marine and estuarine environments and are considered as one of the main causes of

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gastroenteritis in humans. They can also inhabit fresh water and different studies reported the presence of the pathogen in fresh water fish (2-4). Currently, there are 72 species of *Vibrio*, 12 of them are zoonotic to humans (5). The most pathogenic strains of *Vibrio* spp. are *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* due to their contribution in foodborne illness related to seafood products (6). Vibriosis is attributed to the consumption of raw or insufficiently cooked seafood (7). *V. parahaemolyticus* causes at least 30000 food borne infections per year (8). It causes epidemic and sporadic cases of gastroenteritis after ingesting raw or insufficiently cooked seafood, while, *V. cholerae* causes mainly water borne outbreaks and sometimes sporadic cases of diarrhea following eating of food harboring the organism (9-11). *V. parahaemolyticus* gastro-enteritis could be self-limited but still life threatening because infection may lead to septicemia (12).

Toxigenic serotypes O1 and O139 of *V. cholerae* are frequently incriminated in the majority of epidemics (13). Meanwhile, non-O1/non-O139 strains inhabit water environment and cause diarrhea in sporadic cases due to consumption of contaminated seafood (10). Thermo stable direct hemolysin (TDH) encoded by *tdh* and the TDH-related haemolysin (TRH) encoded by *trh* genes are used for determination of pathogenic strains of *V. parahaemolyticus*. (14,15). Hemolysis of red blood cells and cytotoxicity in the host cells are caused by the *tdh* and *trh* genes.

Antimicrobials are commonly used as an effective therapy for infectious diseases in humans and as therapy and prophylaxis in aquaculture, however, some pathogens developed antimicrobial resistance due to extensive use of those drugs (16). This constitutes risk to humans because resistant bacteria are directly transmitted through food to consumers or due to the transfer of resistance associated genes to other pathogens by portable genetic elements (17,18).

Molecular methods are useful for epidemio-logical aspects such as the identification of genetic relatedness of isolates from diverse sources, tracing the source of infection and studying the host range and geographical distribution of a pathogen (19). Enterobacterial Repetitive Intergenic Consensus - Polymerase Chain Reaction (ERIC-PCR) amplifies a specific sequence in the genome of 126 bp which is restricted to transcribed regions (20). ERIC-PCR has been proven in previous studies as a successful method in genotyping different bacterial pathogens (21-23).

This study aimed to determine the occurrence of *Vibrio* spp. in retail fish samples at Sharkia Governorate, Egypt, and in stool swabs from gastroenteritis patients. The virulence, antibiogram and genotyping of *V. parahaemolyticus* isolates were investigated.

Material and methods

Sampling

A total of 400 fish samples (280 shrimps (*Panaeus semisulcatus*) and 120 tilapia (*Tilapia nilotica*) were collected from different retail markets in Sharkia Governorate, Egypt. Brackish water shrimp samples were collected from the Gulf of Suez, while, tilapia samples originated from Nile River. Stool samples (n=100) from patients attending the outpatient clinics at different hospitals in the same study area were also examined. The samples were collected during the period from July 2017 to April 2018. Approval of the study was obtained from the Animal Welfare and Research Ethics Committee, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Isolation and biochemical identification

Vibrio spp. isolation was conducted according to the recommendation of FDA's Bacteriological Analytical Manual (BAM) (24). For enrichment of *Vibrio* spp. muscles from shrimp or tilapia (10 gm) were transferred to 90 ml of sterile alkaline peptone water (Oxoid CM1028B) having pH 8.6, thoroughly homogenized and then incubated at 35ºC±2ºC for 24-48 h (25). A loopful from the enriched homogenate was streaked onto Thiosulfate Citrate Bile Sucrose (TCBS) agar plates (Oxide CM0333B) and then incubated at 35ºC±2ºC for 24 h. Presumptive green or blue green colonies of *V. parahaemolyticus* and yellow colonies of

V. cholerae (5) were purified and then biochemically identified using Oxidase test, TSI agar test, Ornithine decarboxylase (ODC), L- lysine decarboxylase (LDC), Arginine dehydrolase (ADH), β- galactosidase (ONPG), Indole test and Halotolerance test (25,26).

Molecular identification

DNA extraction from biochemically suspected *V. parahaemolyticus* and *V. cholerae* isolates was performed using the QIAamp DNA Mini kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instruction. Isolates suspected to be *Vibrio* species were molecularly confirmed using primers targeting 663 bp of the 16S rRNA specific for *Vibrio* species: F: 5'- CGG TGA AAT GCG TAG AGA T-3', R: 5'- TTA CTA GCG ATT CCG AGT TC-3' (5). Then, PCR targeting 368 bp of *tox*R gene specific for *V. parahaemolyticus* using the primers F: 5'- GTC TTC TGA CGC AAT CGT TG -3', R: 5'- ATA CGA GTG GTT GCT GTC ATG-3' (27) and 304 bp of the *omp*W gene specific for *V. cholerae* with the primers F: 5'- CAC CAA GAA GGT GAC TTT ATT GTG-3', R: GGT TTG TCG AAT TAG CTT CAC C-3') (28).

PCR master mix (25 µl) consisted of 12.5 µl of 2X Dream Taq Green mastermix kit, 5.5 µl PCR grade water, 1 µl of both forward and reverse primers (20 pmol, each), 5µl Template DNA were used. The used primers were supplied from Metabion (Germany). The amplification conditions were; 5 min of primary denaturation at 94ºC, 35 cycles of secondary denaturation for 30 sec at 94ºC, annealing (16S rRNA PCR: 40 sec at 50ºC; toxR PCR: 40 sec at 55ºC; ompW PCR: 40 sec at 59ºC) and extension at 72ºC for 45 sec (16S rRNA and *omp*W PCR) and 50 sec for *tox*R PCR. A final extension was adjusted for 10 min. Positive controls were kindly supplied by the Biotechnology Unit, Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt.

Viruoltyping

The amplification of *trh* (250 bp) and *tdh* (373 bp) virulence genes of molecularly identified *V. parahaemolyticus* isolates was carried out using *trh* (F: 5'- GGC TCA AAA TGG TTA AGC G-3', R: 5'-CAT TTC CGC TCT CAT ATG C-3') and *tdh* (F: 5'- CCA TCT GTC CCT TTT CCT GC -3', R: 5'- CCA AAT ACA TTT TAC TTG G -3') genes specific primers (29). The reaction conditions were 35 cycles of primary denaturation at 94ºC for 5 min, secondary denaturation for 30 sec at 94ºC, annealing for 30 sec at 54ºC, extension at 72ºC for 1.5 min and final extension at 72ºC for 12 min.

Antibiotic susceptibility test

The antibiotic susceptibility of *V. parahaemolyticus* and *V. cholerae* isolates was carried out by Kirby-Bauer disc diffusion method on Mueller Hinton agar. The inhibition zone was measured based on the guidelines of Clinical and Laboratory Standards Institute (CLSI) (30), except for nalidixic acid, ampicillin/salbactam and kanamycin, The *Enterobacteriaceae* interpretation criteria were used. Fourteen antibiotic disks were used and they included; ampicillin (AM, 10 µg), kanamycin (K, 30 µg), nalidixic acid (NA, 30 µg), ciprofloxacin (CP, 5 µg), chloramphenicol (C, 30 µg), amikacin (AK, 30 µg), gentamicin (CN, 10 µg), tetracycline (T, 30 µg), cephalothin (KF, 30 µg), sulfamethoxazole (SXT, 25 µg), cefotaxime (CTX, 30 µg), ampicillin/sulbactam (AS, 20 µg), ceftazidime (CAZ, 10 µg) and erythromycin (E, 15 µg).

E. coli ATCC 25922 was used as a quality control isolate. Multiple antibiotic resistance (MAR) index defined as the ratio of the number of the antibiotics to which *Vibrio* isolates displayed resistance to the total number of antibiotics tested was determined (31). Multidrug resistance (MDR) was defined as resistance of an isolate to at least one agent in three or more antibiotic classes (32).

ERIC-PCR fingerprinting

ERIC-DG111-F 5'-ATG TAA GCT CCT GGG GAT TCA C-3'and ERIC-DG112-R 5'-AAG TAA GTG ACT GGG GTG AGC G-3' primers were used for amplification of repetitive sequences in the chromosomal DNA of *V. parahaemolyticus* isolates using a single amplification profile (20). Based on presence or absence of each band, ERIC-PCR fingerprinting data were presented as a binary code. Dendrogram was constructed by unweighed pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering routine using SPSS, Inc. version 22 (IBM Corp. 2013, Armonk, NY). The Simpson's index of diversity (*D*) was used to measure the discriminatory power of ERIC-PCR as previously described (33). *D* value of more than 0.9 indicates good distinction.

Results

Vibrio spp were molecularly confirmed in 25.5% fish sample and 3% human stool samples (Figure 1A-C). The prevalence rates of *V. parahaemolyticus* versus *V. cholerae* were (8.9% Vs 0.7%) in shrimp samples and (5% Vs 1.7%) in tilapia fish samples. *V. parahaemolyticus* was the only isolated *Vibrio spp.* from 3% of human stool (Table 1).

Table 1: Proportion of *Vibrio* species isolated from shrimp, tilapia and human samples

Samples	Number examined	*Vibrio* spp.*	*V. parahemolyticus***	*V. cholera***
Shrimp	280	78 (27.9%)	25 (8.9%)	2 (0.7%)
Tilapia	120	24 (20%)	6 (5%)	2 (1.7%)
Total	400	102 (25.5%)	31 (7.8%)	4 (1%)
Humans	100	3 (3%)	3 (3%)	0

*The isolates were identified using PCR targeting 16S rRNA specific for *Vibrio* spp.
**The isolates were confirmed by species specific PCR

The *trh* and *tdh* virulence associated genes were molecularly identified in 34 *V. parahaemolyticus* isolates; 25 from shrimp, 6 from tilapia and 3 from human stool (Figure 1D-E). The results revealed the presence of *tdh* gene alone in 4 (16%) isolates from shrimp and 2 (66.7%) isolates from human stool. However, *trh* gene was detected alone in one (4%) isolate from shrimp. Moreover, both genes were noticed simultaneously in one shrimp (4%) and one human stool (33.3%). All isolates from tilapia fish did not harbor any of the investigated virulence genes.

The antibiotic susceptibility testing (Tables 2 and 3) was performed on 38 isolates (34 *V. parahaemolyticus* and 4 *V. cholerae*) against 14 antibiotics. All isolates were found to resist nalidixic acid and erythromycin, while, 81.6% and 73.7% were resistant to sulphamethoxazol and chloramphenicol, respectively. However, susceptibilities to gentamicin (81.6%), ciprofloxacin (73.7%) and ampicillin/ sulbactam and amikacin (71.1%, each) were observed. Multiple drug resistance was recorded in *V. parahaemolyticus* and *V. cholerae* isolates. Out of 38 isolates, 6 (15.8%) were resistant to all 14 antibiotics with MAR index of 1. Twenty of the isolates (52.6%) were resistant to 5-13 drugs with MAR index higher than 0.286 ranging from 0.357-0.928. The average MAR index was 0.678.

Table 2: Results of antibiotic susceptibility tests on *Vibrio* isolates (n=38)

Antibiotic class	Antibiotics	S	I	R
Penicillin	**Ampicillin**	10 (26.3%)	7 (18.4%)	21 (55.2%)
	Ampicillin/Sulbactam	27 (71.1%)	2 (5.3%)	9 (23.7%)
Cephalosporin	**Cefotaxim**	25 (65.8%)	3 (7.9%)	10 (26.3%)
	Ceftazidime	20 (52.6%)	3 (7.9%)	15(39.5%)
	Cephalothine	13 (34.1%)	4 (10.5%)	21 (55.2%)
Aminoglycosides	**Amikacin**	27 (71.1%)	5 (13.1%)	6 (15.8%)
	Gentamicin	31 (81.6%)	1 (2.6%)	6 (15.8%)
	Kanamycin	19 (50%)	4 (10.5%)	15 (39.5%)
Tetracycline	**Tetracycline**	12 (31.6%)	3 (7.9%)	23 (60.5%)
Quinolones	**Nalidixic acid**	0	0	38 (100%)
	Ciprofloxacin	28 (73.7%)	0	10 (26.3%)
Sulfonamide	**Sulphamethoxazol**	5 (13.2%)	2 (5.3%)	31 (81.6%)
Phenolics	**Chloramphenicol**	7 (18.4%)	3 (7.9%)	28 (73.7%)
Macrolide	**Erythromycin**	0	0	38 (100%)

S: Sensitive, I: Intermediate, R: Resistant

Table 3: Antibiotic resistance pattern and MAR index of *Vibrio* spp.

Resistance pattern	Resistance profile	Number of isolates	Number of antibiotics	MAR
I	NA, E, SXT, AM, C, AS, CZ, T, CN, K, CF, G, CP, AK	6*[#]	14	1
II	NA, E, SXT, AM, C, AS, CZ, T, CN, K, CF, G, CP	1	13	0.928
III	NA, E, SXT ,AM ,C,AS ,CZ, T,CN,K ,CF ,G ,AK	1	13	0.928
IV	NA, E, SXT, AM, C, AS, CZ, T, CN, K, CF, G	2	12	0.857
V	NA, E, SXT, AM, C,AS,,CZ, T, CN, K ,CP,AK	1[#]	12	0.857
VI	NA, E, SXT, AM, C, AS, CZ, T, CN, K, CF	2	11	0.786
VII	NA, E, SXT, AM, C, AS, CZ, T, CN,K	2	10	0.714
VIII	NA, E, SXT, AM, C, CZ, T, CN, K, CF	1	10	0.714
IX	NA, E, SXT, AM, C, AS, CZ, T, CN	2[#]	9	0.643
X	NA, E, SXT, AM, C, AS, CZ, T	1	8	0.571
XI	NA, E, SXT, AM, C, CZ, T, CN	1	8	0.571
XII	NA, E, SXT, AM, C, CZ, T	1	7	0.500
XIII	NA, E, SXT, AM, C, AS	2	6	0.428
XIV	NA, E, SXT, AM, C, CN	1	6	0.428
XV	NA, E, SXT, AM, C	1	5	0.357
XVI	NA, E, SXT, C, CN	1	5	0.357
XVII	NA, E, SXT, AM	2	4	0.286
XVII	NA, E, SXT, C	2	4	0.286
XIX	NA, E, SXT,CZ	1[#]	4	0.286
XX	NA,E,SXT	1	3	0.214
XXI	NA ,E	5	2	3.143
XXII	NA	1	1	0.071

Average MAR index= 0.678

* Clinical isolates are included in Pattern I

[#]*V. cholerae* isolates

The genetic similarity of 38 *V. parahaemolyticus* and *V. cholerae* isolates from shrimp, tilapia and human sources was assessed using ERIC-PCR. The amplified fragments ranged between 244 and 1520 bp. A band of 590 bp was commonly present in all *V. parahaemolyticus* and three of *V. cholerae* isolates (Figure 1F). The reaction showed discriminatory index of 0.5107 in typing the isolates. Five profiles namely E1-E5 and two main clusters and one individual isolate were generated at linkage distance 12.5 (Figure 2). Cluster I included three sub-clusters; Ia sub-cluster consisted of *V. parahaemolyticus* isolates from shrimp, tilapia and humans, while, Ib and Ic sub-clusters included *V. parahaemolyticus* from tilapia and shrimp. Three isolates of *V. cholerae* were allocated in one cluster (Cluster II) and one isolate was separately located.

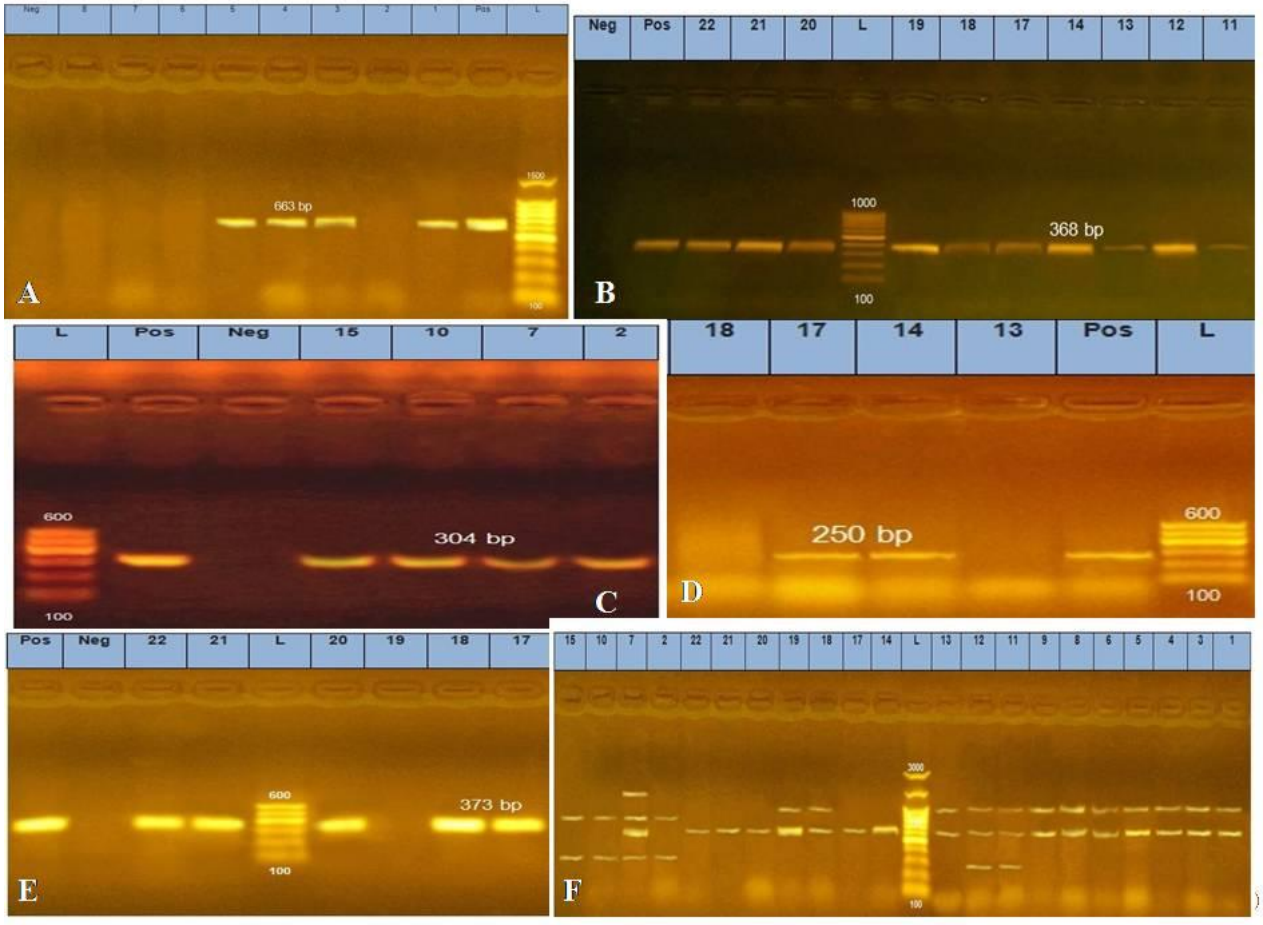

Figure 1: A: 16S rRNA gene amplification for the molecular identification of *Vibrio* isolates from different sources with amplicon size of 663 bp, Ladder: 100 bp. B: *tox*R gene amplification for molecular confirmation of *V. parahaemolyticus* isolates from different sources with amplicon size of 368 bp, Ladder: 100 bp. C: *omp*W gene for the identification of *V. cholerae* isolates with amplicon size of 304 bp, D: *trh* gene in *V. parahaemolyticus* isolates with amplicon size of 250 bp; E: *tdh* gene in *V. parahaemolyticus* isolates with amplicon size of 373 bp. F: ERIC-PCR fingerprinting of *V. parahaemolyticus* and *V. cholerae* isolates, Ladder: 100 bp

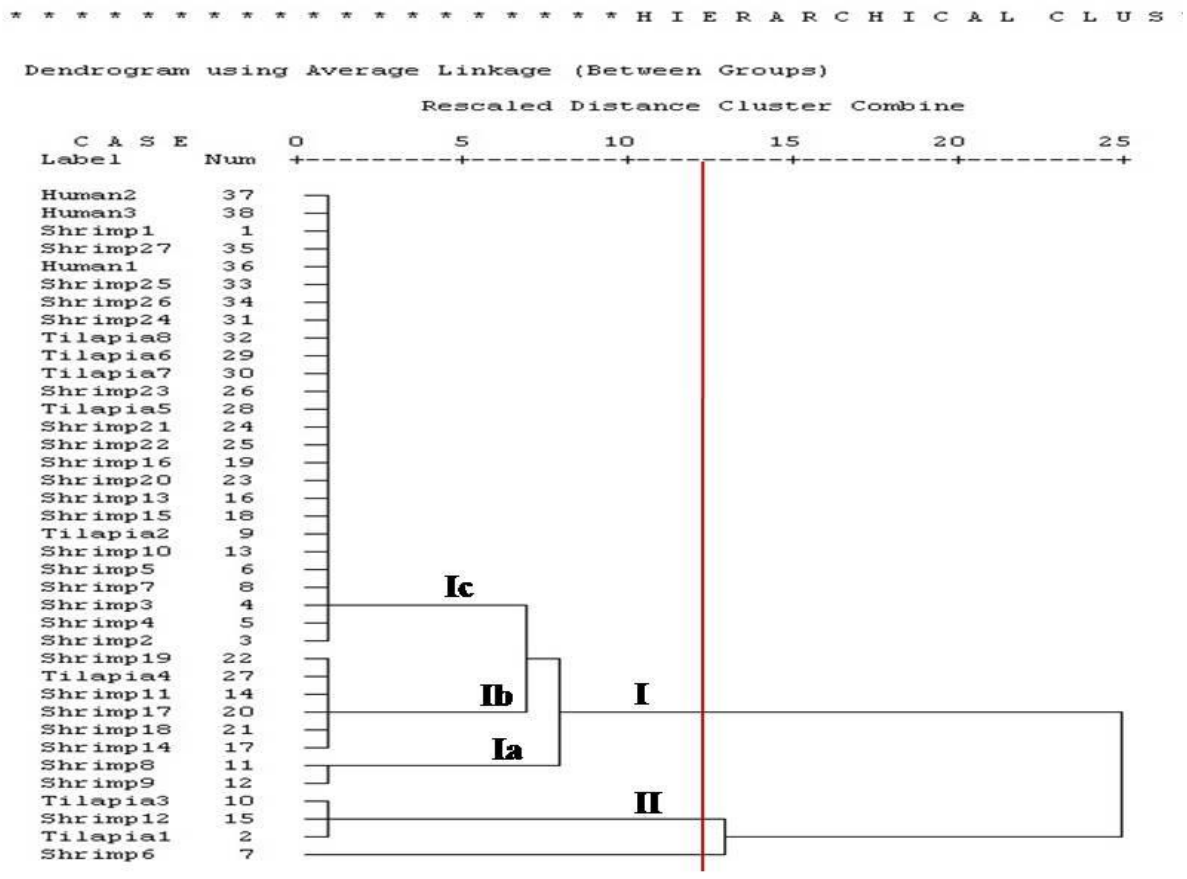

Figure 2: Dendrogram showing the relatedness of *V. parahaemolyticus* and *V. cholerae* isolates from humans and fish sources as determined by ERIC-PCR fingerprinting using the SPSS computer software program

Discussion

Several outbreaks due to ingestion of seafood contaminated with *Vibrio* spp. have been reported worldwide and the source of contamination could be human feces or sewage (34). *V. parahaemolyticus* was isolated in our study from 7.8% of seafood samples (8.9% from shrimp and 5% from tilapia). This was comparable to 8% reported in Netherland (35), 9.3% in Iran (36) and 9.4% in Croatia (37).

Higher isolation rates of *V. parahaemolyticus* from shrimp were reported in different studies; for instance, 32.3% in Senegal (38), 80.8% in Ecuador (39). Moreover, 37.7% (40), 47.9% (41) and 81.7% (8) were reported in China. In Egypt, *V. parahaemolyticus* was isolated from 18.2% of shrimp (42), and 10% from marine water fish (43) in Sharkia Governorate.

V. cholerae was isolated in the current study from 0.7% of shrimp samples compared to 1.5% in Egypt (42) and 2% in Morocco (6). Higher percentages of 9.4% in India (44) and 11.4% in Ecuador (39) were reported for shrimp.

In our study, both species were isolated from freshwater fish samples (tilapia), in accordance, another study in Malaysia reported the isolation of *V. parahaemolyticus* from 24% of freshwater fish samples sold at hypermarkets (3). However, a study in Egypt documented that *Vibrio* spp. other than *V. cholerae* and *V. parahaemolyticus* was recovered from freshwater fish (43).

V. cholerae inhabits some fresh and marine water fish species, and they may spread the bacteria in the aquatic environment and may transmit it to water birds consuming them (45). Hence, fish are considered as a reservoir for *V.*

cholerae and may be responsible for its global distribution. In Burkina Faso, *V. cholerae* were isolated from 6.3% of Tilapia samples (46).

The isolation of *V. parahaemolyticus* from fresh water fish could be due to cross-contamination from marine water fish and ice used for preservation during retail of fish in markets (3). Moreover, improper handling and insufficient hygienic measures in fish markets are also considered risk factors for the contamination of different fish species with *Vibrio* (3). It has been also found that *Vibrio* spp. can survive and grow in freshwater environment as well as in brackish and marine water (47).

The source of samples, methods of identification, study area, season, salinity and temperature during storage or even transportation may influence the variation of *Vibrio* prevalence in seafood (11,48).

Isolation of *V. parahaemolyticus* from 3% of human stool samples coincides with a study in the same geographic area (42). Moreover, isolation of *V. parahaemolyticus* from gastroenteritis patients associated with seafood consumption was previously reported (49,50).

V. parahaemolyticus strains that harbor the *tdh* and/or *trh* genes have a potential of pathogenicity. A proportion of 0.2-3% of *V. parahaemolyticus* strains from environmental sources are considered pathogenic according to studies conducted in different regions (51). In our study, 22.6% of seafood samples harbored *tdh* and/or *trh* genes. In Egypt, 8.3% (42) and 14.3% of *V. parahaemolyticus* from shrimp harbored the *tdh* and/or *trh* genes (48). While, in China, 45.9% isolates were positive for only the *trh* gene (8). The three isolates from clinical cases in our study were positive for the *trh* and *tdh* genes. This was consistent with another study in Egypt in the same study area (42). In China, all the clinical *V. parahaemolyticus* strains were found to harbor the *tdh* gene, and only 77.4% harbored the *trh* gene (41). Isolates lacking hemolysin genes were reported to be pathogenic, thus indicating the presence of other pathogenicity determinants (52).

Antimicrobial resistant bacteria are widely spread due to continuous and extensive use of antibiotics. This is of major concerns in both human and animal health as it reflects the pattern of drug use (8, 53, 54). The bacteria acquired resistance to antibiotics following frequent exposure to antibiotics over time. Resistance is acquired through transfer of horizontal gene mobile genetic elements (55).

In this study we used antibiotics which are mainly used in the treatment of Vibrio infection. High resistance to erythromycin (100%) indicates public health concerns because this drug is used in children treatment (56). The resistance rates were also high to some antibiotics such as sulphamethoxazol, chloramphenicol and tetracycline. This coincides with studies reported in Egypt (42), China (16) and India (9).

Twenty-six of the isolates (68.4%) resisted 5-14 drugs with MAR index more than 0.286 ranging from 0.357-1 with an average MAR of 0.678. The MAR indices of *V. parahaemolyticus* isolates more than 0.2 have been previously reported (42,57,58), thus indicating contamination from high-risk sources including animals and humans causing high risk to fish consumers (15). Hence, antimicrobial resistance testing is important to assess the efficacy of new antibiotics and to guarantee seafood safety. The difference in the MAR indices might be contributed to samples' origin and source as well as the testing methods (59-61).

Different epidemiological studies revealed genetic similarity between clinical and environmental *V. parahaemolyticus* isolates (62,63). ERIC-PCR has been used for typing of *Vibrio* spp. into the species level (63,64).

Our results indicated low discrimination of ERIC-PCR in determining the genetic relationship of *V. parahaemolyticus* and *V. cholerae* isolates. In contrary, ERIC-PCR has been reported as a useful technique for evaluating the genetic relationship of *V. parahaemolyticus* isolates (63). Twenty-seven patterns were reported by ERIC-PCR genotyping of *V. parahaemolyticus* isolates, 12-25 bands with 160-1690 bp size range were discriminated and cluster analysis illustrated close relationship between *V. parahaemolyticus* isolates and discrimination index of 0.98 (23). Bands of sizes 270-, 520-, 660-, and 950-bp

bands were detected in all *V. parahaemolyticus* isolates. Another study in Philippine, showed that ERIC-PCR fingerprints of *V. parahaemolyticus* strains included 6 to 8 amplification bands with a molecular weight ranging from 50-2500 bp, and a shared band of 500 bp was observed in all strains (21). Three clusters were generated and most of the strains were found to be genetically unrelated. The high discriminatory power of ERIC-PCR allowed the reaction to be used for tracing the spread of the strains (21). Moreover, the presence of a common band in all the strains can be used in the production of a diagnostic genetic assay.

Human isolates revealed 100% similarity with environmental isolates from shrimp and tilapia, suggests that environmental strains are the causative agents of clinical cases. This was consistent with the findings of another study (65).

ERIC-PCR genotyping of *V. cholerae* revealed genetic diversity and 31 diverse fragments of DNA extending from 250 to 8000 bp were amplified and the discriminatory index was 0.72 (22). Another study showed also diversity among clinical and environmental *V. cholerae* isolates and produced 16 clusters, in 6 clusters, 14 environmental isolates clustered with eight clinical isolates (65). The heterogeneity of *V. cholerae* isolates was observed in other studies (66,67).

Conclusion

The current study revealed the contamination of shrimp and tilapia in Zagazig fish markets are contaminated with *V. parahaemolyticus* and *V. cholerae* isolates. A proportion of *V. parahaemolyticus* isolates are highly pathogenic and show multiple drug resistance, thus posing risk to human consumers. ERIC-PCR has been shown to be of low discriminatory power, therefore, other molecular techniques should be investigated for determining the genetic relatedness of *Vibrio* isolates. The high similarity of human and environmental isolates and their presence in the same cluster indicated the potential of the environmental isolates to cause human infection.

Conflict of interest

The authors declare no conflict of interest.

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Original Research Article

ABSENCE OR PRESENCE OF *TAPETUM LUCIDUM*: MACRO AND MICROSCOPIC INVESTIGATIONS IN DONKEY (*EQUUS ASINUS*), CAT (*FELIS DOMESTICA*) AND ONE-HUMPED CAMEL (*CAMELUS DROMEDARIUS*)

Hanaa M. El-Ghazali*, Eman A.A. Mahdy

Anatomy and Embryology Department, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt

*Corresponding author, E-mail: drh1980@hotmail.com

Abstract: For explanation eye-shine phenomenon, we used both eyes of five healthy adult donkeys (*Equus asinus*), cats (*Felis domestica*) and one-humped camels (*Camelus dromedarius*). The eyes of a live animal of the three species were photographed under existing light and with a flash. The donkey's pupils appeared black centrally situated and horizontal in direction at daylight. Under flash, the light condensed centrally and the color changed to green or blue**.** The cat's pupils were oval vertically slit-like and colored yellow to red orange under flash. The camel's pupils characterized by presence of a small centrally spot of light with flash. *Tapetum* of the donkey was horizontal triangular in shape under the weak light colored indigo with dark blue spots changed into semicircular appearance under strong light. In the cat, *Tapetum* appeared semicircular in outline with yellow color under weak and strong light. The fundus of camel appeared divided into dark proximal half and lighter distal one under the weak light. Under flash, the two halves appeared transparent white. Microscopically, *Tapetum lucidum* of the donkey was fibrous in its texture while in cat, it was cellular. In camel, there was a brush's membrane and no *Tapetum lucidum*. In donkey, the thickness of the tapetal tissue and the degree of pigmentation in the retinal epithelium differed according to the region of *Tapetum*. The thick tapetal tissue and the unpigmented retinal epithelium combination created the greater reflectance of light. So the absence or presence of *Tapetum*, the tapetal tissue thickness, the degree of pigmentation in the retinal epithelium and the degree of illumination controlled the eye-shine phenomenon.

Key words: eye-shine; *Tapetum fibrosum*; *Tapetum cellulosum*; brush's membrane; choriocapillaries

Introduction

Eye-shine is phenomenon noticed in a wide range of animal species and is attributed to the presence of the *Tapetum lucidum* (1,2). The vision in a low illumination is getting better by the occurrence of an intraocular light- reflective structure; *Tapetum lucidum*, which giving the photoreceptors another possibility for absorbing the reflected beam (3-5). The high sensitivity to the reflected light from the ground depends on the position of *Tapetum lucidum* (6).

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Regarding the site of the reflective materials, *Tapeta lucida* can be categorized into retinal or choroidal tapetum. Retinal tapeta lucida are familiar in fish and some reptiles, while in mammals are seldom occur (1,7,8). The choroidal mammalian tapetum is present inbetween the thin choriocapillary lamina underneath the pigmented layer of the retina and the choroid (1). The choroidal tapetum is classified into fibrous in the horse, cow, sheep, elephant, sirenians and cetaceans and cellular one in carnivores (2,3).

The visual acuity of the horse is good, about half that of humans and double that of domestic cats (9,10) however, that of dromedary camel is higher than cats and rabbits, less than sheep, giraffes, rhesus monkeys and horse, and almost resemble African elephant and buffalos. The eye of the camel has no choroidal tapetum (11).

Tapetum lucidum is previously studied in various animal species but only a few very short reports describing the fibrous tapetum of the horse (3,12) and there is no researches are related to the donkey. So, the main objective of this study is to compare three species, one has no *Tapetum* (camel) and two have different types of it (donkey and cat), describing their macroscopical shapes after exposure to different levels of light, the organization of the tapetal tissue, and the degree of pigmentation of the retinal epithelium along the ocular fundus.

Material and methods

In this study, we used both eyes of five healthy adult donkeys (*Equus asinus*), cats (*Felis domestica*) and one-humped camels (*Camelus dromedarius*) of both sexes and variable ages. The eyes of one-humped camel were obtained from the slaughterhouse of Belbas; the donkeys were collected from various sites all over Sharkia Governorate, while the cats were bought from Pet Shop in Zagazig city, Sharkia Governorate, Egypt.

Anatomical examination

The eyes of a live animal of the three species were photographed using a digital camera with resolution (16.1 megapixels, Sony DSC-W690, 36v and 10x optical zoom) under existing light and with a flash. The donkeys were sedated with Xylazine[2] Hcl at 0.5 mg/kg then narcotized with chloral hydrate[3] 10% at 5 mg/ 50kg i.v while cats were sedated and anesthetized with 1 mg/kg of i.m. xylazine then followed by 5 mg/kg of i.m. ketamine (13). The exsanguinations of donkeys and cats were made through the common carotid artery. Animals were handled in this work following the guidelines of the Institutional Animal Care and the Research Ethics Committee of the Zagazig University, Egypt.

For fixed eyes; both eyes of three animals of the three species were used for gross morphological examination. The eyes were enucleated then were preserved in a mixture of 10% formalin, 3% glycerine and 1% thymol fixative solution to be cut carefully when the eyeball became sufficiently tough without deformation. The eyes were cut at the equator and the vitreous body was removed. The ocular fundus of the posterior half of the eyecup was photographed under ordinary light and with a flash. After removal of the retina, the fundus was rephotographed. The anatomical nomenclature used was based on Nomina Anatomica Veterinaria (14) whenever possible.

Histological examination

The posterior halves of the eyecup of two animals of each species were cut after fixation in 10% neutral buffered formalin for 24 hours into small pieces according to the color, the region of the fundus and the relation to the optic disc as the follow (Figure 1):

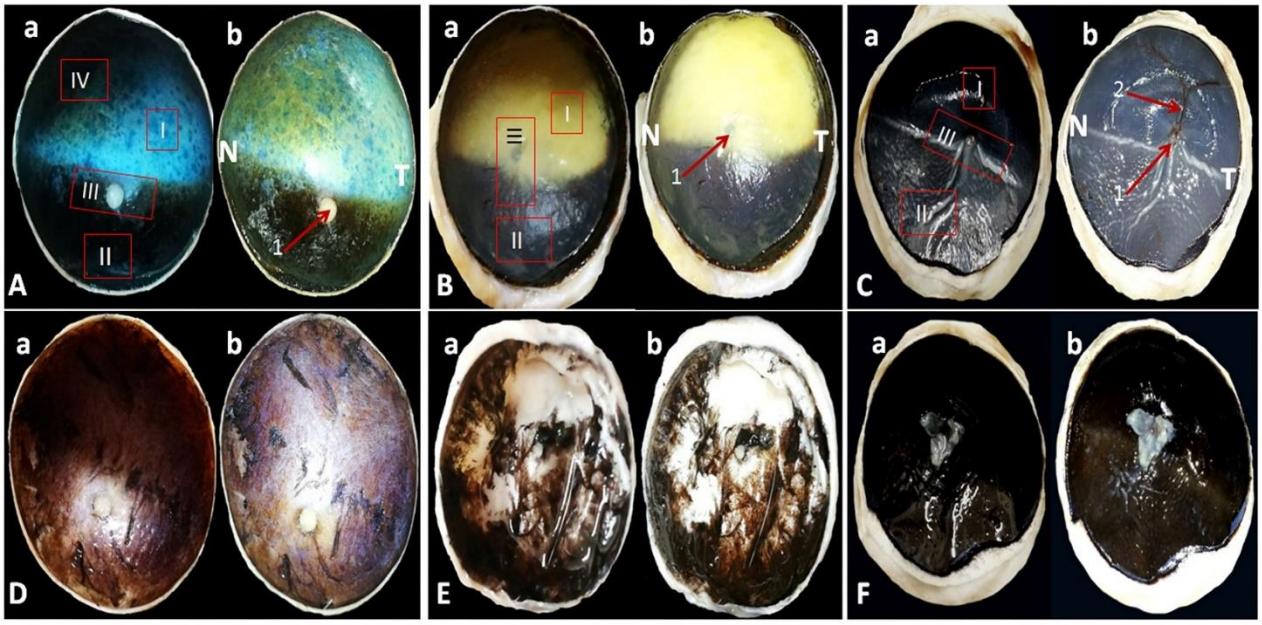

Figure 1: Photomacrographs of equatorial sections of ocular fundus with nasal (N) and temporal (T) ends of donkey without flash (Aa) and with flash (Ab), cat without flash (Ba) and with flash (Bb) camel without flash (Ca) and with flash (Cb) showing the different sites of cutting section for histological examination, colour and shape of Tapetum Lucidum, equatorial sections of ocular fundus after removal of retina of donkey without flash (Da) and with flash (Db), cat without flash (Ea) and with flash (Eb) camel without flash (Fa) and with flash (Fb) showing the differences in colour of the fundus with changes in the degree of illumination

I. Above the optic disc, the indigo colored area with dark blue spots, the yellow area and the dark area in donkey, cat and camel respectively.

II. Under the optic disc, the black area in donkey and cat and the light area in camel.

III. The junction between I and II contained the optic disc.

IV. Above the optic disc, the black area in donkey (changed into green with flash).

The specimens were dehydrated in ascending grades of alcohols, cleared in three changes of Xylol and embedded in paraffin wax for obtaining sections of five microns thickness. The sections were mounted and stained with the following stains: Hematoxylin and Eosin (H&E) stain for general histological demonstration, Crossman's trichrome stain for collagen and muscle fibers and Orcein stain for elastic fibers (15).

Results

Anatomical findings

In daylight, the donkey's pupils appeared black, centrally situated and horizontal in the direction in the brown iris (Figure 2A). Under flash, the light condensed centrally and the color changed into green or blue according to the direction of the light (Figure 2B). Moreover, the cat's pupils were oval vertically slit-like (Figure 2C) and colored yellow to red orange under flash (Figure 2D). The camel's pupils were similar to that of the donkey and the iris was darker in color in daylight (Figure 2E) while under flash there was a small spot of light centrally (Figure 2F).

Figure 2: Photomacrographs of eyes of donkey under day light (A) and with flash (B), eyes of cat under day light (C) and with flash (D) and eye of camel under day light (E) and with flash (F) showing the eye pupil was horizontal in donkey and camel while vertical in cat. The variation of the responsibility to the strong light in the examined species exhibited as green or blue colour in donkey, yellow or orange red in cat and a small light spot in camel

In donkey, *Tapetum* under the weak light was horizontal triangular in shape and was located dorsal to the optic disc with the nasally situated apex (Figure 1A). In the cat, *Tapetum* appeared semicircular in outline (Figure 1B). It was occupied the dorsal half of the fundus and it contained the optic disc in its ventral part (Figure 1B). In camel, the fundus appeared divided into two halves by a line passing below the optic disc (Figure 1C). *Tapetum* was indigo with dark blue spots and yellow in colour in donkey and cat respectively (Figure 1A and B). In donkey, the ventral edge didn't affect by the degree of light, while the dorsal one was indistinct under weak light and was rounded to give *Tapetum* its semicircular appearance under strong light (flash) (Figure 1A). With increasing the illumination degree, the green color appeared in the two third of *Tapetum* toward the nasal end (Figure 1A). In the cat, the ventral edge was horizontal, extended below the optic disc and more curved (bulged) distally at the temporal end (Figure 1B). In camel, under the weak light, the proximal half of the fundus appeared dark and the distal one was lighter. Under flash, the two halves appeared transparent white with large blood vessels extended dorsally and ventrally from the optic disc (Figure 1C). In donkey, the retina was closely attached to the underlying layers. After removing of the retina, the fundus of the eye appeared brown in colour with a rounded white ring around the optic disc by weak light (Figure 1D). While under the strong light it appeared violet and the light concentrated above the optic disc (Figure 1D). While in cat, the retina was easily detached from the underlying layer and the fundus of the eye was dark brown in color (Figure 1E). Also, in camel the retina was easily

detached and had several corrugations (Figure 1C), after the removal of the retina, the underlying layer appeared dark brown in color with a semicircular appearance at the distal half. The dorsal edge of it was horizontal just below the optic disc (Figure 1F).

Histological findings

Tapetum lucidum was located between the choroid layer (Choroidea) externally and retinal epithelium internally in donkey and cat instead, there was a brush's membrane in the same location in camel (Figure 3A, B and C). There was a single layer of large blood vessels between *Tapetum* and the choroid. These vessels send small branches which penetrated *Tapetum* and formed a single layer of capillary network termed the choriocapillaries layer separating *Tapetum* from the retinal epithelium (Figure 3D). In donkey, the tapetal tissue was fibrous in its texture, consisted of collagen fibers with fibroblast cells (Figure 3E and F). The collagen fibers were in the form of parallel bundles (Figure 3F) crossed by the blood vessels to form choriocapillaries layer above its surface (Figure 3D). In the cat, *Tapetum* appeared as brick-like rows of rectangular cells (Figure 3G). The tapetal cells had homogenous cytoplasm and the nucleus was large, oval, well stained centrally located. Two to four nucleoli were seen inside each nucleus. The latter was lightly stained with small dark stain areas scattered throughout the nucleus and inside the nuclear envelope (Figure 3G and H). While in camel, the brush's membrane was a single layer of elastic collagen fibers (Figure 4A). In donkey, the thickness of the tapetal tissue differed according to the region of *Tapetum*. Above the optic disc according to the macroscopic anatomy, *Tapetum* was thick in the region I (Figure 1A) and no pigmentation in the retinal epithelium (Figure 4B). The tapetal thickness decreased in the region (IV) (Figure 1A) and the pigmentation of the retinal epithelium began to appear (Figure 4C). When the tapetal layer abruptly decreased in its thickness in the region II (Figure 1A), the retinal epithelium was highly pigmented (Figure 4D). The nuclear layer of the retina was extended above the optic disc in the area of the junction (region III) (Figures 1A and 4E). In the cat, the tapetal layer appeared thinner at the periphery and above the optic disc (Figure 4F and G), then increased gradually in thickness at the center. *Tapetum* was 10-14 layers of cells in the thickest part. The most external layer of *Tapetum* formed of many cells separated from each other leaving large spaces with few numbers of scattered nuclei (Figures 3G and 4H). The outer dorsal layer (choriocapillary) had flattened nucleus and some cells with an oval nucleus (Figure 5A). The retinal epithelium was unpigmented allover the tapetal layer except for a little extent before the disappearance of the tapetal layer (Figures 4G, H and 5B). The latter was expanded on the optic disc (Figure 4G). In camel, the brush's membrane characterized by rounded dark (heterochromatin) nucleus with one pale nucleolus centrally located (Figures 4A and 5C). In the dark region (I) (Figure 1C) of the fundus, the retinal epithelium was heavily pigmented with few, spaced out and darkly stained melanocytes in the choroid layer (Figure 5D and E). While the light part (region II) (Figure 1C) characterized by few pigmentations in the retinal epithelium with a lot of regular (evenly) distributed melanocytes in the choroid (Figure 5F and G). At the area of the junction and above the optic nerve (*N. opticus*), the nuclear layer of the retina which was present in the donkey was represented by retinal ganglionic layer (Figure 5H).

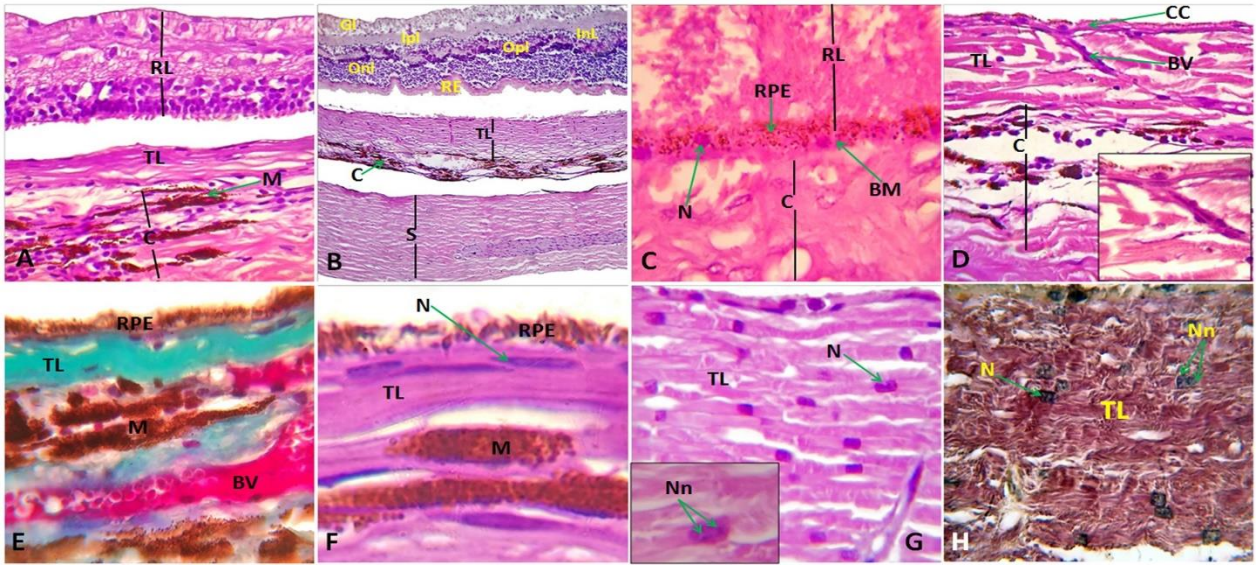

Figure 3: A photomicrograph showing: Retinal Layers (RL), Tapetum Lucidum (TL), Choroid (C), Sclera (S), Retinal Epithelium (RE), Retinal Pigmented Epithelium (RPE), Outer nuclear layer (Onl), Outer plexiform layer (Opl), Inner nuclear layer (Inl), Inner plexiform layer (Ipl), Ganglionic layer (Gl), Brush's Membrane (BM), Melanin granules (M), Blood vessels (Bv), Choriocapillary (CC), Nucleus (N) and Nucleolus (Nn). (A) Donkey and cat (B) Stain: H&E. Obj.x 10: Oc.x 10. (C) Camel Stain: H&E. Obj.x 100: Oc.x 10. (D) Donkey Stain: H&E. Obj.x 40: Oc.x 10 with insert showing higher magnification. (E) Donkey Stain: Crossmon's trichrome Obj.x 100: Oc.x 10. (F) Donkey Stain: H&E. Obj.x 100: Oc.x 10. (G) Cat Stain: H&E. Obj.x 40: Oc.x 10 with insert showing higher magnification of the nucleus. (H) Cat Stain: Crossmon's trichrome Obj.x 100: Oc.x 10

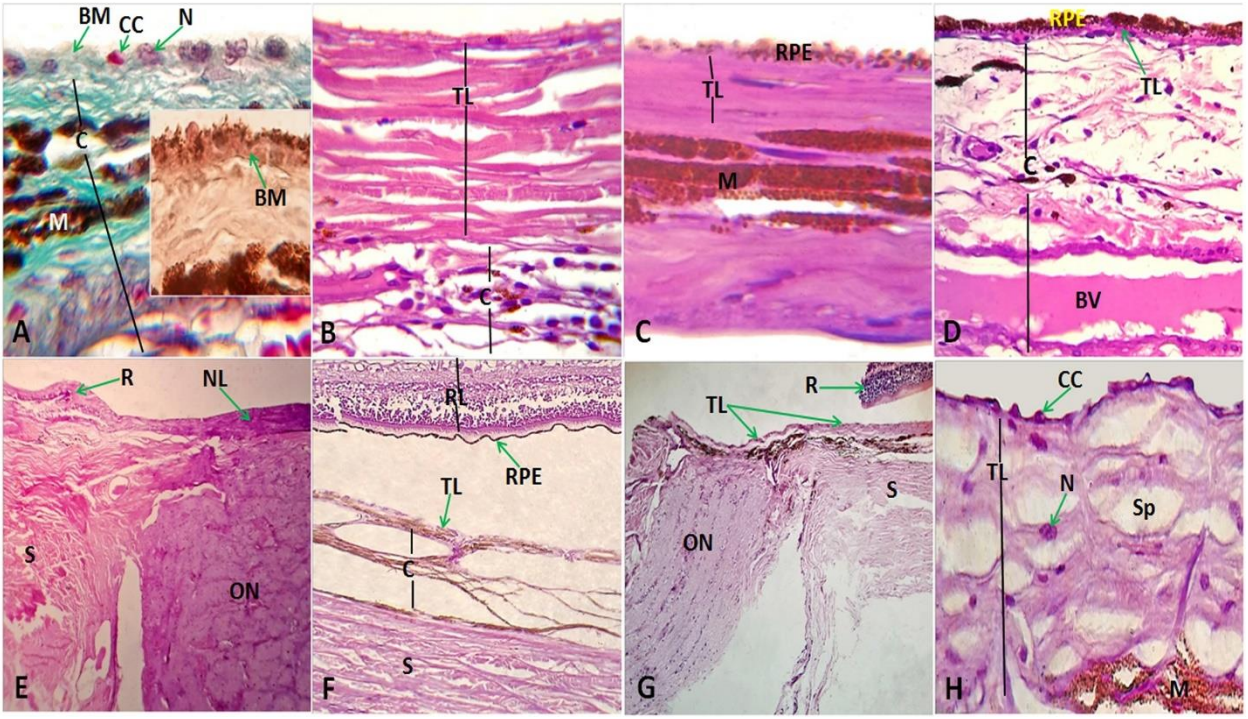

Figure 4: A photomicrograph showing: Retina (R), Tapetum Lucidum (TL), Choroid (C) and Sclera (S) Retinal Layers (RL), Retinal Pigmented Epithelium (RPE), Nuclear Layer (NL), Brush's Membrane (BM), Melanin granules (M), Blood vessels (Bv), Choriocapillary (CC), Nucleus (N), Optic Nerve (ON) and Spaces (Sp). (A) Camel Stain: Crossmon's trichrome with insert showing brush's membrane contained elastic fibers Orcein stain Obj.x 100: Oc.x 10. (B) Donkey Stain: H&E. Obj.x 40: Oc.x 10, (C) Obj.x 100: Oc.x 10, (D) Obj.x 40: Oc.x 10 and (E) Obj.x 4: Oc.x 10. (F) Cat Stain: H&E. Obj.x 10: Oc.x 10, (G) Obj.x 4: Oc.x 10 and (H) Obj.x 100: Oc.x 10

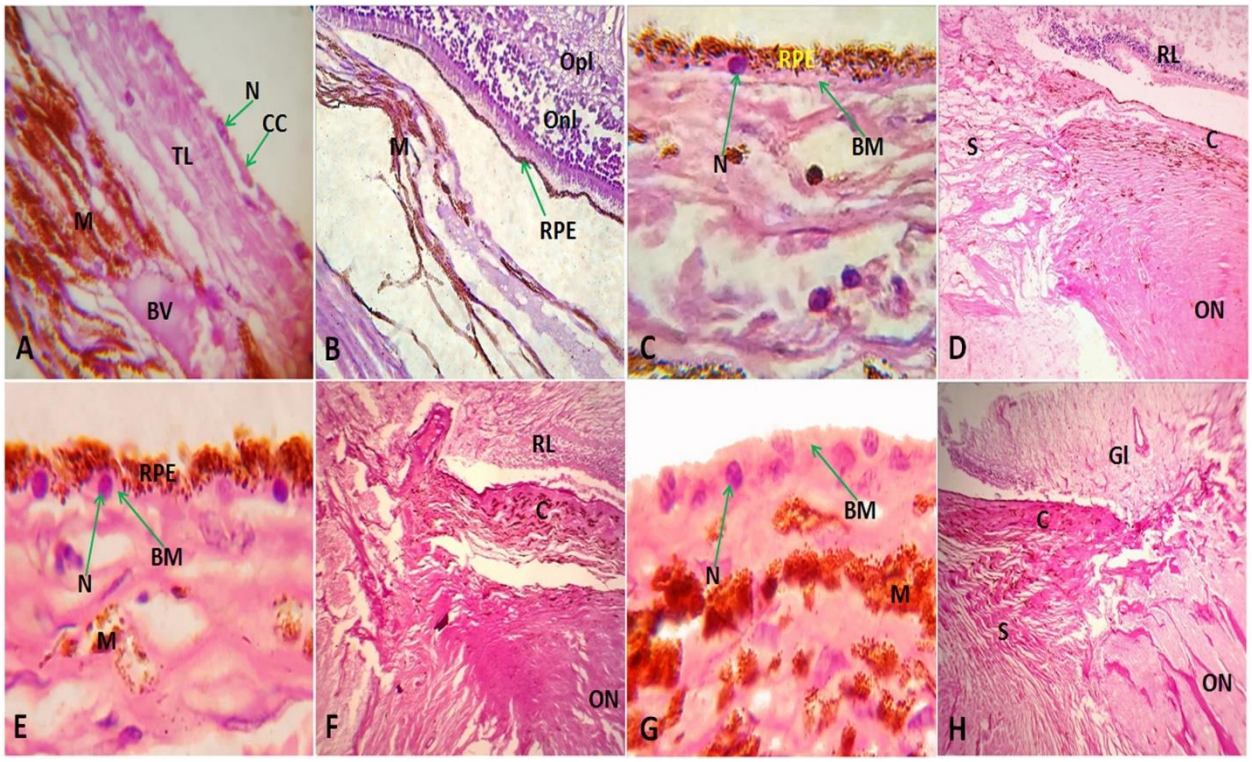

Figure 5: A photomicrograph showing Retinal Layers (RL), Tapetum Lucidum (TL), Choroid (C), Sclera (S), Retinal Pigmented Epithelium (RPE), Outer nuclear layer (Onl), Outer plexiform layer (Opl), Ganglionic layer (Gl), Brush's Membrane (BM), Melanin granules (M), Blood vessels (Bv), Choriocapillary (CC), Nucleus (N) and Optic Nerve (ON). (A) and (B) Cat Stain: H&E. Obj.x 40: Oc.x 10. (C) Camel Stain: H&E. Obj.x 100: Oc.x 10, (D) Obj.x 4: Oc.x 10, (E) Obj.x 100: Oc.x 10, (F) Obj.x 4: Oc.x 10, (G) Obj.x 100: Oc.x 10 and (H) Obj.x 4: Oc.x 10

Discussion

The shape and direction of the eye pupils were differed according to animal species as in donkey and camel, it was elongated and horizontal but in cat, it appeared oval with a vertical direction *Tapetum lucidum* which came in the same line of (16) in the cat.

The colored reflection of the light as green or blue in donkey, yellow to red orange in cat and a small spot of light in camel under strong light were in accordance with the macroscopic findings of the posterior fundus in the present work. Thus, confirmed the presence of *Tapetum lucidum* in donkey and cat and its absence in camel. *Tapetum lucidum* improved the night vision. Humans had poor vision in the night with the comparison to many animals, in part because humans lacked *Tapetum lucidum* (17). The color of *Tapetum lucidum* had been distinguishing in various animal species; yellow in cat, yellow green or green blue in the adult dog, blue green in bovine, sheep and horse (16,18-22).

Regarding the shape and location of *Tapetum lucidum*, in sheep, it appeared as a horizontal strip with lower edge passed through the optic disc while in dog and cat, it was a rounded equilateral triangle with upward apex (18). In our result, *Tapetum* was a horizontal triangular with nasally situated apex by weak light changed into semicircular by flash in the donkey. So, the degree of light affected on the tapetal shape as observed by Shinozaki et al. (20) in sheep and in the horse (21). The tapetal layer in the cat was semicircular and the optic disc was present in its ventral part which disagreed with our result in donkey and Alina et al. (18) who reported that the optic disc of dog was outside the tapetal outlines. On the contrary in the dog (23) clarified that the base of triangular *Tapetum* was in contact with the optic disc.

On the contrary, with our findings in donkey and cat, the posterior fundus of the camel in our

result appeared equally divided with the same color and two different degrees of brightness above and below the optic disc. The clear blood vessels were observed in the fundus of camel more than donkey and cat. In the Sulawesi bear cuscus, which lacked *Tapetum lucidum*, the posterior fundus was dark in color after removal of the retina (24) as our investigations in camel. In our work, the removal of the retina was very easy in cat and camel than in donkey. The dark color of the camel's fundus after retinal removal and the disparity in the degree of coloration after exposure to weak or strong light could be explained as due to the different amounts of melanin granules in the choroid.

The location of *Tapetum lucidum* next to the choroid and separated from the retinal epithelium by choriocapillary layer in donkey and cat in the present work was similar to (3) in cow, sheep, goat and horse (18) in bovine, sheep, dog and cat (22) in Barbary sheep and (25) in sheep and goat.

In the present work, donkey had *Tapetum fibrosum* while in cat, it was *cellulosum* one that came in the same classification manner of (4). While in camel, there was no *Tapetum* on the contrary of the brush's membrane which was well marked the same as Young et al. (26) in the red kangaroo. The presence of the brush's membrane was independent to the presence of *Tapetum*. The choroid consisted of five layers, Suprachoroid layer, Vessel layer, Tapetal layer, Choriocapillary layer and Basal complex or Bruch's membrane (27) in domestic animals, (28) in buffalo, (29) in bovine, (30) in domestic animals and (31) in adult Marwari goat. The histological structure of donkey's *Tapetum* in our study was similar to the general architecture of the fibrous type in bovine and sheep (18), in sheep (20), in horse (21) and in adult Marwari goat (31). In the present work, the cat's *Tapetum* consisted of rectangular cells arranged in layers that were reported to be symmetrical to the microscopic examination of *Tapetum cellulosum* of carnivores (18) and in dogs (19, 23). The brush's membrane of camel had the basic histological structure of this membrane that was reported also by Ramkrishna et al. and Banks (28,32) in some mammals.

Concerning the layers of *Tapetum cellulosum* (18) reported that the tapetal cell layers in the center were 18-20 in dog and 15-20 in cat. These layers also counted by Yamaue et al. (23) in dog and ranged in-between 9 to 12 layers. While in the present study, the layers of cat's *Tapetum* were 10-14 layers of cells in the thickest part. In some areas of *Tapetum* other than the center characterized by an increased thickness that might be due to the spaces between its layers.

The macroscopic appearance of *Tapetum* expressed its functional state and affected by the degree of illumination, pigmentation of the retinal epithelium and the thickness of *Tapetum.* The functional *Tapetum* was noticed macroscopically by weak light. This functional area was microscopically characterized by unpigmented retinal epithelium. Lacking pigmentation in retinal epithelium let light to cross through it then reflected by existed *Tapetum* that was agreed with Previous findings (20) in sheep, (21) in horse and (33) in bovine. Increasing the tapetal thickness was leading to more reflection of light so the horizontal triangle in the posterior fundus of the donkey was the functional part of *Tapetum* which characterized by unpigmented retinal epithelium and the highest thickness. While in cat it was represented by the whole yellow semicircular area of the fundus.

Activity style and the daytime when the animal was conscious and dynamic that decided the quantity of light accessible for vision, considered as a key selective control on the evolution of the vertebrate visual system. Earlier research explained that the functional requirement for vision under photopic and scotopic status selected for divergent eye morphologies in nocturnal, cathemeral and diurnal species because the vertebrate eye under the latter conditions couldn't be optimized for high-quality vision (34, 35). When we analyzed the lifestyle of examined animals with the presence or absence of *Tapetum* and its functional state, we observed that in case of camels as diurnal animals and their eyes were adaptive for improved visual acuity (36), *Tapetum lucidum* was absent. In donkeys that were diurnal animals but active also in other

day times as evening; adapted with human needs; they used the triangular area of *Tapetum* in normal light. Then if the light changed to be strong, *Tapetum* increased in its area and appeared semicircular that agreed with Dwyer (37). He reported that sheep were mainly diurnal but showed some activity as of dawn to evening. According to the shape of its functional tapetal area, sheep got the best mesopic vision in horizontal and antero-inferior visual fields. While the cats usually expressed crepuscular behavior, bordering nocturnal, active at dusk and dawn (38) and their eyes were adaptive for enhanced visual sensitivity (36). They used the whole area of *Tapetum*, which was histologically adapted with their activity pattern and their lifestyle.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

COMBINATION OF GLICLAZIDE DRUG AND LUPIN SEEDS POWDER ALLEVIATE HYPERGLYCEMIA ON INDUCED-DIABETIC RATS RECEIVING HIGH-FAT HIGH FRUCTOSE/SUCROSE DIET

Samah S. Khalil[1]*, Haytham A. Ali[2,3], Hamad A. Al-Saadawy[2], Khalifa El-Dawy[2]

[1]Zagazig University Hospitals, [2]Biochemistry department, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt, [3]Biochemistry department, Faculty of Science, University of Jeddah, Jeddah, Saudi Arabia

*Corresponding author, E-mail: samahsaid75@gmail.com

Abstract: Diabetes mellitus is one of the most prevalent metabolic diseases in the world. Hyperglycemia and lipoprotein abnormalities are the characteristic clinical finding of DM. The most common legume food used in traditional medicine for the treatment of diabetes is lupin seeds powder. The aim of current research is to analyze the therapeutic effects of the combination of both hypoglycemic drug (Gliclazide) and lupin seeds powder on some biochemical parameters (serum blood glucose, serum insulin, glyclated hemoglobin and total lipid profile) and also on mRNA expression level of Glucokinase, Phosphoenolpyruvate carboxykinase (PEPCK), Insulin and Peroxisome Proliferator-Activated Receptor-Gamma (PPAR-γ) genes using relative quantitative PCR. In this study, thirty zucker male rats were divided into two groups: normal control group (six rats) and the other group expose to induce type2 DM by high-fat high fructose/sucrose diet. The diabetic groups were sub classified into 4groups (6 rats in each group), one group diabetic control and the rest was treated with Gliclazide (10mg/kg),combination of Gliclazide(10mg/kg) and lupin seeds powder (1gm/kg) and lupin seeds powder (1gm/kg) only. Combination between Gliclazide (10mg/kg) and lupin seeds powder (1gm/kg) daily for 4 weeks resulted in significant decrease in blood glucose level, glyclated hemoglobin and dyslipidemia (p<0.05), also results in significant increase (P<0.05) in high density lipoprotein-cholesterol(HDL-c) (90.66±3.8), insulin level(4.9±0.058). Also the combination of Gliclazide (10mg/kg) and lupin seeds powder (1gm/kg) tends to return biochemical parameters, lipid profile (triacylglycerol 77±3.05, total cholesterol 132±2) and the transcription of mRNA metabolic genes to normal levels (Glucokinase 1.4±0.1, insulin 1.48±0.25, PEPCK 0.49±0.25, PPAR-γ 3.1±0.2) more than the treatment with Gliclazide drug only, in type 2 diabetic rats. It is concluded that, the combination of Gliclazide hypoglycemic drug and lupin seeds powder has the most hypoglycemic effect when compared to other groups.

Key words: type2 diabetes mellitus; high-fat high fructose/sucrose diet; gliclazide; lupin seeds; total cholesterol

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Introduction

Diabetes Mellitus (DM) is a metabolic disease characterized by the occurrence of chronic hyperglycemia, which affects the metabolism of carbohydrates, lipids, and proteins (1).

In DM, the body can't manage the quantity of sugar (especially glucose) presents in the blood. Glucose is essential to the body in order to perform its vital activity. Carbohydrates convert inside the liver into glucose. The glucose is then secreted into the blood circulation. In the normal condition, a number of hormones control the blood glucose level (2). The main hormone is insulin, which is secreted by the pancreas. Insulin permits glucose to enter from the blood into cells all through the body, where it is utilized for fuel. The cause of diabetes either due to insufficient production of insulin (type 1 diabetes) or inability of proper insulin utilization (type 2 diabetes) (3). In diabetes, blood glucose levels stay high because glucose in the blood can't move effectively into cells. In this case, all cells that need the glucose for fuel suffer from starvation. Moreover, all organs and tissue exposed to destructive effect (4).

Although, there are many pharmaceutical drugs in the markets scientists tends to use medicinal plants to treat diabetes. This is due to the fact that plant medication and natural formulation are often considered to have fewer side effects than manufactured ones (5). The World Health Organization (WHO) confirmed the importance of hypoglycemic drugs originated from plants that are used in traditional medicine. The antihyperglycemic effects of natural plants are due to its ability to renovate the capacity of β-cells of pancreas, expand insulin secretion or reduce glucose absorption. Therefore, curing with medicinal plant drugs are defending β-cells and reducing variation in glucose levels (6). A large portion of the medicinal plants has been found to have antidiabetic impact substances like terpenoids, glycosides, alkaloids, flavonoids. Researchers all over the world will continue to study alternate medications, especially from the plant kingdom, for treating of DM as the disease causes many challenges not only to the doctors but also to the investigators (7).

In traditional medicine, lupin seeds are considered as antidiabetic drugs with antihyperglycaemic activity due to the presence of the protein conglutin gamma (Cγ), a glycoprotein, which has insulin-mimetic activity, allowing it to activate kinases involved in the signaling pathway of insulin, promote glucose transporter-4 (GLUT4) translocation, and contribute to muscle tissue differentiation (8).

Gliclazide is oral antihyperglycemic drugs used for treatment of type2 DM, belongs to a second era sulfonylurea, it acts by increase secretion of insulin from β-cells of pancreas, which is favored due to its rapidly absorbed from gastrointestinal tract (GIT) and lesser liability to cause hypoglycemia and weight gain. Moreover, it has generally cardiovascular protection effects (9).

In the last several years, natural herbal plants had been used for the treatment of DM, but recently scientists discovered new synthetic drugs for the treatment of DM. Advanced researches have been performed to study the effect of both natural herbal plant and antidiabetic synthetic drugs to approach and explore the best result in the treatment of DM. Gliclazide is the most commonly used positive controls in anti-diabetic animal studies. Gliclazide can stimulate insulin secretion by binding to the sulfonylurea binding site and closing the ATP-sensitive potassium channel (10). Other researchers studied the therapeutic effect of combination between lupin seeds and metformin on Type 2DM rats (11).

In the present study, type 2 DM was induced in an animal model using high fat (lard) high fructose / sucrose (HFHF)diet for a period of 12 weeks then the experimental animals were treated with lupin seeds powder and the hypoglycemic synthetic drug, Gliclazide, was used as a positive control.

Material and methods

This experiment was held at Zagazig University Veterinary labs and was approved by the university research committee NO: ZU-IACUC/2/F/24/2018

Animals

Thirty male Zucker rats, with an initial weight of 150+/-50gm, were kept at a suitable temperature of 20-25°C, 12 hours light-dark cycle and free availability of water and diet, they were randomly classified to two groups: normal control (six rats) and diabetic (24 rats).The rats from the normal control group were fed on the standard rat chow and allowed to drink water freely.

Preparation of diabetic rats

Twenty four rats were used for induction of type 2diabetesDM by feeding on HFHF. These rats were given a diet consist of lard (60%) and fructose (17%) added to the standard rat chow while sucrose 10% was added to water supplied to the rats. The experiment lasted for 12 weeks. This model was planned to mimic nearly the cafeteria diet with essentially contain high caloric value (12).

The body weight and blood glucose were taken once weekly for 12 weeks (13). Calculations of the caloric intake of HFHF diet:

Lard contains 8000 Kcal /Kg, Chow contains 3000 Kcal/Kg, and fructose contains 3000 Kcal/Kg.

Animal Grouping

a) Control group fed on normal chow (6 rats)
b) Diabetic groups classified into:
    i. Diabetic control (6 rats) fed on HFHF diet without treatment.
    ii. Diabetic (6 rats) treated with Gliclazide drug 10 mg/kg for four weeks.
    iii. Diabetic (6 rats) treated with Gliclazide drug 10 mg/kg + lupin seeds powder (1g/kg/day) for four weeks.
    iv. Diabetic (6 rats) treated with lupin seeds powder (1g/kg/day) for four weeks.

Experimental design

Preparation of lupin seed powder**:** Dry seeds of lupin of different lots were collected from the local market, mixed and purified from possible impurities and grinded to powder. Lupin powder (1g/kg/day) was given to rats for four weeks, the administration was executed using stomach tube method (feeding needle 18 gauage) (14).

Preparation of Gliclazide powder**:** Gliclazide powder was suspended in 1% (w/v) carboxymethylcellulose (CMC) suspension in normal saline and were given orally to the rats at a dose of 10 mg/kg for 4 weeks, the administration was executed using stomach tube method (feeding needle 18 gauage) (15).

Biochemical assays

After the four weeks of treatment, rats were fasted during the night then blood samples were drawn under phenobarbital 50-100 mg/kg anesthesia intraprotenial injection. The serum levels of glucose, insulin, triacylglycerol, LDL-c, HDL-c, vLDL-c, total lipid and cholesterol was determined as follow:

i. Determination of serum glucose by oxidase method using Spectrum Diagnostics glucose kit (16).
ii. Estimation of serum insulin level using Elisa rat insulin kits method (17).
iii. Ion Exchange Resin method used for quantitative determination of glycated hemoglobin (HbA1c)(18).
iv. The Direct Enzymatic colorimetric Liquid method used for determination of HDL-c, LDL-c (19.
v. Estimation of vLDL-c by Friedewald method (20).
vi. Determination of total lipid by Colorimetric Method (21).
vii. Evaluation of Triacylglycerol by GPO-PAP-enzymatic colorimetric method (22.
viii. Cholesterol liquizyme CHOD-PAP enzymatic colorimetric method used for determination of cholesterol (23).

Tissue samples for relative quantitive PCR (RQ-PCR) Analysis

Immediately after scarifying, livers were collected, weighted and wrapped in aluminum foil then, the tissues were rapidly washed with saline buffer placed immediately in liquid nitrogen container to make snap-freezing of tissue and kept at -80°C until use, for RQ-PCR analysis for the mRNA expression of insulin, Phosphoenolpyruvate carboxykinase (PEPCK),

Peroxisome Proliferator-Activated Receptor-Gamma (PPAR-γ) and glucokinase genes in liver tissue. Total RNA prepared from liver was extracted with easy-RED ™ Total RNA Extraction Kit for animal tissue (Intron Biotechnology cat.No.17063).

Conversion of RNA to complementary DNA (cDNA) was performed using TOPscript™ cDNA Synthesis Kit standard reaction conditions containing 2 µl of 10X TOPscript™ RT Buffer, 1 µl of TOPscript™ Reverse Transcriptase (200 units/µl), dNTP Mixture (2 mM each) 2 µl, Template RNA 1 µl, oligo(dT)18 Primer 1µl, RNase inhibitor (40 units/µl) 0.5 µl, add sterile RNase free water up to 20 µl, then incubated at 42°C (5 mins), 42~60℃ (60 mins), and 95 ℃ (5 mins). The cDNA was stored at -20℃ till analysis. TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Intron Biotechnology cat. No.RT500S) was used for RQ–PCR with standard reaction conditions containing 10 µl of TOPreal™ qPCR 2X PreMIX , template DNA 3 µl, 1.5 µl from forward and reverse primers (5~10 pmol/µl), and sterile RNase free water up to 20 µl. The cycling program was performed in Stratagene Mx3005P qPCR System with the following condition: initial denaturation at 95°C for 15 mins, and 50 cycles (denaturation at 95°C for 15sec, annealing at 60°C for 20sec, elongation at 72°C for 30sec). Table (1) demonstrates primers used for expression of some metabolic genes using RQ-PCR. Primers were synthesized by Bio Basic Inc., Canada. Efficiency of amplification was measured by the slope of a standard curve.

The transcription levels of target genes were normalized to those of GAPDH gene which used as reference gene. The normalized quantity of the target gene was obtained by subtracting cycle threshold for GAPDH from the CT for the target gene (ΔCT sample)(24). The same calculation was performed with controls (ΔCT control). Then ΔΔCT was calculated as the difference of these values (ΔΔCT = ΔCT sample – ΔCT control). Finally, the relative expression was expressed as fold change by $2^{-\Delta\Delta CT}$ relative to control (25).

Table 1: The nucleotide sequences of the PCR primers used to assay gene expression by relative quantitative PCR

Gene	Sequences	Gene bank ID	Annealing Temperature	Reference
PPAR-γ:	F:5′TGTGGACCTCTCTGTGATGG-3′ R:5′-CATTGGGTCAGCTCTTGTG-3'	25664	55°C	(26)
Glucokinase	F:5'-CACCCAACTGCGAAATCACC-3' R:5'CATTTGTGGGGTGTGGAGTC-3'	24385	60°C	(27)
PEPCK	F:5'-AGCCTCGACAGCCTGCCCCAG-3' R:5'CCAGTTGTTGACCAAAGGCTTTT-3'	362282	56°C	(28)
Insulin	F:5'-AGCAAGCAGGTCATTGTTCC-3' R: 5'-TTGCGGGTCCTCCACTTC-3'	24505	65°C	(29)
GAPDH	F:5'-TGCACCACCAACTGCTTAG-3' R: 5'-GATGCAGGGATGATGTTC-3'	24383	55°C	(30)

PEPCK: Phosphoenolpyruvate carboxykinase, PPAR-γ: Peroxisome Proliferator-Activated Receptor-Gamma, GAPDH: Glyceraldhyde3-Phosphate Dehydrogenase

Statistical analysis

Statistical analysis was carried out by SPSS 16 (SPSS, Chicago, Ill) program for windows. Data were expressed as mean ± SEM and was performed using one way ANOVA analysis of variance followed by post hoc test. The criterion for statistical significance was $P < 0.05$.

Results

Regarding body weight (initial and final), in diabetic control group, a significant weight loss was observed compared to normal control animals ($P<0.05$). After treatment with Gliclazide (10 mg/kg) + lupin seeds powder (1g/kg) or Gliclazide(10 mg/kg) alone, the body weight was significantly increased compared to control diabetic animals , but there was no significant change in body weight in

Table 2: The biochemical parameters in in male rats experimental groups

Group	Control	Diabetic Control	Gliclazide	Gliclazide+ Lupin	Lupin	*P* Value
BW	155.33±2.9^{a}	116.33±2.0^{d}	132.67±5.0^{c}	143.33±0.9^{b}	119.0±2.9^{d}	0.00
BG(mg/dl)	73.3±0.88^{d}	339.67±11^{a}	112.67±1.7^{c}	77.30±1.2^{d}	130.0±2.1^{b}	0.00
Serum insulin(µIU/ml)	5.0±0.057^{a}	1.26±0.088^{d}	3.20±0.20^{b}	4.9±0.058^{a}	2.20±0.37^{c}	0.00
HbA1c	4.03±0.38^{d}	13.5±0.59^{a}	6.20±0.12^{c}	3.60±0.15^{d}	7.50±0.37^{b}	0.00
TL (mg/dl)	461.3±16^{d}	1520±34.0^{a}	694.0±38.2^{c}	437.7±9.4^{d}	1056.0±38^{b}	0.00
TC(mg/dl)	125.7±3.7^{d}	314.7±16.7^{a}	173.7±9.2^{c}	132.0±2.0^{d}	232.7±11^{b}	0.00
LDL-c(mg/dl)	79.33±3.3^{d}	325.33±18^{a}	116.3±2.6^{c}	79.0±0.88^{d}	212.3±11^{b}	0.00
HDL-C(mg/dl)	74.0±5.5^{b}	22.67±2.33^{e}	59.0±4.04^{c}	90.66±3.80^{a}	41.0±3.6^{d}	0.00
vLDL-c(mg/dl)	10.5±0.98^{d}	72.6±3.9^{a}	26.67±1.1^{c}	15.40±0.61^{c}	33.4±1.5^{b}	0.00
TAG(mg/dl)	52.7±4.9^{d}	363.0±19.5^{a}	133.3±5.8^{c}	77.0±3.05^{d}	174.3±7^{b}	0.00

Means±SE in the same row and carrying different superscripts are very highly significant different at $p<0.001$.
BW: body weight, BG: blood glucose, HbA1c: glycated hemoglobin TL: total lipid, TC: total cholesterol, LDL-c: low density lipoprotein, HDL-c: high density lipoprotein, vLDL-c: very low density lipoprotein, TAG: triacylglycerol.

group treated with lupin only (Table 2). After induction of diabetes, there was significant elevation in serum glucose, HbA1c, total lipid and cholesterol, LDL-c, vLDL-c and TAG. While, the serum insulin level, HDL-c were significantly decreased in diabetic rats compared to normal control. The results also showed that, treatment with the Gliclazide drug (10 mg/kg) alone or the combination of Gliclazide drug (10 mg/kg) + lupin seeds powder (1g/kg), the blood glucose and HbA1c decreased significantly, while insulin level noticeably increased in treated diabetic groups when compared to diabetic control group. However, adding of lupin powder alone did not induce any change in levels of serum glucose and insulin and HbA1c compared to diabetic control rats (Table2). However, this improvement was more significant in the group treated with the combination of Gliclazide+ lupin seeds compared to those treated by Gliclazide alone. The current data showed that the treatment with the Gliclazide (10 mg/kg) alone and combination of Gliclazide (10 mg/kg) + lupin seeds powder (1 g/kg) significantly decreased total lipid and cholesterol, LDL-c, vLDL-c and TAG when compared to control diabetic rats. However, administration of the Gliclazide (10 mg/kg) alone and combination of Gliclazide (10 mg/kg) + lupin seeds powder (1 g/kg) increased HDL-c. Results also showed no significant change in lupin group compared to diabetic group (Table2). However, this improvement was more significant in the group treated with the combination of Gliclazide+ lupin seeds compared to those treated by Gliclazide alone.

The mRNA expression of glucokinase, Insulin and PPAR-γ genes in HFHF diet diabetic control group livers was significantly down regulated compared to control group. However, the mRNA expression level of PEPCK gene in HFHF diet diabetic group livers were up regulated compared to control group (Table 3).

In this study, the mRNA expression of glucokinase, insulin and PPAR-γ genes in HFHF diet diabetic group livers were significantly up regulated in groups treated with both Gliclazide (10mg/kg) alone and the combination of Gliclazide (10 mg/kg) + lupin seeds powder (1g/kg) compared to diabetic control group. While, the mRNA expression level of PEPCK gene in HFHF diet diabetic group livers were down regulated compared to diabetic control group (Table3). Moreover, the previous changes in the mRNA expressions were more significant in the group treated with the combination of Gliclazide+ lupin seeds compared to those treated by Gliclazide alone ($p<0.05$). Whereas, lupin treated group showed no significant change in mRNA gene expression compared to control diabetic group ($p<0.05$).

Table 3: mRNA expression level of some genes in in male rats experimental groups

Genes	Control	Diabetic control	Gliclazide	Gliclazide +lupin	Lupin	*P* value
Glucokinase	1 ± 0.1^{b}	0.091 ± 0.05^{c}	1.30 ± 0.15^{a}	1.40 ± 0.10^{a}	0.17 ± 0.40^{c}	0.00
PEPCK	1 ± 0.1^{bc}	5.83 ± 1.8^{a}	0.062 ± 0.018^{c}	0.052 ± 0.1^{c}	3.70 ± 1.24^{ab}	0.007
Insulin	1 ± 0.1^{ab}	0.43 ± 0.11^{c}	2.0 ± 0.15^{a}	1.48 ± 0.25^{b}	0.20 ± 0.10^{c}	0.00
PPAR-γ	1 ± 0.1^{b}	0.34 ± 0.5^{c}	2.80 ± 0.30^{a}	3.10 ± 0.20^{a}	0.20 ± 0.05^{c}	0.00

Means ±SE in the same row and carrying different superscripts are significant different at $p<0.05$, very highly significant different at $p<0.001$

PEPCK: Phosphoenolpyruvate carboxykinase, PPAR-γ: Peroxisome Proliferator-Activated Receptor-Gamma

Discussion

The present study demonstrated that administration of HFHF diet caused the development of type2 DM associated with metabolic disorders such as hyperglycemia, glucose intolerance, hyperinsulinaemia, hyper-triglyceridaemia and hypercholesterolemia (31). In the current study, HFHF diet induced the development and progression of the hyperglycemic state by increasing level of blood glucose and glycated hemoglobin due to inadequate secretion of insulin from the pancreas. Moreover, it induced disturbance in total lipid profile such as increased level of total lipid (TL), total cholesterol (32, bad cholesterol (LDL-c), vLDL-c and TAG, and decreased the level of good cholesterol (HDL-c).

This agrees with other researches, which concluded that dyslipidaemia and cardiovascular comorbidities and mortalities are more dominant in patients with Type 2 diabetes (33,34). Importantly, Gliclazide is sulfonylurea derivatives drugs, which is utilized for treatment of type2DM by restraining ATP-dependent potassium chann-els. It is administrated orally and has a modified release form (35).

In the present study, it was observed that administration of Gliclazide drug (10mg/kg) lowered blood glucose, glycated Hb, LDL-c, vLDL-c, TL and TC besides increasing insulin level and HDL-c. Moreover, this was associated with down regulation of mRNA expression level of PEPCK gene, upregulation of mRNA expression glucokinase, insulin, and PPAR-γ genes. An important notice, that Gliclazide caused an only slight increase in body weight.

Weight gain is one of the challenges that face type 2 DM patients due to treatment with oral hypoglycemic drugs as they improve pancreas secretion of insulin and also increase sensitivity of cells towards it (36). According to recent study, scientists proved that treatment with Gliclazide MR cause no significant change in body weight for five years of records, moreover in some trials treatment with Gliclazide MR exhibit reduction in body weight (37). The etiology of weight gain with Gliclazide treatment was not discussed before.

In consistent with the obtained results, other studies concluded that treatment with Gliclazide in type 2 DM improved glycemic parameters, insulin sensitivity and dyslipedemia (38,39). Lupin seeds contain conglutin gamma (Cγ) protein, which applies a hypoglycemic impact. Moreover, it decidedly adjusts proteins engaged in glucose homeostasis. Cγ may conceivably be utilized to oversee patients with debilitated glucose digestion (40).

In the current study, administration of lupin seeds powder 1gm/kg daily cause little change in biochemical parameters but did not reach significance. Another study concluded that lupin seeds powder can lower plasma cholesterol and triacylglycerol levels in hypercholesterolemia laboratory animals (41).

Combination of Gliclazide (10mg/kg)+ lupin seeds (1gm/kg) daily for 4 weeks, noticed that all parameters be liable to shift toward normal states such as body weight and blood glucose, also increase secretion of insulin due to upregulation of mRNA expression of insulin gene and downr egulation of mRNA expression level of PEPCK gene, also increase in good cholesterol (HDL-c), decrease in bad cholesterol (LDL-c), TAG, total cholesterol and total lipid. Moreover, enhancement in the

mRNA expression level of PPAR-γ and glucokinase gene compared to Gliclazide alone treatment group.

According to our knowledge, no other research use the combination of Gliclazide (10mg/kg) with lupin seeds powder (1gm/kg) treatment in animals after induction of type2 DM by high-fat high fructose/sucrose diet in the animal model. However, some researchers used the combination of lupin seeds and metformin (antihyperglycemic drug) in the animal model and found that adding lupin seeds to the antidiabetic drug (metformin) induced more glycemic control and potentiated metformin effects and insulin activity (42,43). In addition, Dove et al. in his study concluded that adding lupin seeds with the antihyperglycemic drug in type 2 diabetic patients reduced blood sugar level, improved glucose metabolism, and increased insulin response (44).

Conclusion

In the present study, combined treatment with Gliclazide (10mg/kg)+lupin seeds powder (1gm/kg) daily for 4 weeks resulted in improvement of total biochemical parameters, lipid profile and enhanced the transcription of mRNA gene more than the treatment with Gliclazide drug only in type 2diabetic rats. It is advisable to incorporate lupin seeds in daily diet regimen of diabetic patients such as combing grinded lupin seeds with wheat flour in bakeries. Moreover, possible future researches on adding lupin seeds in diabetes pharmacotherapy.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

EFFECT OF DIETARY BETAINE AND LOW METHIONINE ON MULARD DUCK PERFORMANCE, BLOOD PARAMETERS AND LIPOGENESIS GENE EXPRESSION

Rania El Sayed[1], Doaa Ibrahim[1]*, Shefaa A.M. El-Mandrawy[2], Doaa M. AbdelFattah[3]

[1]Department of Nutrition and Clinical Nutrition, [2]Department of Clinical Pathology, [3]Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University, Zagazig, 44519, Egypt

*Corresponding author; E-mail: doibrahim@vet.zu.edu.eg

Abstract: The effects of dietary methionine and betaine (Bet) on productive performance, blood biochemical parameters and mRNA expression levels of fat acid synthase (FAS) and acetyl-CoA carboxylase (ACC), were investigated in Mulard ducks which raised in summer season. Three hundred one-day old healthy Mulard ducklings with similar body weight were randomly distributed into six groups with five replicates per treatment and ten ducks per replicate. Six diets were prepared as following: control diet (C); low methionine diet (ML); diet supplemented with Bet (0.25% Bet); diet supplemented with Bet (0.5% Bet); diet supplemented with 0.25% Bet and low in methionine (0.25% Bet + ML); diet supplemented with 0.5% Bet and low in methionine (0.5% Bet + ML). Regarding allover growth performance results revealed that increasing dietary Bet significantly increased (P<0.05) body gain of ducks by 13% when compared with the control diet, moreover both groups fed on 0.5% Bet and 0.5%Bet +ML diet exhibited the improved feed conversion ratio (1.88 and 1.93, respectively) when compared with control (2.13) and ML (2.29) groups. Inclusion of Bet either in control or ML diet significantly increased carcass yield, breast and thigh meat yield percent and decreased breast, thigh skin and abdominal fat percent. Nutrient digestibility was improved by adding Bet even in ML diet. Serum total lipids, triglycerides and total cholesterol constituents were significantly decreased in all group supplemented with Bet. Dietary Bet significantly decreased (P<0.05) mRNA expression of fatty acid synthase, acetyl-coA carboxylase genes, this decline was more obvious in control group with Bet than in ML with Bet. In conclusion, the role of Bet in ML diet was clear by improving productive performance of ducks, thus Bet can partially replace methionine in duck's diet.

Key words: Betaine; methionine; performance; carcass; lipogenesisgenes; Mulard ducklings

Introduction

The production prosperity of meat-type ducks can be achieved by good nutritional manipulations and the adjustment of their nutrient requirements is still required for maximizing productive performance. To enhance the production, sustain animals' health, economize feed-cost, supplementing

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feed-grade amino acids it is required (1,2). In growing chicks, previous studies found that supplementation with methionine (Met) could improve carcass quality and metabolism of protein (3). A corn-soybean meal-based broiler diet is often lack Met and supplementation of synthetic Met is an alternative way to meet the nutritional requirements of the bird. However, the unavailability and higher cost of synthetic Met often forces poultry feed nutritionists to search for alternative Met source. Consequently, reduction added DL-Met by supplementation of another dietary methyl donors may reduce Met requirement. Betaine (Bet), a trimethyl derivative of the amino acid Glycine, naturally extracted from sugar beet bulb and molasses, with low dietary Met, Bet can partially replace apart of Met, and can do its function and is as effective as Met in improving the growth performance and carcass quality of broilers (4), also, it may improve the availability of methionine (5). The main function of betaine as a donor of methyl groups like choline and methionine (6). Betaine can supply methyl groups for transmethylation reactions important for many metabolic processes like synthesis of proteins, energy metabolism (7) and also can partially substitute Met in the diet by providing a methyl group thus of homocysteine converted to methionine (8). Additionally, betaine can regulate fat distribution (9). Moreover, betaine has the potential to improve nutrient digestibility by augmenting the intestinal cells growth and health owing to its osmotic functions (10). An addition function of betaine within tissue is synthesis of carnitine, phosphatidylcholine, and creatine which play a key role inside the cells through protein and energy metabolism (11). In this regard, betaine significantly protects birds against the heat stress (12). Moreover, reduction of fat following to dietary betaine supplementation is more prominent than synthetic Met (13) as well, in adipose tissue, betaine supplementation declined the acetyl-CoA carboxylase (ACC), fat acid synthase (FAS) and malic enzyme activities as well as the FAS mRNA level in pigs decreasing the fat deposition (14). In Mulard ducks, although the effect of betaine on fat deposition was investigated, the molecular mechanism by which adipose tissue is down-regulated by betaine is poorly understood and FAS and ACC expression in Mulard ducks remain unknown. The main functions of betaine are methionine-sparing and fat-distribution effects. Accordingly, this study was conducted to investigate the effects of dietary supplementation of Bet with reducing Met on the performance, nutrient digestibility, carcass quality, fat distribution, blood parameters, mRNA expression level of FAS and ACC genes.

Material and methods

The experimental protocol was approved by Committee of Animal Welfare and Research Ethics Faculty of Veterinary Medicine, Zagazig University, Egypt.

Experimental birds and management

A total of 300Mulard ducks (one-day old) were obtained from a commercial hatchery. On arrival, they were weighed and randomly assigned to six groups, each consisting of five replicates of ten ducks each and raised for 42 days under the stress of Egyptian summer season. The temperature was kept at 33°C up to 3 days of age, and then it was reduced gradually to room temperature. Ducks were reared in naturally ventilated open house with sawdust as litter .

Experimental diets and design

The rearing period was divided into starter (up to 21 days) and finisher (22-42 days). The diets were formulated according to NRC (15) nutrition specification. The ducks were allowed free access to water and experimental diets were in wet mash form. The ingredient composition of the control diet is shown in Table (1). Six dietary treatments were prepared as following: control diet (C); low methionine diet; diet supplemented with 0.25% betaine; diet supplemented with 0.5% betaine; Diet supplemented with 0.25% betaine and low in methionine and diet supplemented with 0.5% betaine and low in methionine. In methionine low groups, no supplemented methionine was added to the diet, where the methionine level,

Table 1: Ingredient composition and calculated nutrient content of basal diets for Mulard ducks

Ingredient (%)	Starting diet (0-21)	Growing-overfeeding diet (22-42)
Corn	50	63.5
Soy bean meal (44)	37.3	21.6
Wheat bran	7	9
Soybean oil	2.5	2.6
Di-calcium phosphate	1.5	1.5
Ca carbonate	0.8	0.9
Na chloride	0.3	0.3
Premix[1]	0.5	0.5
Methionine	0.1	0.1
Calculated nutrient content		
Crude protein	21.67	16.24
Ether extract	2.65	3.07
Crude fiber	3.32	3.27
Metabolizable energy (kcal/kg)	2913	3012
Lysine	1.26	0.85
Methionine	0.42	0.36

Premix1 (per kg of diet): 10000 IU; Vitamin A, 5000 IU; vitamin D3, 16 mg; vitamin E, 11 mg; vitamin K3, 2.5 mg; Vitamin B1, 2.3mg; Vitamin B2, 7 mg; Vitamin B6: 4 mg; Vitamin B12: 14mg; Folic acid:1.3 mg; Biotin:140 mg; Calcium pantotenate: 25 mg; Nicotinic acid: 65 mg; Fe: 75 mg; Cu:9 mg; Mn:65 mg; Zn: 40 mg; I: 0.32 mg; Se: 0.15 mg.

in starter low methionine diet was 0.33% and in grower low methionine diet was 0.26%. Betaine was purchased from (Beta-key, Excentials, Netherland).

Growth performance

The body weight, gain, and feed intake of all groups of ducks were recorded weekly and feed conversion ratio (FCR), protein efficiency ratio (PER) at 21 and 42 d of age and overall performance were calculated.

Digestibility trial

At the end of feeding trail, each group was supplied by its original feed with addition of chromic oxide (0.3%, analytical marker) for 7 days (as a preliminary period) and then the excreta were collected daily on a plastic sheet for another 7 days (as a collecting period). The fecal samples were dried in hot air oven at 60 ºC for 72 h and were grounded. A complete proximate analysis was made for both feed and fecal samples for dry matter crude protein, fat and crude fibre as described by the Association of Official Analytical Chemists (AOAC) (16).

Carcass traits and meat quality

At 42 d of age five ducks from each group were selected at the end of the experiment, fasted overnight, weighed and then sacrificed to obtain weight of the dressed carcass, breast, thigh, and abdominal fat yields expressed as a percentage of live body weight. Samples were stored at -20ºC until analysis. The chemical composition for dry matter, crude protein and ether extract in breast and thigh was done according to AOAC (16). For meat quality measurements, right side of breast meat was used to determine Drip loss (%; proportionate weight loss of a sample hanging in a plastic bag for 48 h at 2°C). The same sample was used for cooking and thaw loss, after storage at −20°C, thaw loss (%; proportional weight loss of a meat sample before frozen storage at −20°C and after overnight defrosting at 4°C and cooking loss (%; proportionate weight loss of a sample after cooking in an open plastic bag in a water bath at 70°C for 40 min followed by cooling).

Serum biochemical analysis

Blood samples were collected at 42 d of age from 5 ducks per group into tubes without anticoagulant for collection of serum and with anticoagulant for haematological parameters . The separated serum was used for determination of total protein (T1949), albumin(A5503), globulin, triglyceride, (TR0100), high-density lipoprotein-cholesterol

(HDL-C), low-density lipoprotein-cholesterol (LDL-C) (MAK045) colorimetrically using kits from Sigma Aldrich, following the manufacturer's instructions.For haematological indices, total red blood cell counts (RBCs), haematocrit (HCT), haemoglobin (HGB),mean corpuscular volume (MCV), white blood cells (WBCs), lymphocytes and granulocytes were measured using an automated blood cell counter(Sysmex IV2000).

Sampling for real-time quantitative RT-PCR

Adipose tissue samples were collected immediately after slaughter, frozen in liquid nitrogen, and stored at −80°C till analysis for gene expression of fatty acid synthase, FAS, acetyl-coA carboxylase, ACC and β-Actin.

Real-Time quantitative RT-PCR for gene expression

Total RNA was isolated from abdominal adipose using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. Before RT-PCR, RNase-free DNase (Promega, Madison, WI) was used to remove contaminating genomic DNA from samples. The total RNA was reverse transcribed into cDNA, using QIAGEN Long Range 2 Step RT-PCR Kit, following manufacturer's directions. One µl of cDNA was mixed with 12.5 µl of 2x SYBR Green qPCR mix with ROX from Bio-Rad (USA), with addition of 5.5 µl of RNase-free water and 0.5 µl of each forward and reverse gene specific primers. The values for the specific targets were normalized using β-Actin as reference gene. The primer sequences from published were used as following of studied genes are the following: FAS gene; F: 5' TGG GAG TAA CAC TGA TGG C 3', R: 5'-GAT GGT GAG GAG TCG GAT-3' (Gene Bank ID, JQ080308), ACC gene, F: 5' TGC CTC CGA GAA CCC TAA 3' R:5'- TCC AGG CTT GAT ACC ACA 3'(Gene Bank ID, JQ080306) β-Actin, F: 5' CAA CGA GCG GTT CAG GTG T-3' R: TGG AGT TGA AGG TGG TCT CG 3'(Gene Bank ID, EF667345).

Statistical analyses

The experimental data were evaluated using One-way ANOVA procedure, post hoc comparisons were applied, whenever appropriate, using Duncan's test. All statistical measures were performed using PASW statistics 18 (SPSS Inc., USA). Statistical significance was considered at $P \leq 0.05$. Fold change was considered using the (2-ΔΔCt) method to quantify mRNA levels.

Results

The effect of replicates was non-significance, so all data was merged together.

Ducks growth performance and nutrient digestibility

A growth response to betaine (Bet) supplementation in control diet or in methionine low (ML) diet is shown in Table (2). Ducks in 0.5% Bet group had the highest ($P< 0.05$) final body weight (FBW), followed by the ducks in (0.25% Bet) and 0.5% Bet with ML groups, then 0.25% Bet with ML when compared with the control group, while ML group recorded the lowest FBW. The all over values of feed conversion ratio and protein efficiency ratio were improved in groups fed 0.5% Bet and 0.5% Bet with ML groups followed by 0.25% Bet when compared with the other groups.

Dietary supplementation of 0.25 and 0.5% Bet to Mulard ducks improved DM digestibility by 2.9 and 3.5%, respectively. The reduction of methionine content in duck's diet with Bet supplementation improved the DM especially with 0.5 % Bet. Moreover, crude protein digestibility significantly increased ($P< 0.05$) in groups supplemented with dietary Bet either in control or ML diet. In relation to fat and crude fiber digestibility, addition of Bet especially 0.5% significantly increased ($P< 0.05$) their digestibility (Table 2). In accordance with this result, crude protein (CP), crude fiber (CF) AND ether extract (EE) digestibility were higher with Bet supplementation either in methionine supplemented group or methionine deficient groups compared to the control group.

Table 2: Effect of two levels of dietary betaine supplementation in normal and low methionine diets on all over growth performance and nutrient digestibility of Mulard ducks (d 42)

	Control	Methionine low (ML)	Betaine 0.25%	Betaine 0.5%	Betaine 0.25%+ ML	Betaine 0.5%+ML	*P*-value
Allover Growth Performance							
Initial body weight, g/bird	59.50±0.24	61.00±0.44	60±0.20	59.80±0.30	60±0.40	60.5±0.40	0.55
Final body weight, g/bird	2909±11.84^{d}	2783±9.27^{e}	3211±6.19^{b}	3354±8.46^{a}	3087±6.11^{c}	3185±3.12^{b}	<0.001
Body weight gain, g/bird	2849 ±11.85^{d}	2722±9.33^{e}	3151± 6.24^{b}	3294 ±8.5^{a}	3027±6.14^{c}	3125±3.12^{b}	<0.001
Feed intake, g/bird	6083±32.65^{bc}	6228 ±34.49^{abc}	6395± 34.78^{ab}	6187±30.07^{abc}	6479±44.77^{a}	6023±40.25^{c}	<0.001
Feed conversion ratio	2.13±0.03^{b}	2.29±0.03^{a}	2.03 ± 0.02^{bc}	1.88±0.01^{d}	2.14 ±0.02^{b}	1.93±0.04^{cd}	<0.001
Protein efficiency ratio	2.25±0.03^{c}	2.10 ±0.03^{d}	2.37 ± 0.02^{b}	2.55 ±0.02^{a}	2.24±0.02^{c}	2.49±0.05^{a}	<0.001
Digestibility %							
Dry matter	89.40±0.12^{c}	87.57±0.18^{d}	92.30±0.12^{b}	92.93±0.09^{a}	91.40±0.85^{b}	92.33±0.13^{b}	<0.001
Crude protein	82.53±0.15^{c}	79.90 ±0.15^{d}	88.33±0.13^{a}	89.40±0.12^{a}	89.33±0.03^{a}	88.70±0.40^{a}	<0.001
Ether extract	68.53±0.19^{d}	67.70±0.15^{e}	73.50±0.06^{b}	75.77±0.09^{a}	70.33±0.03^{c}	70.53±0.03^{c}	<0.001
Crude fibre	32.40±0.06^{b}	28.53±0.19^{c}	32.33±0.15^{b}	33.50±0.07^{a}	32.20±0.1^{b}	32.47±0.09^{b}	<0.001

Values were means ± standard error. Values with different superscripts within a row were significantly different (P<0.05).

Carcass Characteristics and meat quality

Results related to carcass traits and meat quality are shown in Table (3). The carcass weight yield and dressing % were significantly higher (P< 0.05) in Bet supplemented groups even in ML diet when compared with control group or ML group, moreover the percentage of skin was significantly decreased by increasing of dietary Bet. The lowered abdominal fat % was found in group with 0.5% Bet followed by groups supplemented with 0.25% Bet and 0.25 and 0.5% Bet with ML diet, there were no significance difference between the latter groups, when compared with control and ML group. Concerning meat chemical composition, increasing the level of Bet from 0.25 to 0.5% significantly increased (P< 0.05) the breast and thigh dry matter and crude protein content and in contrast, the breast and thigh fat content were significantly decreased. Also, with increasing the level of Bet from 0.25 to 0.5% in ML diet, the protein content was significantly increased in thigh and breast when compared with control group or ML group. The drip loss, thaw loss and cooking loss % of whole carcass meat were significantly lowered for ducklings fed diet supplemented with Bet, moreover reduction of methionine together with betaine decreased these previous parameters, as compared to those fed the control and ML diet.

Gene expression

Data related to mRNA expression of fatty acid synthase (FAS) and acetyl-coA carboxylase (ACC) genes are shown in figure 1. The results revealed that mRNA expression of FAS gene reached its lower level (P< 0.05) in Mulard duck group fed on control diet supplemented with 0.5% Bet. In addition, inclusion of dietary 0.25% Bet, 0.25% Bet + ML or 0.25% Bet + ML significantly decreased (P<0.05) the expression of FAS gene, with no significance difference in latter groups, when compared with control and methionine low groups. In regard to ACC gene expression, the groups of ducks supplemented with dietary 0.25 and 0.5% Bet exhibited the lower expression followed by groups supplemented by dietary 0.25 and 0.5% Bet +ML in comparison with the control and ML diet.

Table 3: Effect of two levels of dietary betaine supplementation in normal and low methionine diets on carcass characteristics, meat chemical composition on dry matter basisand meat quality in Mulard ducks at slaughter (d 42)

	Control	Methionine low (ML)	Betaine 0.25%	Betaine 0.5%	Betaine 0.25%+ ML	Betaine 0.5%+ML	*P*-value
Carcass weight, g	2288 ± 11.51^{d}	2148 ±7.42^{e}	2588 ±7.25^{b}	2725±8. 61^{a}	2467±6.20^{c}	2566±3.15^{b}	< 0.001
Carcass yield g/kg	1047 ± 9.77^{d}	903 ± 8.51^{e}	1047 ± 8.71^{b}	1488 ±8.31^{a}	1236±6. 40^{c}	1337±3.15^{b}	< 0.001
Dressing %	77.32±0.01^{c}	76.38 ±0.15^{d}	77.92 ± 0.03^{b}	79.15±0.05^{a}	77.34±0.07^{c}	77.98±0.03^{b}	< 0.001
Carcass parts, %							
Breast yield	50.72 ± 0.09^{c}	47.15±0.06^{e}	51.69 ± 0.09^{b}	53.44 ±0.07^{a}	50.31 ±0.04^{d}	51.76 ± 0.07^{b}	< 0.001
Breast skinless	40.56 ±0.04^{c}	39.54 ±0.07^{d}	42.52 ± 0.09^{b}	44.65 ±0.05^{a}	40.78±0. 2^{c}	42.28 ±0.1^{b}	< 0.001
Breast skin	12.06 ±0.05^{a}	12.20 ±0.05^{a}	10.72 ±0.06^{c}	10.38 ±0.08^{d}	11.13 ±0.06^{b}	10.74 ±0.05^{c}	< 0.001
Thigh yield	30. 64 ±0.08^{d}	28.71 ±0.1^{e}	31.93 ±0.04^{b}	32.50 ±0.05^{a}	31.42 ±0.5^{c}	31.21 ±0.1^{c}	< 0.001
Thigh skinless	22.32 ±0.04^{c}	22.12 ±0.05^{c}	24.11 ±0.05^{b}	24.50 ±0.09^{a}	23.82 ±0.05^{b}	23.94 ±0.2^{b}	< 0.001
Thigh skin	9.45 ±0.1^{a}	9.58 ±0.03^{a}	7.46 ±0.07^{c}	6.41 ±0.03^{cd}	8.43±0.03^{b}	8.19 ±0.06^{b}	< 0.001
Abdominal fat, %	1.93 ±0.05^{a}	1.87 ±0.03^{a}	1.64 ±0.04^{b}	1.35 ±0.02^{c}	1.58 ±0.03^{b}	1.52±0.05^{b}	< 0.001
Chemical composition, %							
Breast DM	26.78 ± 0.08^{b}	25.7 ±0.06^{c}	26.82 ±0.05^{a}	26.89±0.03^{a}	26.42±0.04^{b}	26.75±0.04^{b}	< 0.001
Thigh DM	28.33 ± 0.07^{a}	27.66 ± 0.08^{b}	28.33 ± 0.07^{a}	28.48 ±0.05^{a}	28.31±0.05^{a}	28.31±0.04^{a}	< 0.001
Breast crude protein	73.49±0.06^{e}	71.23 ±0.03^{f}	75.80 ± 0.04^{b}	76.35±0.02^{a}	73.70±0.09^{d}	74.76 ±0.06^{c}	< 0.001
Thigh crude protein	70.42 ± 0.07^{e}	68.64±0.1^{f}	72.26 ± 0.04^{b}	72.82 ±0.07^{a}	70.87 ±0.03^{d}	71.70 ± 0.04^{c}	< 0.001
Breast ether extract	13.33 ±0.01^{b}	14.66 ±0.08^{a}	12.70 ± 0.07^{c}	10.76 ±0.1^{f}	11.56±0. 05^{d}	11.23 ±0.06^{e}	< 0.001
Thigh ether extract	16.42 ±0.09^{a}	16.48 ±0.1^{a}	15.48 ±0.12^{b}	13.16 ±0.07^{e}	14.52 ±0.11^{c}	13.60 ±0.8^{d}	< 0.001
Meat quality							
Drip loss, %	0.98 ±0.04^{a}	1.04 ±0.08^{a}	0.68 ±0.1^{b}	0.64 ±0.09^{b}	0.76 ±0.05^{ab}	0.62 ±0.06^{b}	< 0.001
Thaw loss, %	4.76 ±0.1^{b}	5.94 ±0.04^{a}	3.72 ±0.11^{c}	3.50 ±0.22^{c}	3.84 ±0.22^{c}	3.64 ±0.18^{c}	< 0.001
Cooking loss, %	13.06 ±0.18^{ab}	13.54 ±0.19^{a}	12.32 ±0.1^{c}	11.5 ±0.26^{d}	12.52±0.1^{bc}	12.06 ±0.09^{cd}	< 0.001

Values were means ± standard error. Values with different superscripts within a row were significantly different ($P < 0.05$).

Table 4: Effect of two levels of dietary betaine supplementation in normal and low methionine diets on serum and blood hematological parameters in Mulard ducks at slaughter (d 42)

	Control	Methionine low (ML)	Betaine 0.25%	Betaine 0.5%	Betaine 0.25%+ ML	Betaine 0.5%+ML	*P*-value
TP, g/ dl	4.18±0.11^{c}	3.58±0.12^{d}	4.9±0.06^{b}	5.2±0.07^{a}	4.2±0.07^{c}	4.7±0.07^{b}	< 0.001
Albumin, g/ dl	1.9±0.18^{ab}	1.48±0.12^{c}	2.1±0.07^{a}	1.84±0.09^{ab}	1.46±0.1^{c}	1.58±0.1^{c}	< 0.001
Globulin, g/ dl	2.3±0.09^{c}	2.1±0.05^{c}	2.7±0.06^{b}	3.3±0.09^{a}	2.7±0.07^{b}	3.14±0.08^{a}	< 0.001
Bet. Homocystine-transferase	1.6±0.07^{c}	1.3±0.05^{d}	2.3±0.09^{b}	2.7±0.07^{a}	2.4±0.05^{b}	2.4±0.05^{b}	< 0.001
Triglycerides, mg/dl	3.7±0.1^{a}	3.6±0.1^{ab}	3.3±0.05^{bc}	3.3±0.1^{bc}	3.4±0.06^{abc}	3.1±0.04^{c}	< 0.001
HDL-C, mg/dl	128.2±0.6^{b}	132.0±0.6^{a}	127.0±0.7^{bc}	123.4±0.5^{d}	125.6±0.7^{cd}	123.8±0.5^{d}	< 0.001
LDL-C, mg/dl	87.8±0.9^{a}	85.8±0.9^{b}	81.2±0.5^{cd}	81.0±0.7^{cd}	82.2±0.7^{c}	79.2±0.4^{d}	< 0.001
Blood hematological parameters							
RBC, M/µl	2.1±0.06^{c}	2.0± 0.07^{c}	2.7±0.06^{b}	3.1±0.07^{a}	2.9±0.04^{ab}	3.0±0.07^{ab}	< 0.001
HGB, g/dl	12.6±0.7^{c}	12.0±0. 2^{d}	15.6±0.1^{b}	17.2±0.2^{a}	16.8±0.2^{a}	17.2±0.2^{a}	< 0.001
MCV, Fl	128.8± 0.6^{d}	123.0±0.9^{e}	150±0.9^{a}	146.4±0.6^{b}	141.6±0.5^{c}	145.6±0.6^{b}	< 0.001
WBCs x10^{3}, M/µl	7.5±0.07	7.3±0.2	7.6±0.09	7.5±0.07	7.5±0.06	7.4±0.08	0.1
Lymphocytes x10^{3}, M/µl	5.6±0.1	5.7±0.07	5.3±0.2	5.3±0.6	5.2±0.9	5.6± 0.7	0.09
Granulocytes x10^{3}, M/µl	2.3±0.1	1.9±0.2	1.8±0.7	1.7±0.8	1.9±0.1	2.0±0.3	0.2

TP: Total protein; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; Values are means ± standard error. Values with different superscripts within a row are significantly different ($P < 0.05$).

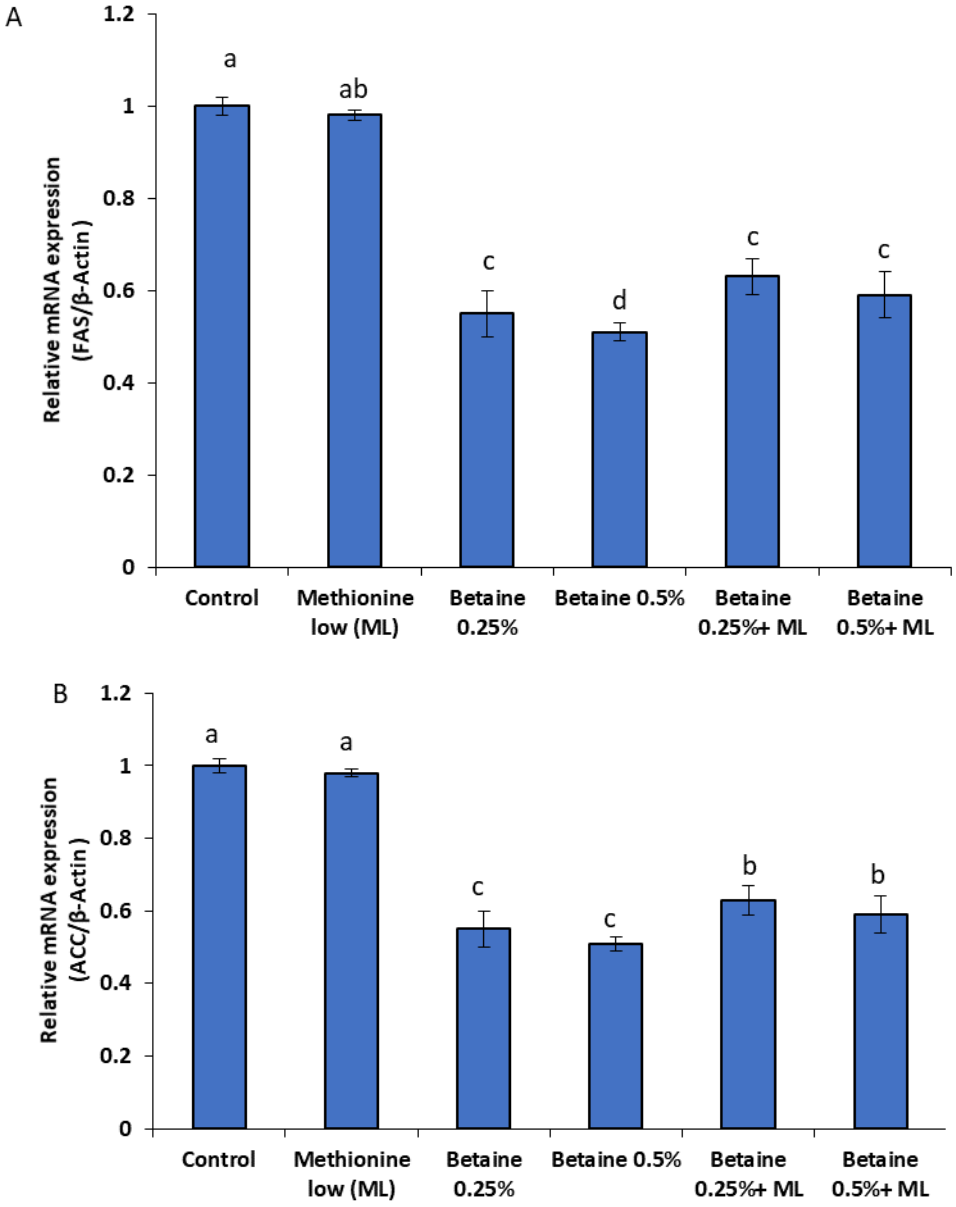

Figure 1: Effect of two levels of dietary betaine supplementation in normal and low methionine diets on the relative mRNA expression A) fatty acid synthase gene, FAS B) acetyl-coA carboxylase, ACC gene of Mulard ducks at slaughter (d 42)

Blood parameters

Data related to serum and hematological constituents are shown in Table 4. Significant interaction was found between supplementation of Bet as methyl donor either in normal diet or methionine deficient diet and total protein. With increasing levels of Bet, the value of total protein was also increased even in methionine deficient diet supplemented with betaine. As serum lipid profile is an important index of lipid metabolism, betaine revealed a lowered significant effect on triglycerides (TG) and low-density lipoprotein (LDL-C) and higher levels of high-density lipoprotein (HDL-C). In addition, increased count of RBCS was observed with addition of betaine in normal control diet or in methionine deficient diet. While there was no significance difference between WBCs, lymphocytes and granulocytes count between different groups.

Discussion

Effects on duck performance and nutrient digestibility

Dietary inclusion of betaine (Bet) up to 0.5% significantly increased gain and feed efficiency of ducks, also our results suggest that betaine can partially replace the methionine in maize-soybean diets of meat-type ducks and resulted in good productive performance. These findings were supported by Su et al. (18) that geese supplementation with betaine (1 g/d/ goose) could increase the final live body weight. Recently, yang et al. (19) reported an improvement in growth performance parameters of goose fed on Met-deficient diets supplemented with betaine (600mg/ kg diet). Likewise, addition of dietary Bet by 1000 mg/kg to broilers chicks significantly enhanced their growth performance in a 10% Met-reduced diet (11). Moreover, Chen et al. (20) suggested that supplementation of dietary Met supplementation at optimum level could increase growth performance in growing birds. In addition, our results are in agreement with reports about using different levels of Bet (0.5, 1 and 1.5g/Kg diet) for Domyati ducks (21) and (0.1, 0.2 and 0.4%) for broilers (22) significantly improved body weight gain and feed utilization (12). The improved performance due to Bet supplementation in ducks might be attributed to several factors as increasing intestinal integrity (23), stimulating growth hormone and insulin-like growth factor-I (24), increasing intestinal immunity (25). Moreover, the osmolytic function of betaine influence positively on the growth, survival and integrity of intestinal cell (26).Thus, it appears that betaine in a marginally methionine low diet could lead to corresponding growth response in ducks, and that betaine could spare a small portion of methionine which agreed with Zhan et al.(27).Observations of current study about digestibility were in agreement with the findings of FariaFilho et al. (28) concerning weight gain and feed conversion ratio of an efficient nutrient degradation and absorption. Also, in monogastric animals' fermentation process in the gastrointestinal system is affected by dietary betaine supplementation (29). The role of betaine in improvement of nutrient digestibility could attribute to its osmoprotective properties, protecting intestinal epithelium and augment the growth of beneficial intestinal microorganism (10). On other hand, poultry lacks fiber-degrading enzymes and the improvement in fiber digestibility indicates that betaine has the potential to stimulate the bacterial fermentation of dietary fiber (30). Also, El-Husseiny (31) reported that in methionine-deficient diet supplementation of 0.05-0.10% to broilers chicks fed on dietary betaine significantly increased the digestibility of organic matter, protein, fat and fiber. According to Rørvik et al. (32), the potential role of betaine on ether extract digestibility can explained by an increased bile acid secretion.

Effects on carcass characteristics and meat quality

The obtained results suggested that it would be ideal to optimize the quantity of supplemental methionine with Betaine, which has a positive influence on carcass meat yield which agreed with Waldroup et al. (33). The results in accordance with Chand et al. (34) who revealed that betaine supplementation significantly ($P< 0.05$) improved dressing percentage and in methionine deficient the breast yield of broilers was increased with Betaine supplementation (5). Similar results were reported with dietary betaine (0, 0.075, 0.150 and 0.225%) inclusion in broilers (35). Other interesting perspective of betaine inclusion in poultry' diets are decreasing carcass fat content associated with increasing the lean meat which may be satisfy consumer needs (22) and this property of betaine may be related to its methyl group donor properties (29),higher availability of methionine and cystine for protein deposition (36) and enhanced utilization dietary amino acids for protein synthesis that may result in fewer amino acids available for deamination and eventual synthesis of adipose tissue (37). Also similar to the present study, Neoh and Ng (38) reported that addition of dietary betaine improved carcass yield and breast percentage as well as reduced abdominal fat. Accordingly, variations

in hormone levels and growth factors involved in the regulation of fat synthesis and degradation, as well as lower activities of lipogenic enzymes, have been observed following dietary betaine supplementation (14). A potential explanation for decreasing abdominal fat of dietary betaine may be due to its major effect on lipid metabolism as a result of activating carnitine synthesis in liver and muscle (27) and synthesis of lecithin which was responsible for fatty acids transport to the mitochondria for oxidation, accordingly, lipid deposition was reduced (29). Awadet al. (12) reported that, followed to feeding of Domyati ducklings on dietary betaine (0.5, 1.0 and 1.5 g /kg) eviscerated carcass was significantly increased by 8.34, 13.73 and 16.92 %, and abdominal fat percentage was significantly reduced as compared to the control.

The results related to meat quality in our study revealed that betaine inclusion in control diet or in methionine low diet improved drip loss, thaw loss and cooking loss percentage. This result is in agreement with those obtained by Alirezaei et al. (11) who reported that adding Bet 1 gm/kg to a Met-deficient diet significantly improved meat quality as betaine function could lead to high water retention in broilers exposed to cyclic heat stress (39) and maintain cell integrity and function by regulating water inside and surrounding the cell (40).

Effects on expression of genes responsible for Lipogenesis

Concerning mRNA expression of fatty acid synthase gene, FAS and acetyl-coA carboxylase, ACC in abdominal adipose, addition of betaine significantly decreased their expression especially with 0.5%, also when increasing dose of betaine to 0.5%, betaine could replace part of methionine in duck's diet. The finding of our results was in accordance with the lipotropic effect of betaine because betaine supplementation might result in a decrease in the lipogenesis, as evidenced by the decrease in fatty acid synthase mRNA expression in abdominal adipose tissue and to decrease the activities of acetyl-CoA carboxylase, in finishing pigs after Betaine supplementation (14). Furthermore, betaine acts as methyl group donor for transmethylation reactions needed for synthesis of carnitine and thus altering animal fat metabolism (5). Moreover, betaine may reduce the mRNA level for fatty acid synthase and adipocyte type acid binding protein (41).

Effects on blood parameters

In this study, dietary Bet supplementation positively affect on total protein level, lipid fractions of serum and RBCs counts even in methionine deficient diet. In accordance with our results, Rao et al. (5) described that following Bet supplementation for broilers, total protein, triglycerides (TG) and total cholesterol, free fatty acids (FFA), low-density lipoprotein cholesterol and high-density lipo-protein cholesterol in serum were improved, particularly at the lower Met concentrations compared with the control group which evidence that described Bet can alter lipid metabolism (22). Additionally, Ratriyanto et al. (10), found that in laying hens, betaine enhanced hormone-sensitive lipase activity, leading to a higher levels of serum FFA through activating the hydrolysis of TG to glycerol thus lower the concentration of TG and cholesterol in serum. The results also correlated with the decreased plasma TG and LDL, as Bet is associated with the carnitine metabolisms, which in turn is involved in mitochondrial transport and the fatty acids oxidation (42).

In addition, Park and Kim (43) found that Bet supplementation resulted in more significant improvement in hematological indicators such as RBCs and platelet count than the heat stress control group (P <0.05). The higher values of hematological indices in the ducks fed a dietary Bet than those under heat stress fed a basal diet, suggested that betaine may contribute to synthesis of methionine and control of osmotic pressure surrounding cells (6,26). Thus, the protective effect of betaine for erythrocytes is clear in the current study by increasing their count, therefore protecting against anemia (hemorrhagic and hemolytic). Whereas the decrease of hemoglobin causes microcythemia. As, there is a correlation between hematocrit and packed red cells

volume; when the hematocrit means decreases, the levels of hemoglobin and hemoglobin become lower, their high value following betaine supplementation indicating preventing effect of betaine from iron deficiency anemia specially in the period of stress as heat (44).

Conclusion

It could be concluded that the dietary betaine supplementation up to 0.5% has a positive effect on the productive performance of Mulard ducks and also regulates lipogenesis gene expressions in adipose tissuesthus controlling body fat accumulation in ducks. Methionine could be partially replaced by betaine and there is a positive interaction between the two compounds which was clearly affect on performance of ducks, regulate lipid synthesis. Hence, addition of 0.5% betaine with 0.1% deficiency in methionine than NRC recommendation can be used in feeding of Mulard ducks.

Conflict of interest

The authors declare no conflict of interest.

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Original Research Article

PREVALENCE OF MULTIDRUG-RESISTANT *Staphylococcus aureus* AND *Salmonella* Enteritidis IN MEAT PRODUCTS RETAILED IN ZAGAZIG CITY, EGYPT

Alaa Eldin M.A. Morshdy[1], Wageh S. Darwish[1]*, Waiel M. Salah El-Dien[2], Sahar M. Khalifa[2]

[1]Food Control Department, Faculty of Veterinary Medicine, Zagazig University, 44519 Zagazig, [2]Food Control Department, Animal Health Research Institute, Zagazig Province Laboratory, Zagazig, Egypt

*Corresponding author, E-mail: wagehdarwish@zu.edu.eg

Abstract: This study aimed to monitor the hygienic status of fresh minced meat, smoked sausage and fresh beef burger (50 samples, each) retailed in Zagazig city, Egypt. Aerobic plate count, total *Staphylococcus aureus* count and most probable number of coliforms have been conducted. The prevalence, antibiotic susceptibility as well as detection of the drug resistance associated virulence genes of *S. aureus* (*mec*A, *bla*Z, *and aac (6') aph (2")*) and *Salmonella* species (*bla*TEM, *tet*A(A), and *flo*R) in the examined meat products have been carried out. The highest mean (log_{10} cfu/g) of aerobic plate counts (5.44±0.11) and most probable number (4.15±0.10-log10 MPN/g) were recorded in minced meat. However, the highest mean of *S. aureus* counts (3.47±0.12-log10 cfu/g) was recorded in beef burger. Aerobic plate counts, most probable number and *S. aureus* counts exceeded the recommendations of Egypt Organization for Standardization by (20, 4 and 16%), (14, 12 and 20%) and (50, 10 and 20%) in minced meat, sausage and beef burger, respectively. *Salmonella* Enteritidis was detected in 4 (8%) beef burger. However, *S. aureus* was isolated from minced meat and beef burger (5 samples, each, 10%) and 4 sausage samples (8%). *mec*A, *bla*Z and *aac(6')aph(2")* were detected in all *S. aureus isolates. bla*TEM, *tetA*(A) *and flor*R were detected in the all *S.* Enteritidis isolates. In conclusion, the achieved results revealed inadequate hygienic measures adopted during preparation of such meat products. Therefore, strict hygienic practices should be followed before serving such products to consumers.

Key words: *S. aureus; Salmonella* Enteritidis; drug resistance; meat products

Introduction

Meat products such as minced meat, sausage and beef burger are considered rich sources for animal derived proteins, essential fatty acids, fat soluble vitamins and minerals such as iron and phosphorus. In addition, such meat products have their unique aroma and flavour which make them highly attractive, especially for children (1). However, such meat products may be on responsible for human illnesses by food-borne pathogens such as *Staphylococcus aureus (S. aureus)* and *Salmonella* species.

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Microbial contamination of meat products may arise from the raw ingredients used in their manufacture, improper handling during transportation, processing, storage and distribution (2). Therefore, evaluation of the hygienic status of meat products is a major task for meat hygiene and food safety sectors in Egypt.

S. aureus enterotoxigenic strains are responsible for foodborne intoxication due to the production of heat-stable enterotoxins (3). *Salmonella* spp. is a leading cause of foodborne infection (4,5). The abuse of antimicrobials in the veterinary field and the use of the same drugs for treatment of both humans and animals had resulted in development of antimicrobial resistant organi-sms. Such organisms may harbour some virulence attributes, which are positively contribute to the development of this multidrug resistance phenomenon (6). However, there is a clear lack of information about multidrug resistant foodborne pathogens in Egypt, in particular among strains isolated from meat products.

Therefore, this study was conducted to evaluate the microbiological quality (aerobic plate count (APC), total *S. aureus* count and most probable number (MPN) of coliforms) of meat products including minced meat, sausage and beef burger retailed in Egypt. Additionally, the prevalence of some foodborne organisms including *S. aureus* and *Salmonella spp.* was investigated. Furthermore, the multidrug-resistance profiles of the identified strains were examined. Finally, the expression of drug resistance-related genes in the isolated organisms was detected using PCR assay.

Material and methods

Collection of samples

One hundred and fifty samples of fresh minced meat, smoked sausage and fresh beef burger (50 samples each) were randomly collected from butcher shops and stores in Zagazig city, Egypt. Samples were kept in an ice tank and then immediately transferred to Food Control Laboratory, Faculty of Veterinary Medicine, Zagazig University, Egypt for bacterial isolation and identification.

Preparation of samples, enumeration and isolation procedures

From each sample, 25g were aseptically homogenized in 225 ml of 1% sterile peptone water (Oxoid CM9) to make a dilution of 10^{-1} and then serial dilutions were followed up to 10^{-7} dilution (7). For aerobic plate count, 1ml of each dilution was pipetted into separate duplicate petri dishes, and then overlaid by 12-15ml of nutrient agar (CM003, Oxoid, England), mixed well by alternate rotation and then let to solidify. Solidified petri dishes were inverted and incubated at 37ºC for 24 h. All colony-forming units (pinpoint size) were counted (8).

For *S. aureus*, isolation and count were done on Baird Parker agar (Biolife, Italy) supplemented with egg yolk-tellurite emulsion (Himedia, India). After incubation at 37ºC for 48 h, colonies (black, shiny, convex, 1–1.5 mm in diameter, and surrounded by a clear halo zone) and/or atypical colonies (black with no zones) presumptive colonies were counted and five colonies were selected and sub-cultured on blood agar plates (Difco Laboratories, Detroit, MI) and incubated for 24 h at 37ºC (8). Gram's stain and biochemical tests were performed on suspected colonies for identification of *S. aureus* (9). For the most probable number (MPN) of coliforms; 1ml of each dilution was inoculated separately into 3 MacConkey broth tubes with inverted Durham's tubes. Then, tubes were incubated at 37ºC and examined after 24 and 48h. Positive tubes showing acid and gas productions in inverted Durham's tubes were recorded as MPN of coliforms (10).

Regarding *Salmonella* spp., original homogenate was pre-enriched in buffered peptone water 1% at 37ºC for 24h. Then 1 ml of pre-enriched peptone water was enriched in Rappaport Vassiliadis broth with soya broth at 41.5ºC. A loopful was streaked on XLD agar, incubated at 37ºC for 24h and red colonies with black centre were enumerated (11). The obtained purified isolates were identified biochemically and serologically (12).

Genomic DNA extraction and PCR analysis

Genomic DNA extraction was done using QIAamp DNA kit according to the manufacturer's instructions. Primer sequences for identification of antibiotic resistance genes were described in Table 1. The target genes of *S. aureus* included *mec*A (encoded for methicillin-resistance) (13)**,** *bla*Z (encoded for β-lactamase-resistance) (13) and *aac (6') aph (2'')* (encoded for aminoglycoside-resistance) (13). For *Salmonella* spp., the targets genes were *bla*TEM (encoded for ampicillin-resistance) (14), *tet*A(A) (tetracycline resistance gene) (15) and *flo*R (florfenicol/ chloramphenicol resistance gene) (16). Uniplex PCR assays were carried out according to Darwish et al. (17). The thermal cycle of the reaction was started with a single 1 min cycle at 94°C, followed by 35 cycles of 10 sec denaturation at 94°C, 1 min annealing (annealing temperatures are indicated in Table 1) and 1 min extension at 72°C and then a final cycle of extension for 7 min was carried out at 72°C. The amplified products were then electrophoresed in 2% agarose gel and stained with ethidium bromide (18).

Table 1: Primers' sequences of the investigated drug resistance associated genes in *S. aureus* and *S.* Enteritidis isolated from different meat products

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Annealing (°C)	Reference
***mec*A**	F-GTAGAAATGACTGAACGTCCGATAA R-CCAATTCCACATTGTTTCGGTCTA A	310	50	(13)
***bla*Z**	F-ACTTCAACACCTGCTGCTTTC R-TGACCACTTTTATCAGCAACC	173	54	(13)
aac(6')aph (2'')	F-GAAGTACGCAGAAGAGA R-ACATGGCAAGCTCTAGGA	491	54	(13)
***bla*TEM**	F-ATCAGCAATAAACCAGC R-CCCCGAAGAACGTTTTC	516	54	(14)
***Tet*A(A)**	F-GGTTCACTCGAACGACGTCA R-CTGTCCGACAAGTTGCATGA	576	50	(15)
***flo*R**	F-TTTGGWCCGCTMTCRGAC R-SGAGAARAAGACGAAGAAG	494	50	(16)

Antibiogram

Antibiotic sensitivity testing of *S. aureus* and *Salmonella spp.,* was performed using single diffusion assay against 11 commercially prepared antibiotic discs (6 mm) with variable concentrations (19).

Statistical analysis

Statistical significance was tested using One way analysis of variance (ANOVA) followed by Tukey-Kramer HSD test (JMP statistical package, SAS Institute Inc., Cary, NC, USA) (P <0.05).

Results and discussion

The microbiological quality of meat products examined reflects the hygienic measures adopted during the preparation and post-processing handling of such products. In the present study, the results revealed the average aerobic plate counts (log_{10} cfu/g) were 5.44±0.11, 5.41±0.08 and 4.07±0.11 in the examined minced meat, beef burger and sausage, respectively (Table 2). Comparing the recorded values with the permissible limits set ensured by Egypt Organization for Standardization (EOS) (20), it was clear that, 20%, 4% and 16% of minced meat, sausage, and beef burger, respectively exceeded that limits. *S. aureus* counts expressed as log_{10} cfu/g was found to be; 3.45±0.20 in minced meat and 3.47±0.12 in beef burger that was significantly (p<0.05) higher than in sausage (2.31±0.19). Moreover, it was found that, 14, 12, and 20% of minced meat, sausage, and beef burger exceeded EOS recommendations (20). The Most Probable Number values (log_{10} MPN/g) of coliforms were higher in minced meat (4.15±0.10), followed by beef burger (2.99±0.12) and sausage (2.12±0.12) that exceeded EOS limits by 50, 20 and 10%, respectively.

Table 2: Hygienic indicators in the examined meat product samples

	Minced meat	Sausage	Beef burger
Aerobic plate count			
Mean ± SE	5.44±0.11[a]	4.07±0.11[b]	5.41±0.08[a]
Range	4.45–6.85	4.00–6.18	4.30–6.60
Exceed PL (%)	20%	4%	16%
S. aureus count			
Mean ± SE	3.45±0.20[a]	2.31±0.19[b]	3.47±0.12[a]
Range	1.80–4.18	1.50–3.78	1.80–3.90
Exceed PL (%)	14%	12%	20%
MPN of coliforms			
Mean ± SE	4.15±0.10[a]	2.12±0.12[c]	2.99±0.12[b]
Range	3.00–5.30	1.00–3.15	1.00–4.15
Exceed PL (%)	50%	10%	20%

Means and ranges of the examined samples are expressed as $\log_{10}$ cfu/g in case of aerobic plate count and *S. aureus* counts and expressed as $\log_{10}$ MPN/g in most probable number count.
Means carrying different superscript letters within the same row were significantly different at $p < 0.05$.
SE: standard error of mean. PL: is the permissible limits of aerobic plate count (5 $\log_{10}$ cfu/g); *S. aureus* count (2 $\log_{10}$ cfu/g) and MPN of coliforms (3 $\log_{10}$ MPN/g) according to Egyptian Organization for Standardization (EOS 2005).

Lower values of hygienic indicators were recorded in sausage compared to minced meat and beef burger that agreed with those recorded in Greece (21). This may be attributed to composition of sausage (minced meat packed in the intestine of animals). These intestines may be insufficiently cleaned, hence, lower the hygienic indicators.

In general, meat products had relatively high microbial contamination indicating inadequate measures adopted during manufacturing of such products. High contamination of meat products was reported in catering establishments in Hay Hassani district-Casablanca, Morocco (22). High microbial loads in the final products may arise from contamination of the contact surfaces of the meat products (23).

Meat products are responsible for a significant number of foodborne illnesses due to ingestion of foodborne pathogens such as *S. aureus* and *Salmonella spp. S. aureus* is considered one of the most important causes of food poisoning worldwide that is responsible for food borne intoxication due to the production of heat-stable enterotoxin.

In the current study, *S. aureus* was detected in 5(10%), 4(8%) and 5(10%) out of the examined minced meat, sausage and beef burger, respectively. This reflects unsatisfactory hygiene measures during handling and processing of meat. Food handlers may be responsible for meat contamination by *S. aureus* as a result of cross contamination from their hands (3). *Salmonella spp.* is a natural inhabitant in the intestinal tract of animals and can contaminate animal carcasses via cross contamination by meat contact surfaces, meat handlers, low hygienic standards, inadequate storage, dust and insects (23). *Salmonella spp*. was isolated only from 2 beef burger samples (4%), the isolated strains were identified as *Salmonella* Enteritidis. Similarly, *S. aureus* and *Salmonella Enteritidis* were isolated from meat products in Greece, Morocco, Algeria and China (21,22,24,25).

Emergence of multidrug-resistance among foodborne pathogens had a worldwide concern due to its public health and economic impacts. For instances, United States Centre for Disease Control and Prevention (CDC) reported that more than two millions of US population is suffered annually from drug resistant organisms (26). In addition, this number was estimated to be 400000 in Europe (27). Development of drug resistance among foodborne pathogens is mainly due to the abuse of antibiotics in the veterinary field including improper use, lack of adherence to treatment guidelines, inadequate dosing and using of therapeutic agents as feed additives (28). Several pathogenic organisms had

evolved some genetic traits to resist antibiotics as an evolutionary protection; such organisms include *S. aureus* and *Salmonella* spp.

In the current investigation, *S. aureus* isolates showed multidrug resistance profiles for AMC, CTX, DA, E, G, ME and S as indicated in Table 3. All isolated strains harboured drug resistance-related virulence attributes including *mecA, blaZ* and *aac(6')aph(2'')*. This result agreed with previous reports on *S. aureus* strains isolated from chicken meat and giblets and ready-to-eat meat products from Egypt and China (3,25). Globally, the proportions of multidrug resistant *S. aureus* especially for methicillin-resistant *S. aureus* (MRSA) combined with one or more antibiotics ranged from 20% to 80% in all WHO regions (29).

Table 3: Antibiotic susceptibility of the isolated 14 *S. aureus* and 2 *Salmonella* Enteritidis strains from meat products examined

		S. aureus			*Salmonella* Enteritidis		
	Disc concentration	**S**	**I**	**R**	**S**	**I**	**R**
Amoxicillin-clavulanic acid (AMC)	30 µg	6 (42.9)	0 (0)	8 (57.1)	0 (0)	0 (0)	2 (100)
Cefotaxime (CTX)	30 µg	6 (42.9)	3 (21.4)	5 (35.7)	1 (50)	0 (0)	1 (50)
Chloramphenicol (C)	30 µg	13 (92.9)	0 (0)	1 (7.1)	0 (0)	0 (0)	2 (100)
Ciprofloxacin (CIP)	5 µg	12 (85.7)	1 (7.1)	1 (7.1)	2 (100)	0 (0)	0 (0)
Clindamycin (DA)	2 µg	10 (71.4)	1 (7.1)	3 (21.4)	1 (50)	0 (0)	1 (50)
Doxycycline (DO)	30 µg	13 (92.9)	0 (0)	1 (7.1)	0 (0)	0 (0)	2 (100)
Erythromycin (E)	15 µg	11 (78.6)	0 (0)	3 (21.4)	1 (50)	0 (0)	1 (50)
Gentamicin (G)	10 µg	5 (35.7)	0 (0)	9 (64.3)	1 (50)	0 (0)	1 (50)
Methicillin (ME)	10 µg	0 (0)	0 (0)	14 (100)	0 (0)	0 (0)	2 (100)
Streptomycin (S)	10 µg	0 (0)	0 (0)	14 (100)	2 (100)	0 (0)	0 (0)
Sulfamethoxazole-Trimethoprim (SXT)	25 µg	13 (92.9)	0 (0)	1 (7.1)	2 (100)	0 (0)	0 (0)

The 14 *S. aureus* isolates were 5 from each of minced meat and beef burger and 4 from sausage.
Values between brackets are the percentages of the isolates showed susceptibility (S), intermediate (I) or resistance (R) to the tested antimicrobials. *mecA, blaZ and aac(6')aph(2'')* were detected in all *S. aureus isolates (n=14).blaTEM, TetA(A) and florR* were detected in the 2 *S.* Enteritidis isolates from beef burger.

All *Salmonella* Enteritidis strains were resistant to AMC, C, DO, and ME. However, only 50% were resistant to each of CTX, DA, E, and G, These strains harboured *blaTEM*, *tetA(A)* and *floR* associated resistance genes. Similarly, multidrug resistant *Salmonella* Spp. were isolated from red meat, poultry meat and processed meat products from Algeria and South Korea (24,29). Multidrug resistant *Salmonella* spp. is associated with invasive infections and increased risk of hospitalization and deaths. Recently, several studies have shown a decreased susceptibility of *Salmonella spp.* to fluoroquinolones, drugs of choice for treatment of *Salmonella*-related gastrointestinal infections. According to WHO statistics (30), the resistance percentage of *Salmonella* to fluoroquinolones had been raised to reach 35% in Africa, 49% in Middle East and 50% in Europe. Therefore, it is highly recommended to reduce the abuse of antibiotics in veterinary field and to find alternatives to antibiotics to be used as feed additives.

Conclusions

The results of this study revealed improper hygienic measures adopted during processing of meat products marketed in Zagazig city, Egypt. Furthermore, some of these meat products were contaminated with *S. aureus* and *Salmonella* Enteritidis. The isolated strains showed multidrug resistance profile. Therefore, strict hygienic measures should be followed during processing of these meat products. In addition, strong legislations should be taken in order to produce meat products of high keeping qualities.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

MONITORING OF ORGANOCHLORINE PESTICIDE RESIDUES IN *OREOCHROMIS NILOTICUS* COLLECTED FROM SOME LOCALITIES IN EGYPT

Alaa Eldin M.A. Morshdy[1], Wageh S. Darwish[1]*, Jehan R.M. Daoud[2], Mohamed A.M. Hussein[1], Maher A.M. Sebak[3]

[1]Food Control Department, Faculty of Veterinary Medicine, Zagazig University 44519, Egypt, [2]Food Control Department, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt, [3]Directorate of Veterinary Medicine, Maragha, Sohag, Egypt

*Corresponding author, E.mail: wagehdarwish@zu.edu.eg

Abstract: Tilapia nilotica (*Oreochomis niloticus*) is the major fish species consumed in Egypt, particularly due to its high nutritive value, palatability and relatively low price compared with other kinds of fishes or red meat. In Egypt, tilapia fish is caught directly from river Nile or cultured in a specified aquaculture. Organochlorine pesticides (OCPs) have been extensively used in Egypt and many African countries in the past century for the control of the agricultural pests. A major character of OCPs is their persistent bio-accumulation in the environment, especially in the food chain, where they can get reach to humans. There is few reports had investigated the current scenario of OCPs contamination in fish in Egypt, particularly in Upper Egypt cities such as Sohag. Additionally, fish is consumed cooked in Egypt not raw like many Asian countries. Therefore, this study aimed at monitoring the residue levels of different OCPs in tilapia fish caught from Upper Egypt (Sohag) and compared with either that from northern part of Egypt (Damietta) or with fish cultured in a control location (Abbasa). Additionally, the effect of different cooking methods (boiling, grilling and pan-frying) on the residue levels of OCPs was investigated. The tested OCPs included pp-DDT and its metabolites pp-DDD and pp-DDE; hexachlorohexanes (HCHs) including α HCH and γ HCH; heptachlor and heptachlor epoxide; aldrin and endrin; chlordane, methoxychlor and hexachloride benzene and were detected using electron capture gas chromatography equipped with Ni63 – electron capture detector. The recorded results revealed that tilapia collected from Damietta had the highest incidence of OCPs' contamination (75%), over than that collected from Sohag (60%) compared to control value (35%). All examined samples had OCPs residues within the maximum permissible limits (MPLs) set by world health organization. Pan-frying had the highest reduction effect on the OCPs' residues followed by grilling and boiling. The public health significance of the examined OCPs was also discussed.

Key words: Tilapia; organochlorine pesticides; cooking methods; Egypt

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Introduction

Fish such as tilapia species (*Oreochromis niloticus*) plays important roles as a food supply for Egyptian population. Fish is rich in high quality protein, essential amino acids, fatty acids and minerals such as calcium and phosphorus (1). Tilapia is the most famous fish species on the Egyptian table due its high palatability and relatively lower price compared with other fish species, red meat and poultry meats.

Organochlorine pesticides (OCPs) are persistent organic pollutants that were used over the past century in Egypt as well as in many African countries including those of the Nile basin for the control of pests and for malaria vector control. OCPs are characterized by their high bioaccumulation in the environment including aquatic system, where they can get reach to aquatic organisms and subsequently to human bodies after ingestion of contaminated fish or water (2,3).

OCPs had detected in the human milk and sera in many countries of the Nile basin including Egypt, Congo republic and Sudan (4-7). Additionally, there were positive association between para, para, dichlorodiphenyl trichloroethane (pp-DDT) and breast adenocarcinoma, reproductive disorders and fetal anomalies (8,9). OCPs are banned in Egypt and most of the world since 1980s (10). However, still some OCP compounds are used illegally to increase the productivity of the agricultural crops and for pest control; and subsequently can find their way into the water body through wastewater drainage systems. Among the most important OCPs are DDTs, hexachloride benzene (HCB), chlordane, heptachlor, aldrin, dieldrin and endrin (11). There are few reports indicating the current scenario of OCPs contamination in fresh water fish as tilapia species in Egypt, particularly in Upper Egypt cities such as Sohag.

Unlike many Asian countries such as China and Japan, fish is consumed cooked in Egypt. However, the effect of different cooking methods such as boiling, grilling and pan-frying on OCPs residue levels are less informed. Therefore, this study was undertaken to firstly monitor the residue levels of twelve OCPs in the edible tissue of tilapia species collected from Sohag (Upper Egypt) and compared with either Damietta from Northern Egypt or a control location (Abbasa aquaculture). Then the recorded levels were compared with the maximum permissible limits of different OCPs set by world health organization (WHO). Secondly, the effects of different cooking methods of fish used in Egypt (boiling, grilling and pan-frying) on the accumulated OCPs were investigated. Lastly, the public health significance of the detected OCPs was discussed.

Material and methods

Research plan guidelines of Zagazig University, Egypt were followed during conducting this research study.

Sampling

Sixty *Oreochromis niloticus* fish samples were randomly purchased from local anglers at Sohag (Upper Egypt), Damietta (Northern Egypt) and from Abbasa aquaculture in Sharkia Govern ate, Egypt that there is little information available about OCPs scenario in the selected locations. Additionally, 15 *Oreochromis niloticus* samples (initially screened as positive for OCPs contamination) were heat-treated via boiling, grilling and pan-frying (n= 5 each treatment) (12). In brief, the boiled group was placed in boiled water at 100ºC for 10 min, the grilled group was placed in an electric oven and grilled at 180ºC for 10 min, the pan-fried group was deep-fried in corn oil at 150ºC for 5 min on each side. Fish samples were homogenous in the weight and length for all sampling sites and had an average weight of 180.0±20.0 g and length of 20.0±3.0 cm. Samples were transferred into the laboratory in a cooled condition. Organochlorine pesticides were extracted and measured at Agricultural Research Center, Dokki, Giza, Egypt.

Detection of organochlorine compounds
Chemicals

Standard OCPs including pp-DDT, pp-DDD, pp-DDE, α HCH, γ HCH, heptachlor, heptachlor epoxide, aldrin, endrin, chlordane, metho-

xychlor and HCB were obtained from Sigma–Aldrich Chemie GmbH (Kappelweg, Schnelldor, Germany). Other chemicals were of the highest quality available and purchased from Merck (Darmstadt, Germany). Florisil was purchased from Silica (Silica Co., USA). All solvents were of pesticide grade. Florisil was activated at 130ºC for 24 h and cooled to room temperature.

Extraction and preparation of samples

Each muscle sample (50g) was mixed with anhydrous sodium sulfate (100g) and petroleum ether for three successive extraction steps (2 min each) (13). Samples were filtered, and then subjected to solvent evaporation using a rotary evaporator at 40ºC until dryness.

Partitioning of the extract

The method of the Association of Official Analytical Chemists (14) was followed during portioning of the extracted samples. The extracted samples were separated with a 100 ml mixture of 80:20 n-hexanes: acetonitrile. The acetonitrile layer was collected after 3 repetitive times of partitioning followed by evaporation on a rotary evaporator to a 10-ml volume that used in florisil cleanup.

Cleanup of the extract

Cleanup of the extracted samples was performed in a chromatographic column containing 20g activated florisil. After cleanup, the resultant eluent was dried on a rotary evaporator and dissolved in hexane to a volume of 10 ml. An aliquot of each extract was transferred to 2-ml injection vials to be ready for the analysis with the electron capture gas chromatography (13).

Determination of organochlorine pesticide residual concentrations

Organochlorine pesticide residues were analyzed using electron capture gas chromatography (Hewlett Packard GC Model 6890) equipped with Ni63 – electron capture detector. GC conditions: HP- 5MS capillary column (30 m length X 0.32 mm internal diameter, X 0.25µm film thickness, carrier gas: N_2 at a flow rate of 4 ml/min; injector and detector temperatures were 230°C and 300°C respectively). The extract was injected into a single inlet that was split into the dual columns. Instrumental settings were as follows: injector and detector temperatures were 230ºC and 300ºC, respectively; the gas chromatography oven temperature program was initiated at 150ºC for 5 min, raised to 170ºC (at a rate of 5ºC/min) and held for 10 min, then raised to 220ºC (at a rate of 10ºC/min) and held for 20 min (with a total run time of 44 min); the injection volume was 1, ll, and the flow rates of nitrogen gas was 20 ml/min.

Quality assurance of analytical procedures

Analysis was done based on the comparison with the created standard curves. To validate the analytical procedures, the standard reference material, SRM 1947 (Lake Michigan Fish Tissue) was similarly processed and analyzed like the samples. The recovery percentages of the tested OCPs ranged from 88% to 106%.

Statistical analysis

All recorded values were normally distributed and expressed as means ± SE. Statistical significance was judged using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey–Kramer HSD test (JMP statistical package; SAS Institute Inc., Cary, NC).

Results and discussion

Organochlorine pesticides were extensively used in Egypt over the past century. These chemicals are characterized by their high accumulation potential, so the occurrence of these OCPs in the muscles of tilapia fish was examined. The results indicated in Figure 1A showed that the tested OCPs were detected in tilapia collected from Upper and Northern Egypt as well as the reference site with varying percentages. Only pp-DDT (25%), ɤ-HCH (15%) and HPT (15%) were detected in the reference site. This may be attributed to the persistence nature of these OCPs on the environment. Tilapia fish collected from Damietta had the highest incidence for the tested OCPs compared with that from Sohag. The total OCPs' residual concentration (ng/g

ww) was very minute (14.2±1.8) in the reference site, when compared with samples collected from Sohag (69.7±8.54) and Damietta (508.83±56.55) (Figure 1B). Similarly, OCPs were detected in tilapia from the Nile basin countries as in Egypt, Ethiopia, Tanzania and Uganda (10,11,15-17).

Fish samples collected from Damietta had significantly (p<0.05) the highest pp-DDT and its metabolites pp-DDD and pp-DDE with average residual concentrations (ng/g ww) of 98.11±11.69, 55.23±7.11 and 44.15±9.76. However, only the parent compound pp-DDT was detected in the fish samples collected from Sohag (21.33±5.12) and Abbasa (12.5±4.55) (Figure 2A). Similarly, fish samples collected from Damietta had significantly the highest residues (ng/g ww) of α-HCH (52.16±6.11) and its isomer ɤ-HCH (121.0±10.25) compared with samples collected from Sohag and Abbasa (Figure 2B). The recorded concentrations in the current study were far below than the established maximum permissible limits (MPL) set by World Health Organization (WHO) (200 ng/g ww for each of DDTs and HCHs) (18). The recorded concentrations of either DDTs or HCHs were comparable to that recorded in Ethiopia and Tanzania (13,15).

Heptachlor and its epoxide metabolite were detected in the examined samples. Samples coming from Damietta had significantly the highest heptachlor and heptachlor-epoxide residues, the average concentrations (ng/g ww) of these chemicals were 18.55±1.44 and 14.33±2.11, respectively. These concentrations were 5.23±0.55 and 3.4±0.56, respectively in samples collected from Sohag. Samples collected from Abbasa had minute concentrations of HPT only (1.2±0.2) as indicated in Figure 3 A. All samples had HPT levels below MPL set by WHO (150 ng/g ww) (16). Heptachlors and its metabolites were also detected in comparable levels in fish consumed in Ethiopia (19).

Drins were tested in the presented study and were not detected in samples collected from the reference site (Abbasa). Aldrin was not detected in any fish sample. Endrin was significantly high in fish samples collected from Damietta (15.65±2.66) when compared with samples collected from Sohag (4.5±0.32) (Figure 3B). It notes worthy to confirm that the recorded drin levels were below MPL set by WHO (150 ng/g ww) (16). Nearly similar concentrations were recorded in tilapia in Ethiopia (13,19).

Chlordane, HCB and methoxychlor were not detected in tilapia caught from the reference site (Abbasa). However, all of these OCPs were detected in fish samples collected from Sohag and Damietta. Fish collected from Damietta had significantly higher levels of these OCPs compared with that from Sohag. The average residual concentrations (ng/g ww) of chlordane, HCB and methoxychlor were 10.1±2.78, 9.44±1.51 and 8.55±2.12, respectively, in samples collected from Sohag and 16.44±1.22, 41.33±6.33 and 31.88±5.21, respectively in fish samples collected from Damietta (Figure 3C). These levels were below MPLs set by WHO (18). The recorded concentrations of these OCPs were corresponding to the levels recorded in Nile tilapia in Ethiopia and Uganda (13,16). The reasons for the high load of OCPs in fish collected from Damietta compared with other sampling sites may be due to the extensive past use of these OCPs in agricultural and non-agricultural activities (10).

As fish is consumed heat-treated in Egypt and many countries worldwide, so the effects of different heat treatments on the accumulated OCPs were investigated and the results were recorded in Figure 4. Pan-frying had the highest reduction effects on such OCPs. The reduction percentages were 80%, 75%, 855 and 90% on the total DDTs, HCHs, HPTs and drins, respectively. Grilling came second after pan-frying with reduction percentages of 50%, 55%, 60%, and 65% on the total DDTs, HCHs, HPTs and drins, respectively. Boiling had the lowest reduction percentages compared with other common cooking methods in Egypt as indicated in Figure 4. These results go in agreement with Mahmoud et al. (20) who observed a clear reduction in the OCPs levels in the edible offal of cattle under different cooking methods. Therefore, it is highly recommended for fish consumers to have their fish efficiently cooked before consumption, especially in areas with high OCPs' contamination.

Organochlorine pesticides were detected in low concentrations in the present study. However, such chemicals are lipophilic and can accumulate in the breast milk to find their way into infants leading to endocrine disrupting effects (21). Additionally, such toxicants can cross the placental barriers leading to spontaneous abortion, fetal anomalies and other reproductive tract related cancers in both sexes (8,9). Therefore, continuous monitoring plans for OCPs residues in foods should be followed. Additionally, strict legislations should be adopted to avoid the illegal use of OCPs in the agricultural activities.

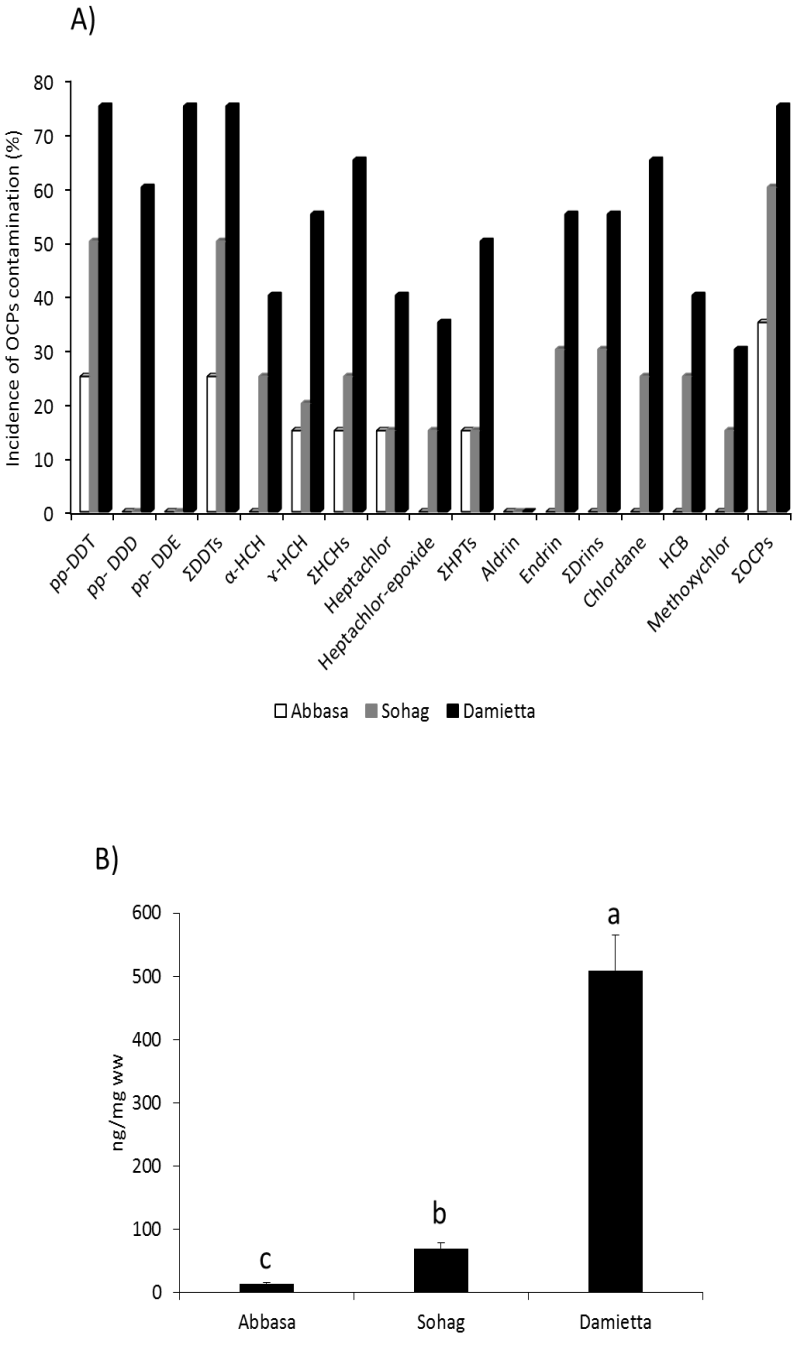

Figure 1: Incidence of *Oreochromis niloticus* OCPs contamination from some localities in Egypt. A) Frequency (%) B) Total OCPs concentrations (ng/g ww) in the examined *Oreochromis niloticus* from different locations in Egypt. Data represent means ± SE (ng/g ww) (n=20 from each location). Columns with different superscript letter are significantly different at $p < 0.05$

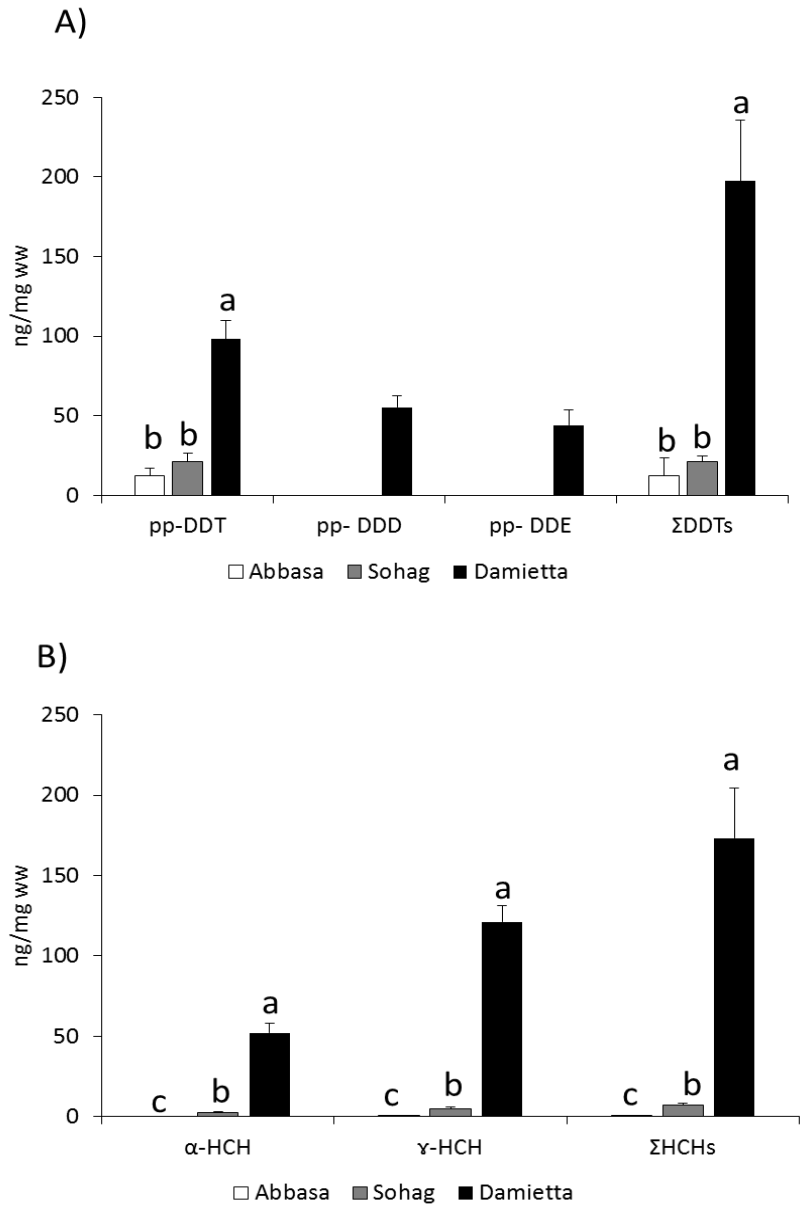

Figure 2: Levels of DDT and HCHs in *Oreochromis niloticus* marketed in Egypt. A) Total DDT and its metabolites B) Total HCH and its isomers residues in the examined *Oreochromis niloticus* collected from different locations in Egypt. Data represent mean ± SE (ng/g ww) (n=20 each from each location). Columns that carry different superscript letter among the same chemical are significantly different at $p < 0.05$

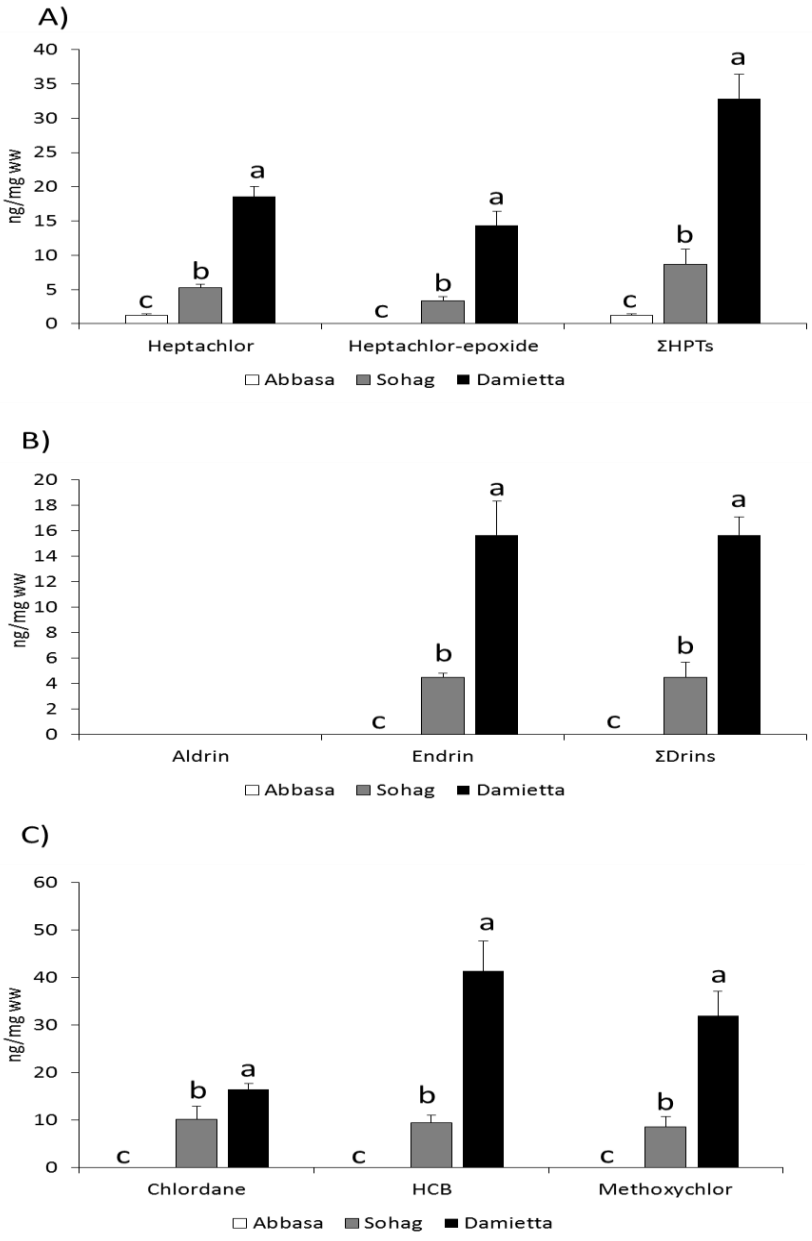

Figure 3: Levels of heptachlors, drins, and other OCPs in *Oreochromis niloticus* marketed in Egypt. A) Total heptachlor and its epoxide metabolite B) Total drins C) Chlordane, HCB and methoxychlor residues in the examined *Oreochromis niloticus* collected from different locations in Egypt Data represent mean ± SE (ng/g ww) (n=20 each from each location). Columns that carry different superscript letter among the same chemical are significantly different at $p < 0.05$

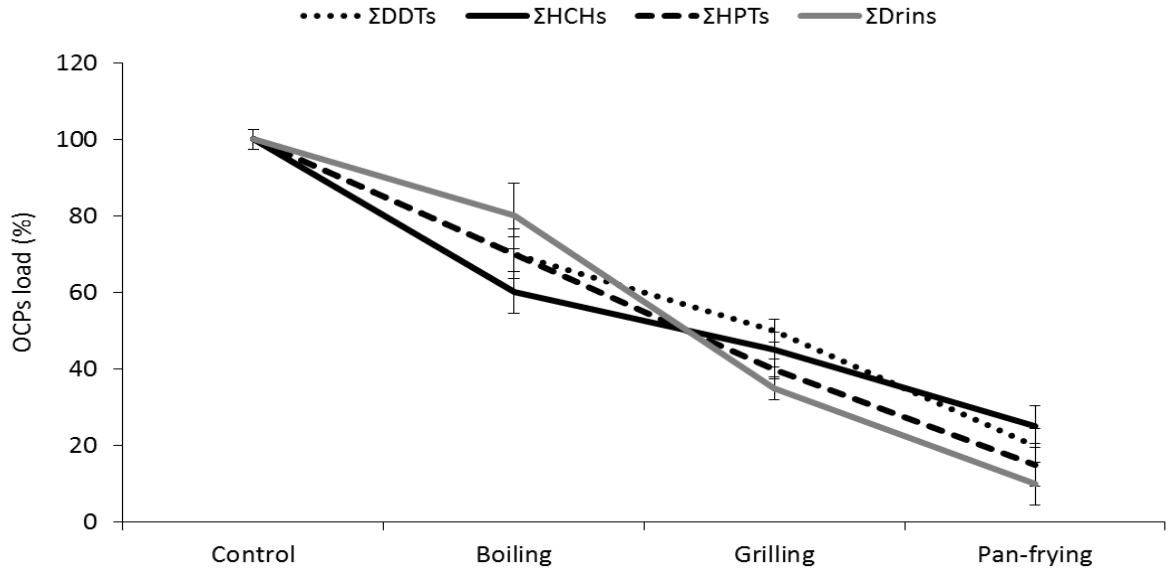

Figure 4: Effects of different cooking methods on total OCPs levels in the examined *Oreochromis niloticus.* Data show the effects of different cooking methods (boiling, grilling and pan-frying) compared with the non-heat treated (control). Data represent mean ± SE (%) for the load of the total OCPs in the examined tilapia samples collected from Damietta (n=5 each treatment)

Conclusion

The achieved results revealed contamination of tilapia fish with OCPs at low concentrations, but did not exceed the maximum permissible limits. Therefore, continuous monitoring studies for the levels of OCPs in foods of animal origin should be continued in Egypt. Efficient cooking of fish, particularly, grilling and pan-frying strongly reduced the residue levels of OCPs in fish. Thus, it is highly advisable to expose fish to efficient heat treatment, especially in areas with high OCPs contamination.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

INFLUENCE OF GAMETE CO-INCUBATION TIME, SIRE AND SPECIAL ADDITIVES ON THE *IN VITRO* FERTILIZATION OF CUMULUS-ENCLOSED OR DENUDED BUFFALO OOCYTES

Maha EL Gebaly, Hany Abdalla*, Hussein Amer, Abu Bakr Hazza

Department of Theriogenology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Sharkia, Egypt

*Corresponding author, E-mail: lotfi_hany@yahoo.com

Abstract: The present study was designed to investigate the influence of cumulus removal before the *in vitro* fertilization step and the impact of co-incubation time, sire, and additives to the fertilization medium on the efficiency of *in vitro* fertilization of buffalo oocytes. *In vitro* matured oocytes were fertilized either as cumulus-oocyte complex (COCs) or after removal of cumulus cells (denuded). Cumulus-enclosed or denuded oocytes were co-incubated with sperm cells for 6, 12 or 18 h (experiment 1), fertilized with sperm cells from one of three sires (experiment 2) or fertilized in medium supplemented with 20µg⁄ml heparin, 5 mM theophylline or a mixture 20µM penicillamine, 10µM hypotaurine and 1 µM epinephrine (PHE) (experiment 3). In all experiments, a group of oocytes was fixed and stained to evaluate the fertilization pattern (penetration, normal and abnormal fertilization rates) and the rest were cultured up to 8 days to assess the developmental competence (cleavage and blastocyst yield). In all experiments, removal of cumulus cells before fertilization step significantly retarded the fertilization pattern and the developmental competence. Various co-incubation times did not significantly influence the fertilization pattern or the developmental competence of denuded or COCs. However, 6 h tended to decrease the abnormal fertilization (15.74±1.70 vs 28.46±6.06, P=0.069) and to improve the blastocyst/oocyte (11.70±3.41 vs 5.53±1.75) and the blastocyst/cleavage (27.14±6.19 vs 11.98±3.81, P=0.082) when compared with 18 h. Sperm cells from the three sires resulted in similar fertilization pattern and developmental competence in COCs and denuded oocytes. In COCs and denuded oocytes, PHE tended to improve the blastocyst/oocyte (7.44±2.58 vs 14.67±4.29) and blastocyst/cleavage (18.43±6.08 vs 33.13±9.27) in comparison to heparin (P>0.05). Thus, none of the investigated factors could counteract the adverse effect of cumulus removal otherwise, the addition of PHE showed promising results but it need further investigations.

Key words: cumulus; co-incubation time; additives; fertilization pattern; developmental competence

Introduction

Limited efficiency of superovulation embryo transfer in buffalo gives the *in vitro* fertilization protocol (IVF) further importance in this species (1). Research in the field of buffalo IVF aims to maximize the normal fertilization pattern and the developmental competence of

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presumptive zygotes (2). The fundamental steps of IVF protocol are oocyte maturation, sperm preparation, fertilization and embryo culture. During each step, there are numerous factors influence the protocol efficiency (3). Different laboratories have exerted a lot of efforts to define the optimum conditions of buffalo IVF protocol (4). Otherwise up today most of the applied conditions are adapted from cattle protocol.

Conditions during fertilization step not only influence the fertilization pattern but also the developmental competence of presumptive zygotes. These conditions include gamete co-incubation time (5), sperm cell concentration (6,7), basic fertilization medium (8,9), sperm preparation and capacitation methods (10,11), additives to fertilization medium (12,13) and sires themselves (6,10). Moreover, interactions between the effects of sire and sperm cell concentration (6,14), sire and co-incubation time (15), sire and additives to fertilization medium (16), sperm cell concentration and cumulus existence (7), co-incubation time and basic fertilization medium (17,18), special additives and sperm cell concentration (13) have been reported in different species.

Most of the laboratories co-incubate buffalo gametes for 16-24 h, while fewer laboratories apply 6 h co-incubation time (3). Prolonging the co-incubation time to 16 h has improved the fertilization rate, but further prolongation behind16h has decreased the blastocyst/oocyte rate in buffalo (5). In cattle, maximum cleavage and blastocyst rates have been achieved after gamete co-incubation for 8 h with no further improvement after longer co-incubation time 12 and 16 h (15). Moreover, extending the co-incubation time from 5 to 10 h significantly improved the cleavage and the blastocyst rates, while further extension to 15 or 20 h significantly improved the cleavage but not the blastocyst rate (19).Basic fertilization medium is usually supplemented with special additives as heparin, caffeine, theophylline or a mixture of penicillamine, hypotaurine, and epinephrine (PHE) (6,13). These additives induce capacitation like changes in sperm cells, enhance sperm cell motility and inhibit the reactive oxygen species (6,13,16,20). Supple-mentation of fertilization medium with PHE and theophylline has increased the fertilization and the blastocyst development rates (13). Moreover, incorporation of 10μg heparin/mL into the IVF medium has increased the fertilization rate of some sires, while increasing heparin concentration to 100 μg/ml has improved or retarded the fertilization depending on the sire (6).

Common IVF protocol involves fertilization of cumulus-enclosed oocyte (COCs) followed by complete removal of the cumulus before embryo culture. Fertilization of cumulus-free oocytes (denuded) has negatively affected the fertilization pattern and the developmental competence in buffalo (7) and cow (21-23). However, partial removal of some cumulus layers before fertilization has increased the fertilization and the blastocyst development rates (24).Furthermore, fertilization of denuded cattle oocytes in cumulus cells conditioned medium or in presence of loose cumulus cells slightly improved the cleavage rate but it did not reach the COCs cleavage rate (21,22). Recently, oocyte cryopreservation has been widely practiced and it has been reported that oocyte denudation before vitrification has improved the survival rate but reduced the fertilization and the development rates (25). This point out the urgency of optimizing the fertilization conditions of denuded oocytes. In this direction, previous reports have attempted using cumulus cells conditioned medium, adding free cumulus cells or co-fertilizing denuded oocytes with COCs (21,22,25-27). However, to our knowledge, a few reports have proposed the modification of other fertilization conditions to improve the fertilization and developmental competence of denuded oocytes (7,26,28).

Therefore, the present study was designed to evaluate the effect of cumulus removal before fertilization step. Additionally, we investigated whether modification of co-incubation time, using semen from different sires and supplementation of fertilization medium with heparin, theophylline or PHE could improve the fertilization and the developmental competence of oocytes fertilized as COCs or denuded.

Material and methods

Chemicals and media

All chemicals used for preparation of different media were purchased from Sigma Chemical CO. (St. Louis, MO, USA) with exception of sodium bicarbonate, sodium pyruvate, EDTA, and sodium lactate that were purchased from Oxford (Maharashtra, India).

The basic medium used for oocyte maturation was Hepes-buffered tissue cultures medium 199 (TCM-199, M7528) and the basic medium for sperm preparation and fertilization was Brackett and Oliphant medium (BO) (29). The BO medium was composed of 112.0 mM NaC1 (,S-5886) 4.02 mM KC1 (P-5405), 2.25 mM $CaC1_2$ (C-7902), 0.83 mM NaH_2PO_4 (S-5011), 0.52 mM $MgCI_2$ (M-2393), 37.0 mM $NaHCO_3$ (S-07990), 13.9 mM glucose (S-2837), 1.25 mM sodium pyruvate (S-08302), 30 µg/ml penicillin G sodium (P- 3032) and 3 mg/ml bovine serum albumin (A -6003).

The basic culture medium was synthetic oviduct fluid (SOF) contained 107.70 mM NaCl, 7.16 mM KCl,.119 mM KH_2P0_4 (P-5655), 1.71 mM $CaCl_2$, 0.49 mM $MgCl_2$, 25.07 mM $NaHCO_3$, 3.30 mM Na lactate (L-7900), 0.33 mM Na pyruvate, 1.50 mM glucose, 5 mg/ml bovine serum albumin, 100 units/mL penicillin G sodium and 50 µg/ml streptomycin (S-1277) (30).

Oocyte collection and maturation

Buffalo oocytes were aspirated from all antral follicles in abattoir-derived ovaries. Oocytes with homogenous cytoplasm and surrounded with ≥2 layers of compact cumulus were selected for further experiment. A group of 10-15 COCs was matured in a 100µl droplet of TCM-199 supplement with 10% heat-inactivated fetal calf serum (F-6178), 0.2mM sodium pyruvate and 50µg/ml gentamycin sulfate (G -3632) at 38.5 ºC in 5% CO_2 under humidified air for 24h. At the end of maturation and before the *in vitro* fertilization, some oocytes were denuded by vortexing in TCM-199 supplemented with 10IU/ml hyaluronidase (H-4272) enzyme while others were used as COCs.

Sperm preparation and fertilization

For separating motile sperm cells, frozen-thawed sperm cells were allowed to swim up in BO medium supplemented with 0.5mM caffeine (C -0750) for 20 minutes at 38˚C. Harvested sperm cells were washed in fertilization medium that constitutes of BO medium supplemented with different additives according to the experiment. After washing in fertilization medium, *in vitro* matured oocytes were fertilized in 100µl droplet of fertilization medium at 38.5ºC in 5% CO_2 under humidified air. The duration of the sperm-oocytes co-incubation was varied according to experiment. The final sperm concentration in fertilization droplet was $5x10^6$/ml.

Experimental design

Experiment 1: The effect of different co-incubation time and its interaction with cumulus cells existence.

In this experiment, 266 denuded oocytes and 288 COCs were co-incubated with the sperm cells for 6, 12 or 18 h. The fertilization medium was BO medium supplemented with 20µg/ml heparin (H3149).

Experiment 2: The effect of different sires and its interaction with cumulus cell existence.

In this experiment, sperm cells from different sires were used to fertilize 271 denuded and 293 COCs in BO medium supplemented with 20µg/ml heparin for 18 h.

Experiment 3: The effect different additives to IVF medium and its interaction with cumulus cells existence.

In this experiment, 367 COCs and 280 denuded oocytes were fertilized in BO medium supplemented either with 20µg/ml heparin (28), 5 mM Theophylline (T-1633) (31) or a mixture of 20 µM penicillamine (P-4875), 10 µM hypotaurine (H-1384) and 1 µM epinephrine (E-4250) (32). The sperm oocytes co-incubation time was 18 h.

In all experiments, a group of fertilized oocytes was stained for assessment of the fertilization pattern, and the rest were cultured for 8 days to assess the developmental competence.

Assessment of fertilization pattern

After 18 h from the beginning of fertilization, the cumulus and sperm cells were removed through vortexing in Dulbecco′s phosphate buffered saline supplemented with 10 IU⁄ ml hyaluronidase enzyme. Denuded oocytes were fixed in aceto-ethanol (acetic acid: ethanol 3:1 v/v) and then stained with 1% orcein in 45% acetic acid (28). Oocytes with no pronucleus were defined as unfertilized while those with two pronuclei were defined as normally fertilized. Oocytes with one or more than two pronuclei were defined as abnormally fertilized.

In vitro culture and assessment of developmental competence

At the end of gamete co-incubation, the attached sperm cells or cumulus -if present- were removed by vortexing. Presumptive zygotes were washed several times in modified SOF medium then transferred into 200µl of SOF contained a monolayer of cumulus cells. The basic SOF was supplemented with 5% fetal calf serum, 100µg/ml cysteine (C-03147), 50µg/ml myoinostiol (I-7508), 30 µl/mL essential (P-6766) and 10µl/ml non-essential amino acid (M-7145) (13,30).

For preparation of cumulus monolayer, cumulus cells collected during oocyte collection were washed several times in culture medium. The final cell concentration was adjusted to 1×10^5⁄ml. The culture droplet was incubated for 48 h before culturing the presumptive zygote. The zygotes were cultivated at 38.5°C under 5% Co2 in humid air. On day four, half of the droplet (i.e 100µL) was replaced with 100 µL of conditioned SOF supplemented with 10% FCS. Therefore the final concentration of FCS in the culture droplet beginning from day 4 was 7.5%. The cleavage rate was assessed on day 2 and the morula and blastocyst rates were assessed on days 5 and 8, respectively.

Statistical analysis

All experiments were replicated three times. The effects of all variables under investigations were statistically analyzed by ANOVA using the GLM procedures of the IBM SPSS software (Version 16.0; IBM Corp., Armonk, NY, USA). In all experiments, the model included the fixed effects of either the co-incubation time (6, 12 and 18 h) or the different sires (1, 2 and 3) or the different additives (heparin, theophylline and PHE) in addition to the type of oocytes (COCs vs denuded). The model also investigated the interaction between different *in vitro* fertilization conditions and cumulus cells existence. Multiple mean comparisons were done using Duncan Multiple Range Test. All values are presented as the mean ± standard error. The differences and interaction were considered significant if $P < 0.05$.

Results

Effect of cumulus removal before IVF step

In all experiments, removal of cumulus cells before the *in vitro* fertilization step adversely affected the fertilization pattern and the developmental competence of buffalo oocytes (Tables 1-3). The penetration rate did not differ significantly between denuded oocytes and COCs in all experiments ($P > 0.05$). In experiment one, in comparison to denuded oocytes fertilization of COCs resulted in significantly higher normal fertilization, cleavage and morula rates ($P \leq 0.05$) and similarabnormalfertilization, blastocyst/oocyte and the blastocyst/cleavage rates. In experiment 2 and 3, removal of cumulus cells prior to fertilization significantly decreased the normal fertilization, the cleavage, the morula, the blastocyst/oocyte and the blastocyst/cleavage rates and significantly increased the abnormal fertilization rate (Tables 1- 3).

Effect of co-incubation time and its interaction with cumulus cells existence

Theco-incubation time did not significantly influence the fertilization pattern or the developmental competence of buffalo oocytes (Table 1). However, 6 h of co-incubation tended to none significantly improve the blastocyst/cleavage (27.14±6.19 vs 16.76±5.61 and 11.98±3.81; P=0.082). Moreover, prolonging the co-incubation time to 12 and 18 h tended to none significantly increase the mean value of the abnormal fertilization (23.12±2.31 and 28.46±6.06 vs 15.74±1.70; P=0.069).

Table 1: The effect of gamete co-incubation time and its interaction with cumulus cells existence on *in vitro* fertilization pattern and *in vitro* development of buffalo-oocytes (mean ± SE)

Factors		Pattern of fertilization				In vitro development				
		No. of oocytes	Penetration	Normal fertilization	Abnormal fertilization	No. of oocyte	Cleavage	Morula	Blastocyst/ oocyte	Blastocyst/ cleavage
Co-incubation Time	6 h	81	66.71± 2.59	50.97±3.98	15.74 ±1.70	76	40.67±4.55	16.98±3.85	11.70±3.41	27.14±6.19
	12 h	101	68.14 ±5.21	45.03±6.33	23.12±2.31	72	42.50±5.22	14.76±2.40	7.93±2.84	16.76±5.61
	18 h	121	69.79±4.90	53.82±15.5	28.46±6.06	103	43.72±5.44	13.21±1.99	5.53±1.75	11.98±3.81
Cumulus cells existence	Denuded	143	63.96 ±4.61	37.16±4.66^{b}	26.80±4.9	123	34.32±3.13^{b}	11.39±1.47^{b}	5.65±1.60	15.88±4.48
	COCs	160	72.47±1.22	62.71±10.37^{a}	18.08±1.58	128	50.27±3.22^{a}	18.57±2.20^{a}	11.13±2.42	21.37±4.43
Interaction*			0.96	0.45	0.64		0.90	0.95	0.78	0.89

Values with different superscript at the same column were significantly different within the same factors ($P < 0.05$).

*P value of interaction between different co-incubation time and cumulus cell existence. Values > 0.05 indicate no interaction between the two factors

Table 2: The effect of different sires and its interaction with cumulus cells existence on *in vitro* fertilization pattern and *in vitro* development of buffalo-oocytes (mean ± SE)

Factors		Pattern of fertilization				*In vitro* development				
		No. of oocytes	Penetration	Normal fertilization	Abnormal fertilization	No .of oocytes	Cleavage	Morula	Blastocyst/ oocyte	blastocyst/ cleavage
Sires	1	125	70.73±4.15	45.85±5.96	24.21±4.73	95	35.98±4.67	15.81±3.68	11.65±3.54	28.96±8.16
	2	98	65.79±3.01	46.04±4.72	19.74±4.20	70	42.51±5.04	17.90±3.09	10.86±2.93	27.14±6.19
	3	104	73.92±2.42	43.74±6.82	30.17±6.16	72	37.61±7.18	13.82±4.00	9.40±4.81	23.80±10.70
Cumulus cells existence	Denuded	154	66.26±2.60	33.96±3.11^{b}	32.29±4.45^{b}	117	26.73±2.34^{b}	8.60±1.76^{b}	4.35±1.45^{b}	16.93±5.90^{b}
	COCs	173	74.03±2.76	56.27±2.66^{a}	17.12±1.81^{a}	120	50.67±2.05^{a}	23.09±1.64^{a}	16.92±2.71^{a}	36.93±5.77^{a}
Interaction*			0.856	0.680	0.718		0.300	0.782	0.801	0.795

Values with different superscript at the same column are significantly different within the same factors ($P < 0.05$).

*P value of interaction between sires and cumulus cells existence. Values > 0.05 indicate no interaction between the two factors.

Table 3: The effect of different additives to fertilization medium and its interaction with cumulus cells existence on the *in vitro* fertilization pattern and *in vitro* development of buffalo-oocytes (mean ± SE)

Factors		Pattern of fertilization				*In vitro* development				
		No. of oocytes	Penetration	Normal fertilization	Abnormal fertilization	No. of oocytes	Cleavage	Morula	Blastocyst/ oocyte	Blastocyst/ cleavage
Additive	Heparin	152	68.77±4.04	40.99±6.20	27.77±3.66	102	34.43±4.43	12.99±3.09	7.44±2.58	18.43±6.08
	Theophylline	109	71.93±4.16	60.26±17.73	28.33±5.24	88	36.66±4.93	13.19±3.18	10.23±3.20	24.13±5.97
	PHE	107	74.17±4.47	48.17±5.93	26.00±4.17	89	39.73±4.60	18.78±4.74	14.67±4.29	33.13±9.27
Cumulus ells existence	Denuded	161	69.27±3.70	35.21±4.50^{b}	34.06±2.60^{b}	119	29.37±3.27^{b}	9.88±2.23^{b}	5.74±2.16^{b}	16.14±5.95^{b}
	COCs	207	73.97±2.10	64.41±9.54^{a}	20.68±2.52^{a}	160	44.52±2.27^{a}	20.10±2.83^{a}	15.83±2.36^{a}	34.33±4.30^{a}
Interaction*			0.84	0.36	0.84		0.97	0.56	0.84	0.94

Values with different superscript at the same column were significantly different within the same factors ($P < 0.05$).

*P value of interaction between different additives and cumulus cells existence. Values > 0.05 indicate no interaction between the two factors

There was no interaction between cumulus cell existence and co-incubation times for penetration, normal fertilization, abnormal fertilization, cleavage, morula, blastocyst/ oocyte, and blastocyst/cleavage; respectively.

Effect of different sires and its interaction with cumulus cells existence

Semen from the three sires under investigation resulted in a similar fertilization pattern and *in vitro* developmental competence

of buffalo oocytes. The differences among sires were very narrow in all judgment criteria (Table 2). There was no interaction between sire and cumulus cell existence.

Effect of different additives and their interaction with cumulus cells existence

Different additives used in the currents study did not influence the fertilization pattern or the developmental competence of buffalo oocytes. However, the addition of heparin to fertilization medium significantly decreased the normal fertilization rate in comparison to theophylline and PHE (40.99±6.20 vs 60.26±17.73 and 48.17±5.93). Fertilization of the oocytes in medium supplemented with PHE resulted in none significantly higher blastocyst/oocyte (14.67±4.29 vs 10.23±3.20 and 7.44±2.58; P=0.10) and blastocyst/ cleavage (33.13±9.27 vs 7.44± 5.97 and 18.43±6.08; P=0.16) compared to medium supplemented with heparin and theophylline.

Different additives used in the current study did not influence the fertilization pattern or the developmental competences of buffalo oocytes fertilized as COCs or denude. There was no interaction between the additives and cumulus existence for penetration, normal fertilization, abnormal fertilization, cleavage, morula, blastocyst/oocyte and blastocyst/cleavage; respectively.

Discussion

Undoubtedly research in the field of buffalo IVF aims to define the optimum conditions that maximize normal fertilization and later developmental ability. In the current study, removal of cumulus cells before fertilization step did not influence the penetration rate in all experiments. Some studies have stated a significant reduction in the fertilization rate after fertilization of denuded oocytes (23,26), while others showed no significant difference (33). On the other hand, higher fertilization rate after fertilization of partially denuded oocytes has been reported (24). In the current study, denudation of the oocytes before fertilization increased the abnormal fertilization which is in agreement with most of the previous studies (28,33). Taken the previous results together, it seems that enclosing the oocytes with cumulus cells during fertilization is important to achieve normal fertilization otherwise defining the necessary threshold volume of cumulus requires further investigation. During fertilization cumulus cells increase sperm motility, influence acrosomal reaction, enhance sperm-zona binding capacity, prevent abnormal spermatozoa from entering cumulus matrix and/or create optimum microenvironment around the oocytes (34).

Additionally, removal of cumulus cells before fertilization step retarded the developmental competence of buffalo oocytes. This result is in agreement with previous reports in cows (22,23) and buffaloes (7). However, partial removal of cumulus cells before fertilization has improved the blastocyst rate(24).Lower cleavage and blastocyst/oocyte rates after fertilization of denuded oocytes may be a sequel of lower penetrationrate, while lower blastocyst/cleavage rate may be a subsequent to higher abnormal fertilization rate. Furthermore, cumulus cells enclosed the oocytes may protect the oocyte or the presumptive zygotes from the hazers of oxidative stress (21) or secret embryotonic substances that improve later developmental competence (22). The positive impact of cumulus cells during fertilization step was restricted to cumulus cells enclosed the oocytes while loose cumulus cells did not have the same effect (26). Otherwise, the addition of loose cumulus cells or using cumulus cells conditioned medium to denuded oocytes slightly improved the cleavage rate but it did not reach the efficiency of COCs (21,22).

In the current study, we observed a tendency for higher abnormal fertilization and lower blastocyst yield after extending the co-incubation time above 6 h in oocytes fertilized as COCs or denuded. Maximum fertilization rate has been achieved after 8 h (26) and maximum cleavage, blastocyst/oocyte or blastocyst/cleavage have been achieved after 6 h (35), 10 h (19) in cow and 16 h in buffalo (5) with no further improvement after longer gamete co-incubation. Moreover, polyspermia has been decreased when sperm cells were removed from oocytes surface at 6-12 h after

fertilization (28). Variation among studies may be due to the interaction between co-incubation time with other factors as sires them self (15), sperm preparation method (36) and fertilization medium (17). Additionally, the current study didn't detect an interaction between co-incubation time and cumulus cells existence which is in agreement with a previous study stated that timing of sperm penetration was not modified by the cumulus (26). In addition to higher polyspermia, prolonged co-incubation time may adversely affect the developmental competence of presumptive zygotes through increasing the potential damage induced by higher reactive oxygen species (37) or sperm metabolic product (38).

The current study revealed no differences in the ability of the sperm cells from different sires to fertilize or support embryonic development of buffalo oocytes as previously reported (6,10,15,16,19). Differences among sires were attributed to different characteristics (39), different sperm-zona binding ability (40) or interaction with non-sire factors as sperm cell concentration (41), co-incubation time (15) and additive to fertilization medium (16)**.** The three bulls used in the current study showed similar performance in denuded and COCs. This may be due to the fact that the three sires used in the current study were selected sires based on their performance on IVF.

Numerous reports have stated the beneficial effect of heparin (6,16), PHE (13,32) and theophylline (31) on the fertilization pattern and the developmental competence with no previous report has compared the efficiency of the three additives under the same experimental conditions. The currentstudyshowedthat the blastocyst/cleavage and the blastocyst/oocyte were nearly duplicated after using PHE mixture in comparison to heparin otherwise this difference did not reach a significant level. This promising result was clear in denuded oocytes. Heparin, theophylline and PHE mixture improve the sperm motility, sperm activity, prolong the sperm lifespan and induce capacitation like changes in the sperm cells (6,13,16,20). Additionally, hypotaurine in the PHE may inhibit the reactive oxygen species (42). Since cumulus cells are responsible for protecting the oocytes from reactive oxygen species (43), the antioxidant activity of PHE mixture may be responsible for improved developmental competence after fertilization of denuded oocytes. Further investigation of this result is indicated.

Conclusion

In conclusion, removal of the cumulus cells before fertilization significantly retarded the fertilization pattern and the developmental competence and none of the investigated factors could counteract this adverse effect. However, supplementation of fertilization medium with a mixture of PHE showed promising improvement in the blastocyst yield after fertilization of denuded oocytes. Moreover, co-incubation time longer than 6 h is not necessary even it may have an adverse effect.

Conflict of interest

None of the authors have any conflict of interest to declare

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Original Research Article

DETECTION OF DNA ALTERATIONS IN MUSCOVY DUCKS (*CAIRINA MOSCHATA*) NATURALLY INFECTED WITH HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS SUBTYPE H5N1

Iman E. El-Araby[1], Reham M. ElBakrey[2], Haytham Ali[3,5], Mahmoud H.A. Mohamed[2], Shimaa M.G. Mansour[4], Amal A.M. Eid[2]*

[1]Department of Animal Wealth Development, [2]Department of Avian and Rabbit Medicine, [3]Department of Pathology, [4]Department of Virology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt, [5]Department of Animal and Veterinary Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, Muscat, Sultanate of Oman

*Corresponding author, E-mail: amalaeidvet@gmail.com

Abstract: Highly pathogenic avian influenza virus (HPAIV) subtype H5N1 is circulating in Egypt since 2006, with escalating apprehension about its possibility to become more transmissible amongst humans. In this study, three serial outbreaks of HPAIV H5N1 in domestic Muscovy ducks in Sharkia Governorate, Egypt were investigated. Nervous signs with 62% mortality were observed in Muscovy ducklings. Gross examination revealed severely congested meningeal vessels, hemorrhages on the duodenum, pancreas, and coronary fat. Perivascular lymphocytic cuffing, gliosis and vacuolation of the neuropil were observed in the brain microscopically. Viral antigens were identified in the neurons and the glial cells of the cerebral cortex, submucosal Meissner's plexus neurons of the intestine and the hepatic Kupffer cells by immunohistochemistry. The HPAIV subtype H5N1 was isolated from different duck tissues in 66.7% of examined duck samples. Using RAPD-PCR fingerprinting, there were different patterns in the DNA of Muscovy ducks naturally infected with AIV (24, 48 and 72 hours post appearance of clinical signs) compared to uninfected birds. Differences in RAPD-PCR profiles between infected and uninfected ducks, and genomic instability percent (37.7%±1.76) pointed to the incidence of DNA alterations induced at 24 hours following the appearance of clinical signs. Further in *vivo* and in *vitro* experiments need to be done to determine the relative importance of these findings.

Key words: GTS %; H5N1; RAPD-PCR; Egypt

Introduction

Influenza A viruses belonging to family Orthomyxoviridae, are enveloped, negative sense, segmented single-stranded RNA viruses. They are categorized into 18 haemagglutinin and 11 neuraminidase subtypes (1). According to the pathogenicity of avian influenza viruses (AIVs) in chickens, they are classified into highly pathogenic and low pathogenic viruses (2). Since 2006, highly pathogenic avian influenza virus (HPAIV) subtype H5N1 is circulating in Egypt. Uncontrolled outbreaks in both vaccinated and non-vaccinated flocks as a result of the continuous mutation (3) and evolution of new viruses has been encountered.

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Consequently, massive financial losses in the poultry industry, and proved human cases were reported (4). Beside, the uniqueness of the influenza virus replication that is unusual for an RNA virus is its dependence on nuclear functions as well as interaction with host cell, partially due to the contribution of a variety of cellular activities, resources and biosynthesis as well as transport machinery during the course of infection (5).

Ducks are considered as a crucial reservoir for AIV (6). In Egypt, ducks are nearly all reared in the backyard sector. Earlier, greenish diarrhea, nervous signs, and/or sudden death were recorded among infected ducks. The segmented RNA genomes of AIVs are unstable in duck inhabitants, being constantly rearranged by reassortment (7). Several molecular surveillances revealed close relatedness of HPAI viruses from backyard ducks and humans (8-10). Additionally, the AIV may be enduring genetic diversity, evolving into multiple genotypes. Viral and host factors are equally sharing in continuous evolution of influenza viruses (3). Acute fatal disease caused by systemic replication of influenza virus is commonly recorded with HPAIV infections (11).

Random Amplified Polymorphic DNA (RAPD) is considered as a reliable molecular technique that has benefited from the advent of the PCR (12) and used for detection of DNA alterations. The ordinary use of RAPD involves detection of differences between strains and individuals in organisms (13,14). This study is reporting the application of RAPD on DNA of brain tissues under the impact of natural HPAIV infections among Muscovy ducks in Sharkia Governorate, Egypt. We attempted to use this approach to assess differences between genome of AIV infected ducks and uninfected ones to appraise the effect of AIV infection on DNA alteration.

Material and methods

Viruses

The H5N1 HPAIV was isolated from Muscovy duck tissues during three outbreaks in Sharkia Governorate, Egypt during 2014-2016. Samples were collected for virus isolation and histopathological examination. The virus isolation from prepared tissue samples was done in nine to eleven day-old embryonated chicken eggs (ECEs) via the allantoic route following OIE directions (15). Allantoic fluids were harvested and examined with rapid HA assay using 10% chicken RBCs. At least three blind passages were carried out for the sample to be negative. The viruses were identified using reverse transcriptase-polymerase chain reaction (RT-PCR).

RNA extraction and RT-PCR

The RNA was extracted from HA-positive allantoic fluids, using Gene JET^{TM} RNA purification kit (Thermo scientific, UK). The RT-PCR was carried out using VersoTM One Step RT-PCR Kit (Thermo scientific, UK) and specific primers for the H5 gene (16) and N1 gene (17). Duck hepatitis virus type 1 (DHV-1) infection was tested by RT-PCR with 3D gene specific primers (18).

Pathology and Immunohistochemistry

The birds were necropsied and different tissue samples were collected and fixed in 10% formalin. Samples were processed in a series of alcohol concentrations, cleared in xylene, and embedded in paraffin. Five micron sections were stained with hematoxylin and eosin (H&E). For immunohistochemistry (IHC) staining, consecutive sections were mounted on positively charged microscope slides and stained using a mouse-derived monoclonal anti-influenza A nucleoprotein (NP) clone A1 and A3 (NR4282, BEI Resources; Manassas, VA) as a primary antibody at 1:200 dilution. Specific bindings were seen using the Dako EnVision system (DakoCytomation Inc. Carpinteria, CA, USA) and hematoxylin as a counter stain. Brown color appeared as a positive signal at the site of binding (19).

DNA extraction and RAPD-PCR

Three Muscovy duckling flocks were investigated using RAPD-PCR to assess the effect of AIV infection on cellular DNA. Brain tissues were collected from ducks infected with AIV at 24h, 48h and 72h following appearance

of clinical signs. Samples from uninfected Muscovy ducks were collected as well. The infection was confirmed by virus isolation, RT-PCR, and IHC in brain tissues. The DNA was extracted from AIV infected (n=27) and uninfected (n=9) Muscovy duck brains using Genomic DNA purification kit (Thermo scientific, UK). Eight random primers; OPA-08-14: GTG ACG TAG G; OPA-10-14: GTG ATC GCA G; OPA-20-14: GTT GCG ATC C; OPB-01-14: GTT TCG CTC C; OPB-11-14: GTA GAC CCG T; OPB-15-14: GGA GGG TGT T; OPC-01-14: TTC GAG CCA G; OPE-19-14: GTT GCG ATC C (Operon Technologies Inc, USA) were used for RAPD-PCR. The technique was carried out in 0.2 ml PCR tubes using DreamTaq Green PCR Master Mix (Thermo scientific, UK). The cycling conditions were as follows; 5 min at 94°C, 1 min at 94°C, 1 min at 32°C, and 2 min at 72°C, then 7 min at 72°C for final extension at the end of 40 cycles. The amplified products were run on 1.5% agarose gels. Scoring process was done; number 1 was given to the samples that produced the band and 0 to the samples that failed to produced it. Every separate DNA effect seen in the RAPD-PCR profiles (absence of bands and presence of new bands in comparison with uninfected profiles) was considered in order to assess any DNA alteration. The genomic template stability percent (GTS %) was calculated for each infected duck with the chosen primer as follows: $GTS\ (\%) = (1 - a/n) \times 100$ where "a" is the number of RAPD polymorphic profiles detected in each infected duck and "n" is the number of total bands in uninfected ducks (20).

Statistical analysis

The data were analyzed with one-way ANOVA and comparison of means was done using Duncan's multiple range tests and expressed as mean ± standard error.

Results

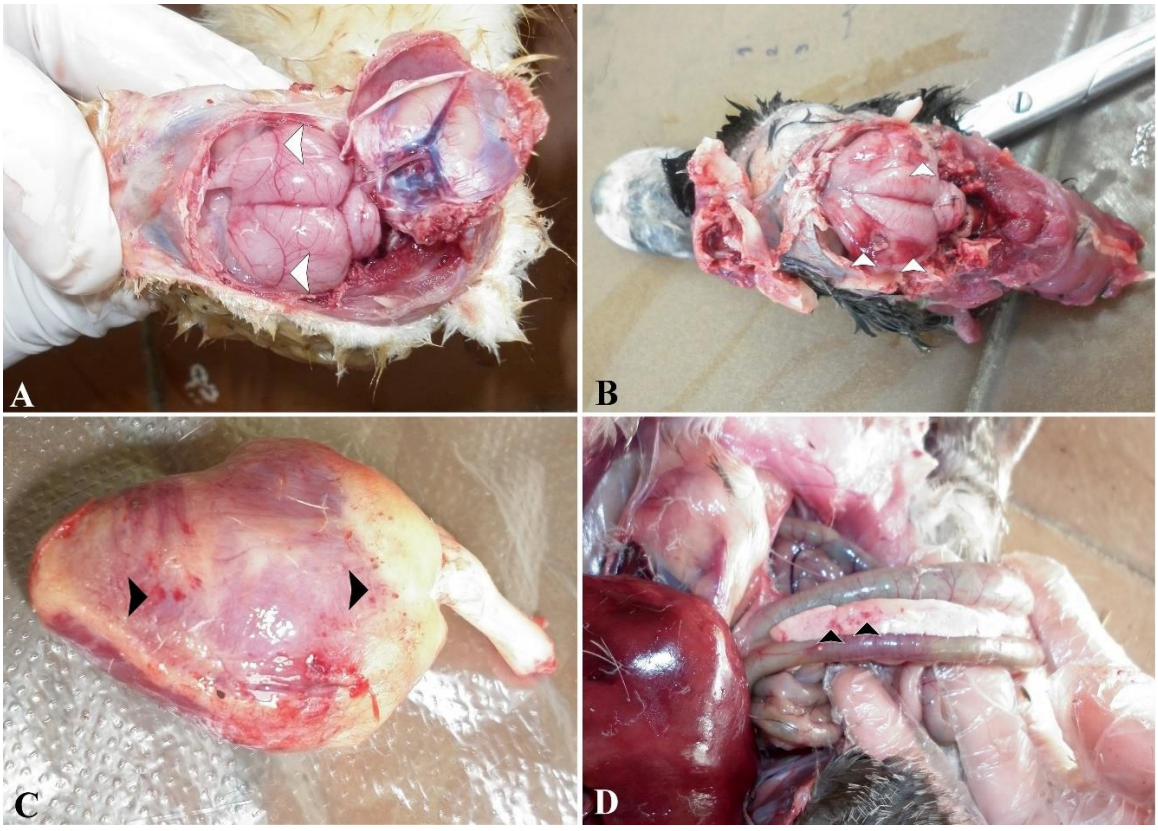

Figure 1: Muscovy ducks infected by HPAIV subtype H5N1. (A) Brain, showing severely congested meningeal vessels. (B) Brain, showing bilateral meningeal hemorrhage. (C) Heart, showing hemorrhages on coronary fat and epicardium. (D) Pancreas, showing petechial hemorrhages

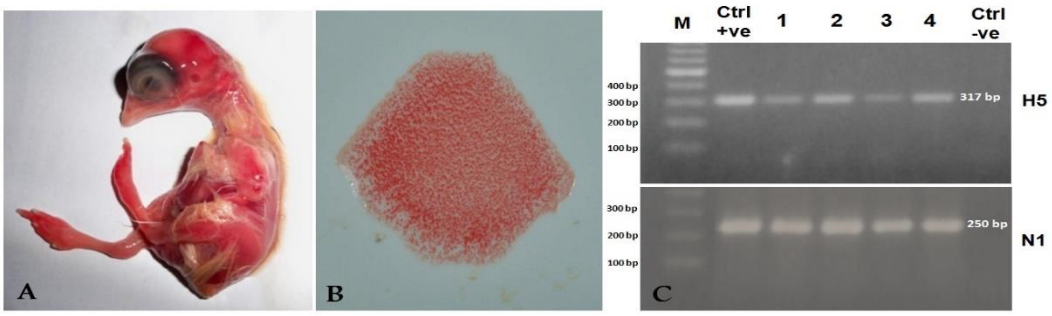

Figure 2: Virus isolation on embryonated chicken eggs and identification of AIV by RT-PCR. (A) Infected chicken embryos inoculated with the supernatants of processed brain of sampled ducks; the embryos died within 48 hrs with hemorrhages and congestion. (B) Infected allantoic fluids were screened with rapid HA assay for detection of hemagglutinating virus(s); positive hemagglutination of chicken RBCs. (C) PCR amplification for HA gene (upper) and NA gene (lower) showing bands of ~317 bp and 250 bp in size respectively. First lane: Molecular ladder, Lane 1-4: Positive samples, Ctrl +ve: Known Positive HPAIV H5N1, Ctrl-ve: Control negative

Case history, clinical signs and gross findings

The clinical signs of the examined ducks were torticollis, lack of coordination, seizures, greenish diarrhea, loss of appetite and sudden deaths. Within 2-3 days of the illness onset, the mortality rates were 37.5-63%. Gross examination revealed severely congested meningeal blood vessels, friable brain tissues (Fig. 1A) and bilateral meningeal hemorrhage (Fig. 1B) in 66.7% of examined ducks. The lungs appeared edematous, congested and severely hemorrhagic. Petechial hemorrhages on the coronary fat and the epicardium were observed (Fig. 1C) in 11% of diseased birds . Congested heart and hepatosplenomegaly were observed in 72% of birds. Petechial hemorrhages on the pancreas were seen in 22.2% of diseased ducks (Fig. 1D). Mild lesions consisting of petechial hemorrhages on the proventriculus, duodenum and ceca were observed in 16.7% of infected ducks.

Virus isolation and identification

The embryo mortalities in infected ECEs occurred within 24-72 hours post-inoculation. Depending upon embryo lesions (Fig. 2A) and mortality, 66.7% of examined duck samples showed evidence of AIV infection. The collected allantoic fluids demonstrated agglutination of RBCs in rapid HA assay (Fig. 2B) in 66.7% of ducks and further identified by RT-PCR for HPAIV subtype H5N1 (Fig. 2C). The HPAIV was isolated from different duck tissues including, brain, lungs and intestine. Duck hepatitis virus type 1 infection was excluded in original liver tissues and allantoic fluids by the RT-PCR negative result.

Histopathology and immunohistochemistry

Lesions were observed mainly in the brain, liver and pancreas. The brain sections showed gliosis, extravastaed erythrocytes in the neuropil (Fig. 3A) and perivascular lymphocytic cuffing with neuronal degeneration (Fig. 3B). The liver sections showed multiple focal lymphocytic aggregations particularly in the portal areas. Multiple focal lymphocytic aggregations were seen in the pancreas. Pulmonary edema and congestion of pulmonary blood vessels were observed. The findings were similar in all examined flocks. Pericarditis with multiple lymphocytic aggregates and hemorrhages were seen (Fig. 3C). Focal aggregations of lymphocytes in the submucosa of the proventriculus were also observed in few birds (Fig. 3D). Positive IHC staining was detected in the cytoplasm and the nucleus of the neurons, in the ependymal and glial cells of the brain (Fig. 3E) and in the submucosal Meissner's plexus neurons of the intestinal tissues (Fig. 3F). Viral antigen was also identified in the hepatic and Kupffer cells.

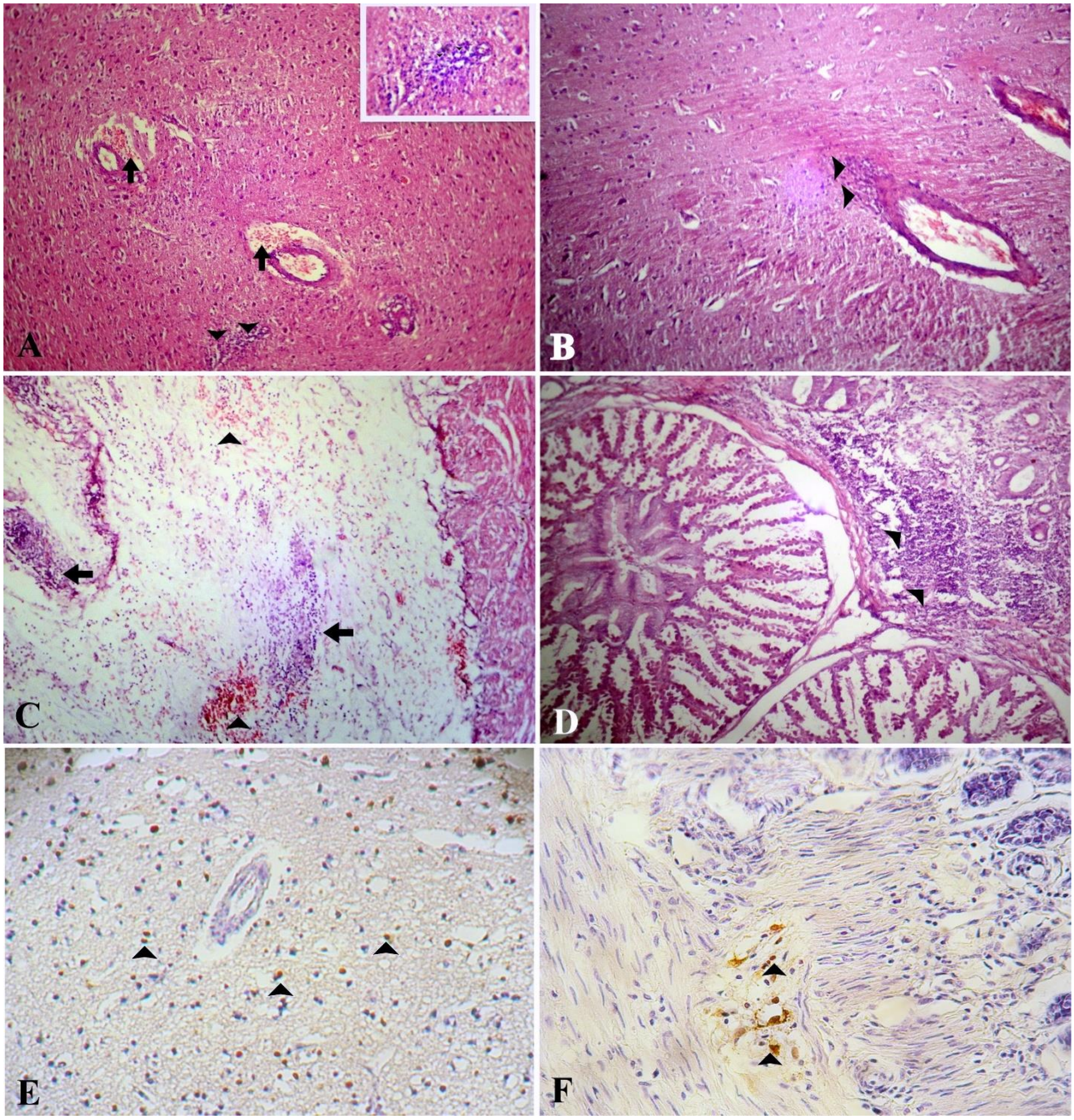

Figure 3: Microscopic lesions and positive viral antigen cells in naturally infected Muscovy ducks by HPAIV subtype H5N1. (A) Brain, extravastaed erythrocytes in the neuropil (arrows) with focal glial cells proliferation (arrow heads), H&E X100; Inset: Higher magnification, H&E, X400. (B) Brain, perivascular lymphocytic cuffing (arrow heads) with neuronal degeneration, H&E X100. (C) Pericardium, severe edema with multiple focal lymphocytic aggregates (arrows) and hemorrhages (arrow heads), H&E X100. (D) Proventriculus, showing severe lymphocytic infiltrations in the submucosa (arrow heads), H&E X100. (E) Brain, positive immunostaining of the neurons and glial cell, IHC, X100. (F) Intestine, positive immunostaining of the submucosal Meissner's plexus neurons (arrow heads), IHC X100

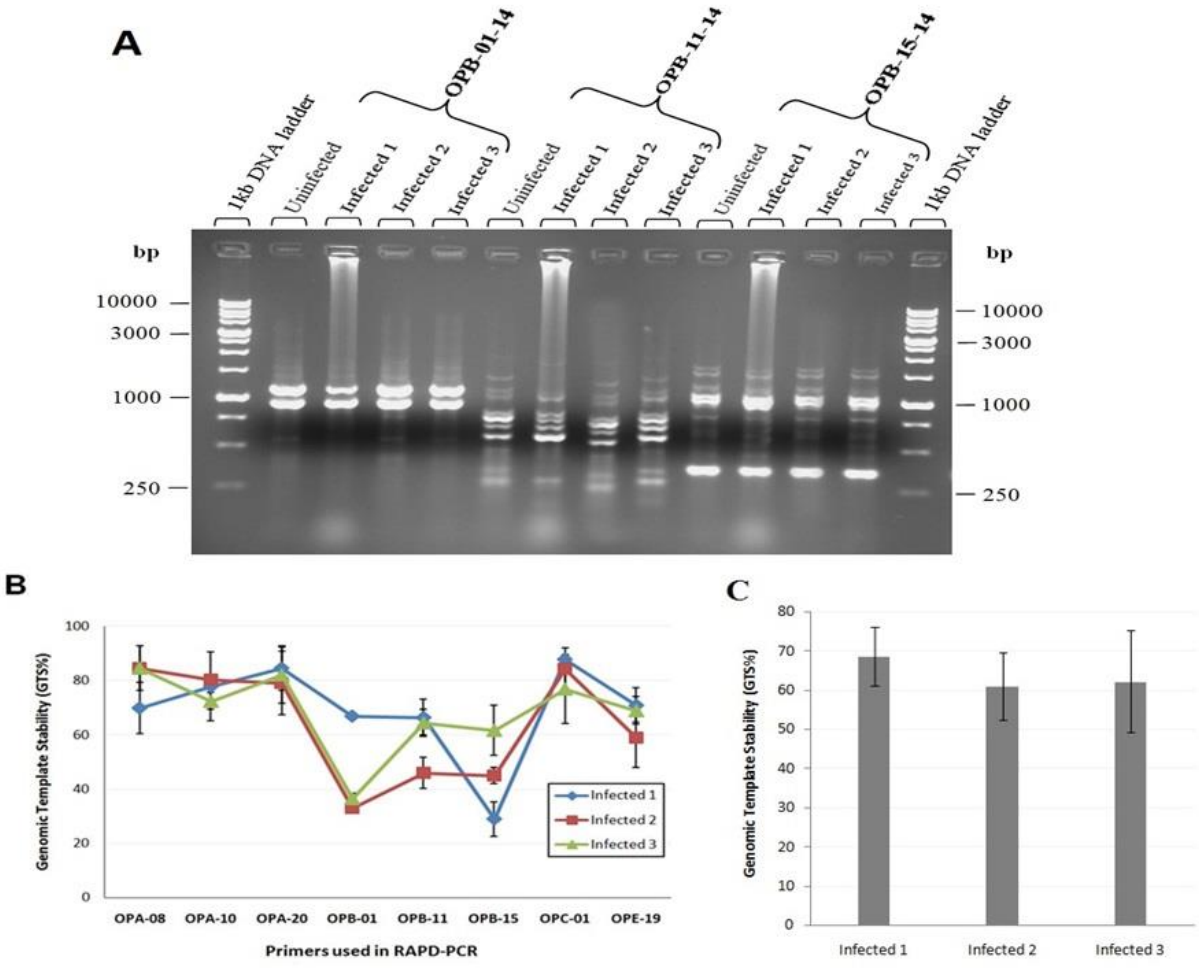

Figure 4: RAPD-PCR analysis of AIV infected at 24h (Infected 1), at 48h (Infected 2) and 72h (Infected 3) following appearance of clinical signs and uninfected Muscovy ducklings. (A) RAPD-PCR profiles of AIV infected and uninfected Muscovy ducklings. (B) Genomic template stability in brain of ducklings infected with AIV using different RAPD primers. The data shown are the mean ± Standard errors (Error bars) of the mean values from nine infected ducks per each flock. (C) Changes of GTS in brain of ducklings infected with AIV in relation to uninfected ones. The data shown are the mean ± Standard errors (Error bars) of the mean values from 8 primers

RAPD-PCR

The pooled DNAs from AIV infected ducks at 24h, 48h and 72h following appearance of clinical signs were further evaluated for any changes in comparison with uninfected ones. The gel electrophoresis showed the presence of numerous bands between 181 and 1980 bp (Fig. 4A). The variations in the RAPD profiles are expressed as reduction in GTS, a qualitative assessment refer to the change in the number of RAPD bands generated by infected ducks, regarding the profiles of uninfected ducks. The GTS% in infected ducks was shown in Fig. 4B and 4C. The statistical analysis revealed no significant differences in GTS% in infected ducks at different time points.

Discussion

Overtime, HPAI H5N1 viruses have evolved and changed their pathogenicity in ducks (21,22) with more severe disease pattern in Muscovy ducks comparing with Pekin ducks (23-25). In Egypt, the role of ducks in virus evolution is still unknown and needs thoroughly investigation particularly after the

emergence of 2.2.1.2 clade in 2014-2015, where the ancestor virus of this clade maybe a duck-origin virus (26). This study focused on the natural outbreaks of HPAIV-H5N1, by exploring the macroscopic and microscopic lesions as well as distribution of viral antigen in tissues in Muscovy ducks along the last three years.

It has been shown that ostriches and turkeys infected with low pathogenic AI viruses were expressed age-related differences in susceptibility (27,28). Conversely, HPAI viruses produce elevated morbidity and mortality in gallinaceous domestic poultry regardless of age (11,29). In consistency, here, the natural infection with HPAIV in domestic Muscovy ducks induced severe lesion and rapid deaths with high mortality rate up to 63% in 6 days old ducklings. Systemic infection was demonstrated by isolation of the HPAIV from multiple duck tissues. The increased virulence of HPAIV in ducks interferes with the disease control such is explains by persistence of HPAI virus shedding for longer periods among vaccinated ducks. Consequently the virus can perpetuate through the surrounding environment with increasing the transmission opportunity to both birds and human (22,30).

In the present study, the infected ducks showed an indicative clinical picture for AIV and/or DHV-1; although the infection with DHV-1 was excluded by negative RT-PCR results. The commonly observed lesions in Muscovy ducks were necrotic pancreatitis beside meningeoencephalitis. The capability of the HPAI H5N1 viruses to get entry to and replicate in duck brain tissue is likely to be a strong determinant in induction of morbidity and mortality (31,32). The findings in this study of high percentage of ducks (66.7%) showing nervous signs, beside high intensity of virus NP as noticed by IHC in the brain tissues, confirm the neurotropism affinity of AIV. Combination of neurological and myocardial dysfunction with multiorgan failure is involved in the pathogeneses of HPAI H5N1 viruses. Here, 11% of the affected ducks demonstrated gross and microscopic changes in the heart. Similar results were reported (33).

The current study shows that the viral nucleoprotein antigen was detected in the neurons and glial cells nuclei which strengths the speculation of virus replication in brains. Viral replication in other tissues was recognized by staining of viral antigen and associated histological lesions (34). The HPAI H5N1 virus formed a fulminating and speedy fatal systemic disease in gallinaceous birds experimentally infected with HK/220/97 (19). Revealing of viral nucleoprotein in the peripheral nervous system is not registered in the H5N1 naturally infected birds (35). Nonetheless, in our study, the nucleoprotein of H5N1 was detected in the intestinal submucosal plexusamong other tissues indicating the pantropism affinity of H5N1 avian influenza viruses.

In Egypt, the outbreaks and high mortality in both chickens and domestic ducks are indicated by continuous circulation of HPAI H5N1 in both commercial and backyards poultry industry (26,36,37). We attempt to monitor the genetic changes in the brain of Muscovy ducks after AIV infection using RAPD-PCR. The loss of bands can reveal an alteration in the sequence of DNA caused by mutations which might be induced as a consequence of the infection (38). A loss of band at 380 bp was observed in ducks infected with AIV at 48 and 72 h following appearance of clinical signs with primer OPE-19-14 indicating changes in this genomic DNA region. Such variation in electrophoretic patterns reflect the DNA alterations which could be attributable to point mutations, or structural rearranging resulting from genotoxins, affecting the primer sites and/or interpriming distances (39-41). Also, presence of new PCR products could be the result of the instability of the genomic template correlated to the level of DNA damage, and the effectiveness of the DNA repair and replication (42,43). Interestingly, a unique band at 1700 bp was observed in ducks infected with AIV with primer OPB-01-14. Moreover, GTS%, statistical qualitative analysis, could allocate the correlation of genomic stability variations with AIV infection at different time points. Here, the GTS% in infected ducklings is 62.3%±1.76 indicated the DNA alterations induced with

AIV infection in ducklings as early as 24 hour following appearance of clinical signs. Infection with influenza virus was accompanied by generation of excess reactive oxygen species (ROS) (44); so, specific oxidant sensitive pathway and apoptosis are activated (45). Consequently, further studies need to be done for measuring ROS release from AIV infected cells.

Conclusion

Generally, our findings indicate that HPAIV H5N1 is still circulating in Egypt amongst Muscovy backyard ducks causing high mortalities particularly in ducklings. Meanwhile, the RAPD profile analysis in conjunction with the evaluation of GTS% would prove a powerful investigation tool to monitor the genome changes following AIV infections. The study demonstrates the possible DNA alterations induced by AIV infections in brain tissues of ducks using the RAPD-PCR.

Conflicts of interest

All authors declare that they have no conflicts of interest.

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Original Research Article

ASPROSIN: A NOVEL BIOMARKER OF TYPE 2 DIABETES MELLITUS

Asmaa Elnagar[1]*, Hussein I. El-Belbasi[1], Ibrahim F. Rehan[2], Khalifa El-Dawy[1]*

[1]Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University, 44511 – Zagazig, [2]Department of Animal Behavior and Husbandry, Faculty of Veterinary Medicine, South Valley University, Qena, 83523, Egypt

*Corresponding authors, E-mail: dr_smsma21488@yahoo.com

Abstract: Type 2 diabetes mellitus has a deadly toll on human health. Therefore, more attention needs to be paid for the creation of biomarker to indicate the newly diagnostic type 2 diabetes and effective therapy. It well known that adipose tissue of mammals can store energy and secrete such hormones. Therefore, obesity is associated with hyperinsulinemia and insulin resistance. Recently, newly identified glucogenic hormone named “asprosin” has been developed in white adipose tissue, encoded by the gene *Fibrillin 1* (*Fbn1). Fbn1* is a 230-kb gene with 65 coding exons. These exons encode a 2,871-amino-acid long proprotein called *proFibrillin* which is proteolytically cleaved near its *C*-terminus by the enzyme furin convertase to give *Fbn1*, a member of the *fibrillin* family, in addition the 140-amino-acid long protein hormone asprosin. It has a metabolic role during fasting condition as it induces liver to secrete glucose for maintaining homeostasis as well as centrally stimulates appetite. It was reported that plasma asprosin concentrations increased in human/mice with type 2 diabetes compared with controls. However, it was significantly reduced in patients with neonatal progeriod syndrome who had characteristic features of low appetite and extreme leanness. This syndrome is due to the truncated mutation in *Fbn1*. Although the great biological role of asprosin *in vivo*, it still limited in research, particularly in the therapy of type 2 diabetes. This study aimed to provide an overview of asprosin and its possibility to be used as a novel biomarker of type 2 diabetes and obesity.

Key words: Asprosin; type 2 diabetes mellitus; *Fbn1* gene

Introduction

It was recorded that the prevalence of diabetes is expected to rise to 10.5% after twenty years (1). It is well known that adipose tissue can store energy and secrete bioactive adipokines (2-8). So, obesity is associated to hyperinsulinemia and insulin resistance (9,10). Several adipokines as adiponectin, resistin, and leptin are involved in the inflammation progress, homeostasis of glucose, and insulin sensitivity (2,3). Recently, asprosin has been discovered as a novel hormone found in white adipose tissue (WAT) of mammals (11-15). It is named asprosin, a Greek word for white “aspros”. It is well known that WAT is the original source of plasma asprosin (11). It is secreted from WAT after fasting (12,15,16) for maintaining thermal energy standards among daily meals (13). In addition it has recently been known as a glucogenic hormone that regulates glucose homeostasis (11). Moreover, asprosin has a genetic background as encoded by *Fibrillin1* gene (*Fbn1*), the precursor of a newly

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described glucogenic hormone (16-19). After analysis of *Fbn1* mRNA expression, the previous researchers confirmed the highest expression levels in WAT of humans and/or mice, suggesting the important function of *Fbn1* in adipogenesis (11,17). Therefore, asprosin is considered as a highly sensitive biomarker of type 2 diabetes mellitus (T2DM) and obesity (11,13,15).

Role of adipose tissue in glucose homeostasis

Adipose tissues can centrally control energy homeostasis. The secretion of adipokines can modulate storage of energy and essential for dissipation. New adipokines sourced from WAT, brown adipose tissue (BAT) and beige adipocytes have been detected (11). Detection of the sensitive receptors for adipokines is highly important for confirming hypothesis of the fat-derived signaling pathways that associated with energy homeostasis.

WAT represents nearly 27% of human body weight, is a key tool of endocrine system. Therefore, leptin therapy for patients with common lipodystrophy who regularly progress chronic hepatic steatosis, insulin resistance and DM has been permitted by Food and Drug Administration, 2014 (20). Even though generalized lipodystrophy is regularly linked to insulin resistance. Moreover, neonatal progeriod syndrome (NPS)-patients developed unknown symptoms of partial lipodystrophy as they kept insulin sensitive and euglycaemic. In 2016, the team discovered mutations in distinguished gene that encodes *proFbn*, named *Fbn1*, in NPS-patients. This genetic mutation was related to the asprosin levels and the *C*-terminal cleavage product of *proFbn2* (11).

Asprosin is not only secreted by WAT but also secreted by other tissues (11). For instance, mammals also have BAT which can dissipate the energy in the form of heat in order to get rid of hyperthermia and obesity. BAT has a smaller mass compared to WAT inside the body of human (e.g., ~60 g of BAT in the adulthood period of human) (21). It could be contributed as an additional endocrine organ to whole-body homeostasis. On the contrary, it was recorded that secretory molecules from BAT, named 'batokines' commonly include fibroblast growth factor 21 (FGF21), vascular endothelial growth factor A (VEGFA), neuregulin 4 (NRG4), and bone morphogenetic protein 8B (BMP8B). Altogether, is confirming the physiological importance of BAT in endocrinology (22).

It was also noticed that "beige adipocytes" is the inducible form of thermo-genic adipocytes in adult humans (23). The differentiation of this kind of adipocytes can be stimulated by exposure to cold, sever exercise, bariatric surgery or cancer cachexia; moreover, the biological importance of these adipocytes was to modulate the energy of whole-body and glucose homeostasis (24). We therefore might understand that batokines can partially mediate the metabolic improvements as well as obtained by increasing beige fat mass.

Moreover, by 2016 the researchers supported the aforementioned hypothesis. They obtained beige adipocytes from the capillaries of subcutaneous WAT of human. Then, mice with diet-induced obesity were implanted with adipocytes (25). They showed that implantation of mice with beige adipocytes of human was a reason of lower levels of fasting blood glucose, higher glucose tolerance and lower hepatic steatosis, 50 days after implantation than controls whose were implanted with matrigel as a vehicle. The improved glucose tolerance after implantation of beige adipocytes was positively correlated with the upregulation of glucose turnover rate and the secretion of adiponectin from the transplanted human beige adipocytes.

The highest *Fbn1* mRNA expression level was found in adipose tissue due to increasing asprosin levels. Moreover, the genetic ablation of adipose tissue in mice (Bscl2$^{-/-}$ mice) cause a two-fold reduction in circulating asprosin in comparable to wild-type mice (11). Furthermore, mature adipocytes in culture were able to secrete asprosin. All these findings concluded that the adipose tissue was the original source of asprosin. It is an important addition for the discovery of newly signaling molecules from adipocytes which can modulate energy homeostasis.

Definitely, detection of particular receptors of asprosin might be a new gate in biochemical

area of research to enable us identifying their target tissues/cells and to understand the mechanisms of action of asprosin. Therefore, adipose tissues can centrally control energy homeostasis due to secretion of adipokines. Meanwhile, it modulates energy storage and has the responsibility for dissipation.

Chemical structure of asprosin

Asprosin consists of 140-amino-acids protein (11,15,16). It is considered long *C*-terminal cleavage product of *proFbn* via the activation of the protease furin (26,27). Moreover, mature *Fbn1* acts as the extracellular matrix component. Meanwhile, asprosin is encoded by the two exons (65-, 66-encoded amino acids) of *Fbn1*. For instance, the exon 65 encodes 11-amino acids, whereas exon 66 encodes 129-amino acids. Together, those two exons showed an ideal model of higher vertebrate evolutionary maintenance score compared with the other *proFbns* coding sequence. In 2016, the researchers did their extensive efforts and created an asprosin-specific monoclonal antibody (mAb) and confirmed its sensitivity using *Fbn1* in wild-type and null cells (11). Combination of immunoblotting of human plasma and the mAb indicated that a single protein ran on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 30 kDa, whereas bacterially produced recombinant asprosin (r-asprosin) ran at 17 kDa, (Figure 1) (11). Asprosin is anticipated to have three *N*-glycosylation sites as post-translational protein modifications. However, that was limited in bacteria. This clearly explained the molecular weight difference between mammalian and bacteria-produced asprosin. Therefore, the expressed asprosin from mammalian cells can form a protein which was secreted into the media and therefore ran on SDS-PAGE at 30 kDa (same molecular weight) (19). After the SDS-PAGE analysis, the researchers detected asprosin in human serum/plasma, cell lysates, media of mouse embryonic fibroblasts, and cell/tissue lysates of cultured adipocytes in WAT of mice. We suggest that the structural complexity of asprosin is a challenge for researchers to continue their experiments and therefore makes it limited in research.

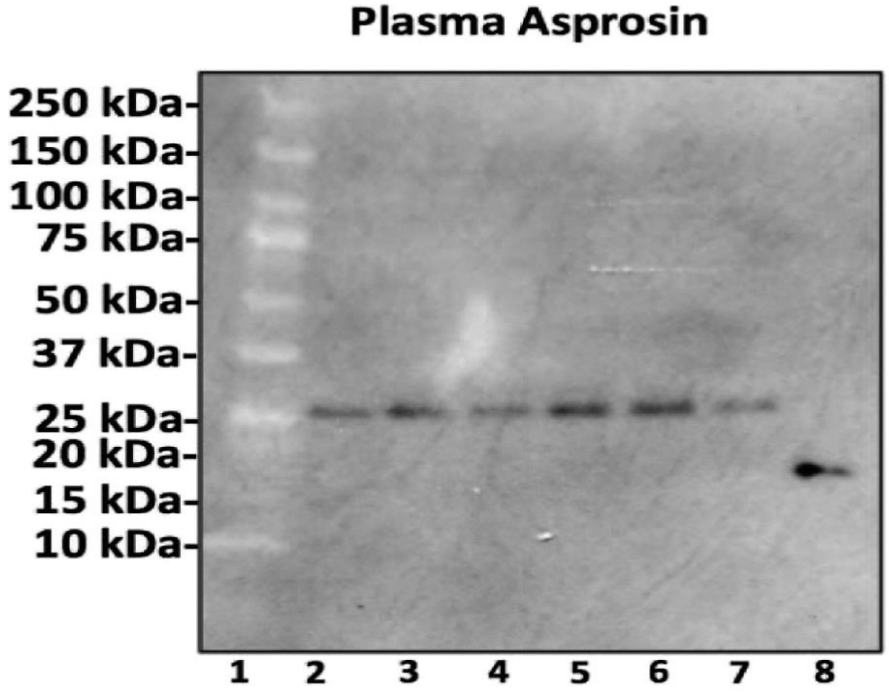

Figure 1: Asprosin immunoblotting on six individual human plasma samples (lanes 1-7), whereas bacterially produced recombinant asprosin was used as a positive control (lane 8), and the molecular weight marker (lane 1). As referenced by Romere et al. (11)

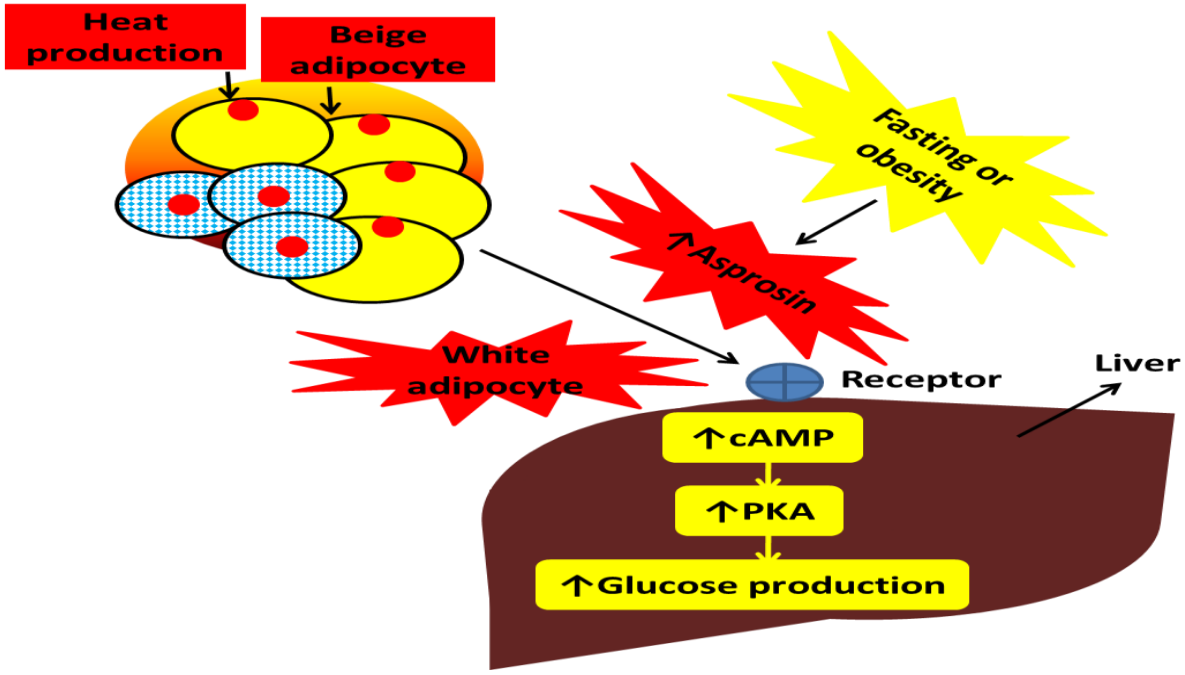

Figure 2: Schematic cartoon shows the modulation of plasma glucose homeostasis and insulin sensitivity through adipokines. The figure is drawn by the authors of this manuscript. cAMP: cyclic adenosine monophosphate receptor and PKA: protein kinase A

Mechanism of action of asprosin

Asprosin binds to the hepatocyte surface to initiate hepatocyte glucose release into the bloodstream (11,18) by crossing the blood brain barrier to activate orexigenic agouti-related neuropeptides ($AgRP^+$ neuron) via a cyclic adenosine monophosphate (cAMP)-dependent pathway (16,28). This mechanism inhibits releasing of anorexigenic proopiomelanocortin (POMC)-positive neurons in a gamma-aminobutyric acid (GABA)-dependent manner in order to stimulate appetite and to store adiposity (14). Asprosin initiates the functional role of the liver during fasting time and is critical for normal neural function among daily meals. Therefore, there is a systematic coordination among the fasting condition, appetite stimulation and hepatic glucose release via asprosin, through distinct mechanisms in both liver and hypothalamus (11). For better understanding, see our schematic cartoon in Figure 2.

Metabolic effect of asprosin

It was reported that levels of asprosin increased in the blood under fasting "a base line of glucose condition" in healthy humans and/or rodents while decreased by feeding "a high glucose condition" (11,15,16,29). Hence insulin resistance is a direct cause of T2DM, asprosin occurs with excess adiposity because WAT can modulate glucose metabolism and maintain energy homeostasis, regardless enhancing or impairing insulin action (2,4). Our schematic cartoon in Figure (3) showed the direct effect of asprosin on liver glucose level and the indirect effect on insulin release. In addition, it seems likely that obesity is dramatically associated to a constellation of T2DM and metabolic syndromes (30-32). Therefore, asprosin has a great role in glucose metabolism as well it has a negative feedback-loop by suppressing circulating asprosin (11,14).

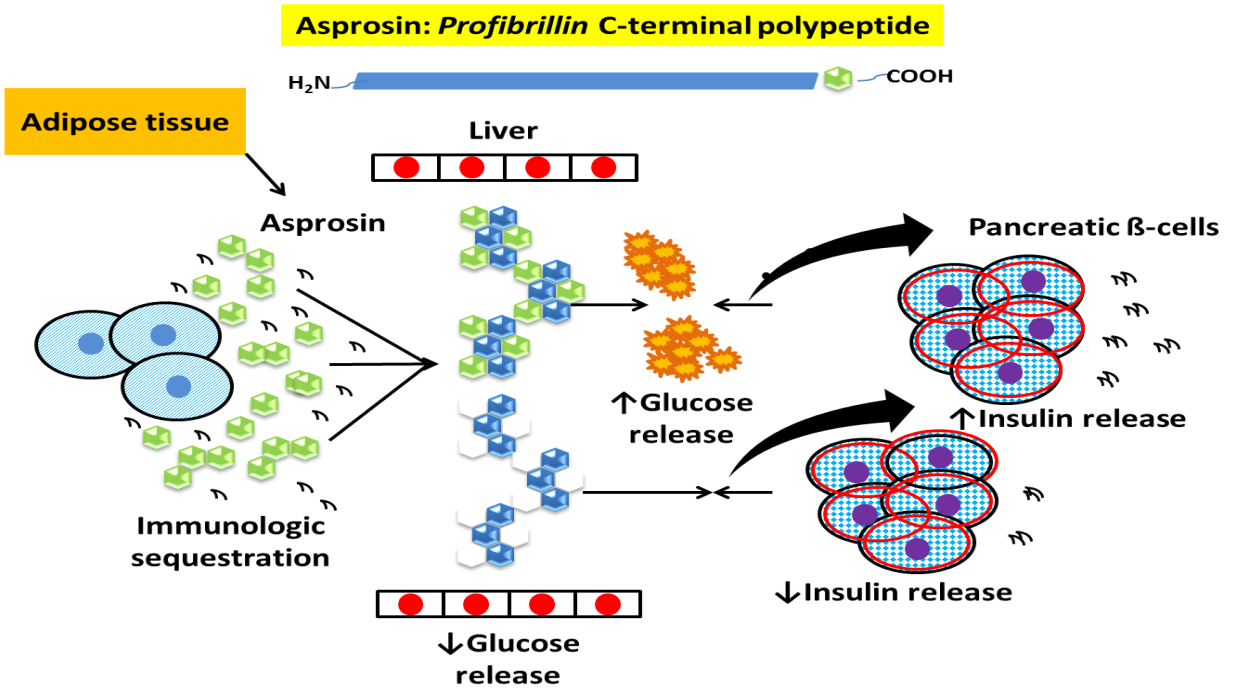

Figure 3: Schematic cartoon shows the role of asprosin in control hepatic glucose release to indirectly influence the insulin release. The figure is drawn by the authors of this manuscript

To evaluate circulating asprosin concentrations in daily basis, mice were kept at 12hr light/12hr dark program for one week to provide the optimum environmental condition, and further were put in a continuous darkness. Plasma was then isolated from these mice in 4hr intervals and then subjected to asprosin enzyme–linked immunosorbent assay (ELISA) analysis. The researchers at that time noticed that circulating asprosin showed circadian oscillation with a sharp decrease in levels equivalent with feeding time (11). In the contrary, fasting overnight in humans, mice and rats led to increase plasma asprosin. In the same study, they found a direct influence of asprosin on liver glucose while a slight influence on insulin compensatory mechanism. Moreover, inflammations are conveyed to contribute to the pathogenesis of DM (11,33). For instance, previous study indicated that asprosin stimulated liver glucose release by using cAMP as a second messenger, which was also included in the inflammatory responses (34-36). *Fbn1* exhibited a 70% down regulation in plasma asprosin in mgR hypomorphic mice. Thus pro-inflammatory cytokines were altered and contributed to the formation of inflammatory diseases, like aortic aneurysms (37). It was wondering whether circulating asprosin controlled glucose metabolism through inflammation. The high sensitivity C-reactive protein (hsCRP) levels dramatically increased among normal glucose regulation (NGR), impaired glucose regulation (IGR), and newly identified T2DM (nT2DM) groups and reached statistical significance in nT2DM-patients (14). However, a single dose of r-asprosin was administrated to mice giving a significant upregulation in blood glucose levels by stimulating hepatic glucose release (11). Therefore, they wondered whether the changed asprosin might be a real cause of glucose dysregulation, because the asprosin was an adipokine secreted by the adipose tissue. Moreover, T2DM is usually linked to the malfunction of adipose tissue which may then lead to unsuitable adipokine secretion. Although the researchers at that time did their great efforts, they had some limitations. First, they cannot consider the alteration of asprosin as a real cause of T2DM. Second, the influence of population characteristics and environmental factors on the secretory platform of adipokines

gave a negative impact to be applied on other populations. Third, not the all parameters correlated to the plasma asprosin levels were analyzed in their study. Finally, the circulating concentration of asprosin may also be associated with its catabolism, which was not evaluated in their study. Therefore, we suggest that future studies are required to assure correlation of asprosin and its potential mechanism, the insulin resistance and *β*-cell function for more clarification.

Relationship between asprosin and fasting glucose and triacylglycerol

High level of adiposity indicates a dysfunction of adipokines and considered as an evidence of metabolic diseases (5,6). It was reported that there was a positive correlations between circulating asprosin and adiposity-related parameters in T2DM-patients, including body mass index (BMI), waist circumference (WC), and waist-hip ratio (WHR) (15). This means increasing asprosin concentrations, BMI, WC, and WHR would be increased accordingly. However, several stepwise regression analysis represented that these adiposity indexes had no relation to asprosin concentrations. It was represented that the change of serum adiponectin levels and chemerin concentrations in T2DM was clearly independent of obesity (38). Moreover, it was found that the adipokine level inside the tissue is probably imbalanced to the amount entered the bloodstream (39). Therefore, it was reported that circulating asprosin had a potential influence on lipids metabolism.

Asprosin concentrations were positively correlated with triacylglycerol (TAG) regardless in healthy or in T2DM groups. Likewise, a study reported that a dyslipidemia was a common status in patients with high releasing of hepatic glucose in the insulin-resistant liver (40,41). Considering the liver as the major target organ of asprosin and therefore insulin resistance can direct us to the evidence of dyslipidemia. Therefore, asprosin was associated to the pathogenesis of lipids disorder. However, it was reported that the crude glucose area under the curve (AUC) of the rate of change (ROC) curve indicated that asprosin was not the only parameter for analyzing T2DM of different age and sex (15). To avoid the limitations of previous studies, the accuracy of asprosin for T2DM diagnosis should be done in a large-scale analysis of prospective cohort study *in vitro* as well as *in vivo*. Also it should include a comparison among NGR, IGR, nT2DM and pre-diabetes-patients, in addition to underlay the relationship between serum asprosin and the physi-opathologic mechanism of T2DM to be more elucidated. Meanwhile, serum asprosin concentrations were not sexually dimorphic in contrast to other adipokines like leptin and adiponectin (42,43). It well indicated that asprosin concentrations might not be influenced by male/female sex hormone. Altogether, is confirming the biological role of plasma asprosin in the improvement of T2DM. In addition, serum asprosin was positively linked to adiposity-dependent parameters in T2DM-patients.

Deficiency and increase of asprosin levels in the bloodstream

Asprosin was detected into the plasma/serum of humans, mice and rats. It is significantly reduced in NPS-patients due to the truncated mutation in *Fbn1* gene (i.e., loss of the *C*-terminal cleavage product of *proFbn)*, giving a critical role of asprosin (11,44). Despite NPS-patients had inslulin sensitivity, they had lipodystrophy with a low appetite and extreme leanness. This case was identical by mice having similar mutations and was completely rescued by asprosin compared with controls (11). It was reported that plasma asprosin concentration is decreased in [NPS-patients of genetic depletion, homozygous MgR mice of genetic depletion and immunologic sequestration in mice] while increased in [adenovirus-mediated overex-pression, injection of r-asprosin and nT2DM]. By this, there was a correlated change in circulating glucose and insulin (11,15). In mice, plasma asprosin stimulated circadian oscill-ation which rises during overnight fasting and are impaired with the beginning of feeding time (11). In the same study, subjecting mice to streptozotocin therapy ablated pancreatic *β*-

cells as well as impaired circulating asprosin compared with controls.

A standard curve had been established using r-asprosin to estimate plasma as well as media levels. A high specificity of the asprosin sandwich ELISA had been displayed using media from wild-type mice and *Fbn1*$^{-/-}$ cells. Asprosin was found in plasma of humans, mice and rats at regular nanomolar levels (11). Surprisingly, in the same study, NPS-patients showed a superior down regulation in plasma asprosin concentrations than those of heterozygous genotype (either wild-type mice as control subjects or patients that had heterozygous truncations of *proFbn* linked *N*-terminal) to undertake mRNA nonsense-mediated decay. Therefore, the mutation of *proFbn* NPS-patients cells has a negative reflection on asprosin release from the wild-type mice allele. Then the overexpression of the truncated mutation of *proFbn* in wild-type mice cells was examined. They found that there was an interference with the capability of those cells to release asprosin to the media in to the overexpression of other protein, like green fluorescent protein (GFP).

Therefore, circulating asprosin was clearly reduced in NPS-patient due to the truncated mutation in *Fbn1*.

To detect plasma asprosin concentration in T2DM-patients and controls, fasting overnight of 11 control subjects and 23 T2DM-patients was done and then examined 2-hs meal tolerance test in the early morning (12). The meal included 460 Kcal (e.g., 56.5g of carbohydrates, 18 g of protein and 18 g of fat). Collection of blood had been done prior and 2-hrs consequently after eating meals. To detect the level of plasma asprosin in T2DM-patient, glycosylated hemoglobin (HbA1c), fasting plasma glucose (FPG) and other biomarkers. The result in that study indicated that fasting asprosin concentrations in controls were significantly lower than those in T2DM-patients (p<0.05). Moreover, in T2DM-patients, postprandial had significant reduction of asprosin levels with a negative correlation to fasting insulin levels (p<0.05). However, asprosin levels and other parameters including FPG showed no significance. Therefore, we concluded that plasma asprosin concentrations increased in human/mice of type 2 diabetes compared with controls.

Liver is the confirmatory main target of asprosin to increase circulating glucose

After applying cell autonomous glucose and examining insulin tolerance tests, mice had been treated with a single dose of r-asprosin. The mice clearly indicated a slight alteration of glucose uptake as a positive feedback of insulin in peripheral organs, like fat or muscle (11). However, it indicated a great alteration of glucose levels for re-implicating the liver. Performing the hyperinsulinemic-euglycemic clamp confirmed that the liver was the major location of asprosin function. This test uniquely represented that plasma asprosin levels were elevated which led to increase hepatic glucose release. However, peripheral organs had no ability to upregulate glucose as a feedback response to insulin. Then the researchers exposed the mouse hepatocytes to high concentrations of r-asprosin or GFP for 2hr. As their results, the media from cells exposed to asprosin showed an increase in glucose levels in a dose-dependent manner, suggesting a potential influence of asprosin on hepatocytes. In conclusion, liver was found to be the main and direct target of asprosin to increase plasma glucose.

Labeled r-asprosin with iodine-125 (I125) was prepared, and then intravenously injected in mice, consequently after a single-photon emission computerized tomography (SPECT) scans in order to detect the sites of accumulation. A typical amount of free I125 or I125-asprosin was used as a control. That amount was boiled for 5 min in order to stimulate the wastage of the asprosin tertiary structure. After analysis, data represented that I125-asprosin trafficked initially to the liver. In addition the tertiary structure of asprosin was necessary for the liver function (11). By this, liver trafficking, gamma counting of blood and viscera demonstrated that plasma r-asprosin levels were down regulated in parallel to the upregulation of liver glucose levels. To test plasma half-life after subcutaneous injection, scientists of the same study above followed the

protocol of a sandwich ELISA-analysis pointing the *N*-terminal histidine (His)-tag to the r-asprosin protein at the following time interval, 15, 30, 60, and 120 min. After the data analysis, plasma His-tagged asprosin indicated a half-life of maximally 20 min and a peak level of 50 nM for 20 min after injection in case of infusion of I125-asprosin. Moreover, to test the specific binding of asprosin to hepatocytes, mouse hepatocytes were incubated with the excess amount of asprosin-biotin conjugates, and then washed with PBS to finally examine the relative level of biotin at the hepatocyte surface. Asprosin connected to the hepatocyte surface in a dose-responsive manner. Repeating the same protocol in the presence of a 100-fold excess unconjugated asprosin ended the effect, indicating a kind of competition and high sensitivity for potential receptor-binding sites. Altogether, is confirming the quick response of asprosin receptors with a high affinity, to bind the hepatocyte surface.

As a result, bacterially produced r-asprosin held the metabolic activity which exhibited by the endogenous produced counterpart. Meanwhile, the alteration of plasma asprosin was enough to raise blood glucose and insulin concentrations. Mice were observed to deny eating along the experiment. It is well known that this single asprosin dose resulted in an immediate signal in plasma glucose levels. However, the real cause was compensatory hyperinsulinemia at the 15 min, which normalized the plasma glucose concentrations by 60 min after injection. Likewise, this hypothesis was found in mice which were exposed to an overnight fasting although the level of the consequential plasma glucose curve was dramatically decreased. It was probably due to fasting-induced reduction of glucogenic substrates. Therefore, liver was the main target organ of asprosin because of its primary role to store glucose (e.g., glycogen) and quickly upregulated into the bloodstream during fasting. Interestingly, asprosin gave no influence on plasma concentrations of catabolic hormones (e.g., glucagon, catecholamines and glucocorticoids) in order to stimulate liver glucose release.

Asprosin upegulates cAMP and activates protein kinase A in the liver

It was reported that mice exposing to a single dose of 30-mg r-asprosin for 20 min – 50 nM peak level, upregulated the cAMP and activated protein kinase A (PKA) in the liver (11), as illustrated in a schematic Figure 2. Similarly, that was done after incubation of mouse hepatocytes with r-asprosin for 10 min. Moreover, hepatocyte PKA activity was upregulated due to the binding of r-asprosin, as what they had noticed the same story in liver glucose production. Interestingly, the physiological role of asprosin on either hepatocyte glucose release or PKA activation was ended by suramin, a general heterotrimeric G protein inhibitor. Moreover, asprosin-mediated hepatocyte glucose release might be ended using cAMPS-Rp which acts as a competitive antagonist of cAMP binding to PKA. Glucagon and catecholamines also engaged to the identical intracellular signaling axis, thus the researchers in the same study wondered what is the reason for inhibition of the glucagon receptor as well as the *B*-adrenergic receptor on the stimulation of hepatocyte glucose release through asprosin?. They understood that the corresponding inhibitors clearly ended the influences of glucagon as well as epinephrine. However, they had no evidence of the ability of asprosin to stop hepatocyte glucose release. It comes by the fact that asprosin used such cell-surface receptor system which is distinctly different from those used by glucagon and/or catecholamines. Due to insulin induces a reduction in intracellular cAMP through stimulation of the Gai pathway, the same researchers examined whether insulin might interfere with asprosin effect on hepatocyte PKA activity and glucose release. The researchers reached to the answer through an increase in intracellular cAMP. In conclusion, it was confirmed that asprosin upregulates hepatocyte glucose release by binding the G protein-cAMP-PKA axis *in vitro* as well as *in vivo*. Moreover, it was noticed that insulin suppressed asprosin-mediated hepatocyte PKA activation and led to glucose release.

The immunological role of asprosin in metabolic-syndrome-associated hyperinsulinism

It was reported that circulating asprosin concentrations were altered in controls with insulin resistance (11). Likewise, alterations were shown in two mouse models of insulin resistance (either fed diet-induced obesity or with obese gene (OB) mutation). Interestingly, a single dose of an intraperitoneal injection of asprosin as a specific mAb was enough to reduce circulating asprosin concentrations. The effect started 3 and 6hr post injection, with a back recovery within 24 hr. Both *ad-libitum*-fed (following 2hr fasting for making a synchronization program) of mouse insulin resistance represented a sharp reduction in circulating insulin along with euglycemia). That was immediately with decreasing circulating asprosin. To examine the influence of loss of asprosin on liver glucose release regardless the possible insulin compensatory mechanism, mouse hepatocytes was treated with the asprosin-specific Ab before the incubation with asprosin. As anticipated, the asprosin-specific Ab ended asprosin-mediated liver glucose release, whereas a nonspecific control Ab had no influence. To confirm immunologic sequestration as a legitimate dysfunction, *Fbn1* was examined in homozygous MgR mice (which had 20% of the wild-type *Fbn1*) (45). However, MgR mice had a 70% downregulation in circulating asprosin. It displayed a two-fold deficit in plasma insulin upon 2-hrs fasting, whereas keeping euglycemia (as the findings observed with immunologic sequestration of asprosin in *ad-libitum*-fed mice). However, upon a full-day fasting time, a physiologic situation was limited insulin release from mice. Moreover, MgR mice demonstrated fasting hypoglycemia, indicating that insulin's buffering mechanism requires to be blocked through a long fasting duration to reveal the dowregulation in circulating glucose stimulated by asprosin dysfunction. Therefore, to confirm this theory, the scientisits performed a hyperinsulinemic-euglycemic clamp research study on MgR mice. Those mice had been fasted for 18hr. It was reported that a higher acute deficit in liver glucose release in MgR than wild-type mice (11). This result was similar to clamp results indicating an upregulate in hepatic glucose production upon the efficiency of asprosin. As anticipated, clamp study could not demonstrate a change in whole-body glucose disposal, indicating that, the influence of asprosin on glucose homeostasis was poor to be served as hepatic glucose release stimulation. Therefore, a single subcutaneous injection of asprosin after overnight fasted MgR mice was enough to totally reduce the insulin. Moreover, the insulin reduction displayed in MgR mice is due to a reduction in circulating asprosin not due to the expression levels of functional *Fbn1* protein. However, any alteration of circulating insulin concentrations is indirectly reflected on the change in hepatic glucose production.

The ability of asprosin to release key phenotypes of Fbn1NPS/$^{+}$ mice

To entirely release the hypophagia phenotype of *Fbn1*NPS/$^{+}$ mice, a single subcutaneous dose of r-asprosin was injected. It indicated that NPS-linked hypophagia is due to a lower circulating asprosin and not due to genetic mutation of *Fbn1* (16). In the same study, a single intracerebroventricular (ICV) injection of r-asprosin in *Fbn1*NPS/$^{+}$ mice mainly had the ability to restore the firing level of AgRP^{+} neurons. Moreover, it mainly restored the resting membrane potential to the AgRP^{+} neurons of recombinant GFP (rGFP)-injected wild-type mice. To confirm, the mouse-NPS-linked down regulated in AgRP^{+} neuron membrane potential as well as firing regularly were fully released by a simple incubation of the hypothalamic slices with r-asprosin but not with rGFP. In conclusion, we thought that NPS-linked hypophagia and depressed AgRP^{+} neuron activities are related to a reduction in asprosin. The asprosin concentration in the blood is essential to keep regular appetite feeling and AgRP^{+} neuron function.

Crossing asprosin via blood–brain barrier of human/mice and initiating the appetite

To evaluate asprosin levels in rat, cerebrospinal fluid (CSF) was prepared, followed by asprosin-specific sandwich ELISA analysis (16). Asprosin was found in CSF at concentrations up to four-to five-folds which then decreased below that was previously reported (11). Likewise, asprosin concentration in the CSF was upregulated by overnight fasting. To evaluate whether circulating asprosin might cross the blood brain barrier and enter the CSF, rats were intravenously injected with r-asprosin. This r-asprosin had *N*-terminal His-tag and examined for the evidence of the His-tag in the CSF using ELISA technique. Thus one hour post-intravenously injection, a strong signal for the His-tag in the CSF which was fitted by six-folds upregulated in asprosin of CSF. Then, to determine whether asprosin stimulates appetite, a single dose of bacterially/ mammalian-expressed r-asprosin or rGFP was subcutaneously injected to wild-type mice. Asprosin-injected mice revealed a higher food intake than GFP-injected mice over the next-day hours regardless r-asprosin preparation was used. Importantly, mamm-alian-produced asp-rosin was twice the molecular weight of bacterially produced asprosin, and as anticipated previously (11). This alteration was mainly because of the glycosylation of the mammalian-produced protein. The plasma half-life of the mammalian-produced asprosin was up to 145 min (in comparison to 20 min for the bacterially produced asprosin), with a 60-μg subcutaneous dose producing a peak plasma levels of 40 nM. Remarkably, there was a hidden phase of a few hours after injection of asprosin and prior to the orexigenic influence was noticed, which distinguished the influences of asprosin from those of more acute-acting orexigenic subjects like ghrelin (46). Dependable on the observation of the influence of subcutaneous injection of asprosin, an orexigenic influence has been found on asprosin after entering it to the CSF through ICV-injection. To realize the chronic responses, wild-type mice were treated with subcutaneous doses of r-asprosin for 10-days interval. Moreover, the result suggested a hyperphagia, and no significance in energy, triacylglycerol content in feces, or body weight. A slightly downregulated in lean mass was observed as a result of the stress of daily injections. A significant upregulation in adiposity was also observed, which confirmed an orientation of the energy homeostasis equation in approval of increased energy intake. After using adenovirus-mediated hepatic human *Fbn1*, it was noticed its overexpression which caused by two-fold upregulation in plasma asprosin (11). Furthermore, it showed the same hyperphagic response and increasing in body weight. However, there were no significant values in energy expenditure or in lean mass. Surprisingly, there was a high level in adiposity once the mice were fed normally, and this influence was clearly abundant when subjecting the mice to a high-fat diet. Therefore, plasma asprosin can enter into the CSF through blood brain barrier to initiate appetite.

It was reported that NPS-patients of congenital lipodystrophy can affect the face and extremities (11). A novel heterozygous 3′ mutations in the gene encoding *Fbn1* in those patients had been identified after whole-*exome* sequencing. By which, expression of a truncated *proFbn* protein had been detected as well as loss of the *proFbn* carboxy-terminal cleavage products. Moreover, in another study, a virtual phenocopy of NPS with hypophagia was the real cause of the presence of a heterozygous *Fbn1-NPS* mutation in mice and further low adipose mass and body weight were identifed (16). Furthermore, the concentration of orexigenic agouti-related neuropeptides ($AgRP^+$ neuron) activity was decreased in mice (28). It was reported that circulating asprosin concentration was significantly higher in IGR and nT2DM-patients compared with those in NGR human, especially in IGR group (14). Meanwhile, in the same study plasma asprosin levels were significantly correlated with parameters of glucose metabolism, obesity, lipid profiles, insulin resistance, and β-cell function, (Table 1). However, in the same study, the trends in the alteration of first phase of glucose stimulated asprosin secretion were greatest differences of insulin secretion among

three groups. Meanwhile, researchers revealed that the plasma asprosin levels in IGR group were the highest one among all (11). That confirmed the presence of a strong protein biomarker which will predict prediabetes. For more understanding, previous study demonstrated that the plasma asprosin was pathologically elevated in human as well as in both diet-and genetic-induced animals of insulin resistance. Consistently, other researchers found a strong positive relationship between plasma asprosin concentrations and insulin resistance. Therefore, the asprosin-related glucose dysregulation might be targeted to the role of asporsin in insulin resistance. In addition, pancreatic β-cell malfunction was considered as another important pathway in the development of T2DM (47,48). Moreover, several adipokines have been reported to interact with the function, proliferation, death and failure of β-cell (49). However, the pattern of first-phase glucose-stimulated asprosin secretion was not correspondent with insulin secretion. By which, the glucose regulation role of asprosin might be independent of its impact on the first-phase insulin secretion. Therefore, the plasma asprosin might also contribute to β-cell malfunction and cause the glucose intolerance. In conclusion, plasma asprosin levels are increased in nT2DM, suggesting the critical roles of adipokines in the pathogenesis of T2DM.

Table 1: General clinical and laboratory parameters in people of NGR, IGR and nT2DM according to Wang et al. (14)

No.	Parameter	NGR	IGR	nT2DM
1	Sex (M/F)	52 (17/35)	40 (15/25)	51 (27/24)
2	Age (year)	54.73 ± 11.90	54.00 ± 9.62	53.73 ± 10.06
3	BMI (kg/m^2)	22.76 ± 3.61	23.86 ± 3.08	24.73 ± 3.55^{b}
4	Wc (cm)	81.08 ± 9.05	88.18 ± 6.48^{b}	87.71 ± 9.36^{b}
5	WHR	0.86 ± 0.06	0.87 ± 0.07	0.90 ± 0.06^{bd}
6	SBP (mmHg)	120.98 ± 14.04	125.03 ± 12.76	126.75 ± 13.71^{a}
7	DBP (mmHg)	75.42 ± 9.36	75.75 ± 9.14	79.10 ± 11.51^{a}
8	FPG (mmol/l)	5.19 ± 0.34	6.68 ± 0.25^{b}	8.27 ± 1.92^{bd}
9	2hPG (mmol/l)	4.70 ± 0.60	6.90 ± 1.17^{b}	11.79 ± 3.50^{bd}
10	HbA1c (%/mmol/mol)	5.55 ± 0.32/37	6.01 ± 0.41/42	7.78 ± 1.89/62^{bd}
11	FINS (mU/l)	5.92 ± 3.59	7.18 ± 3.79	8.25 ± 4.14^{b}
12	HOMA-IR	1.38 ± 0.88	2.14 ± 1.16^{b}	3.02 ± 1.64^{bd}
13	HOMA-β	71.03 ± 41.79	44.83 ± 22.80^{b}	38.92 ± 22.97^{b}
14	AUC	498.95 ± 481.04	165.86 ± 109.59^{b}	100.96 ± 113.99^{b}
15	AIR	62.89 ± 61.81	17.17 ± 11.69^{b}	10.93 ± 13.07^{b}
16	GDI	1.61 ± 0.45	1.30 ± 0.31^{a}	0.97 ± 0.26^{bc}
17	TC (mmol/l)	4.41 ± 0.98	4.73 ± 1.02	4.79 ± 1.08
18	TAG (mmol/l)	1.20 ± 0.50	1.98 ± 1.40^{b}	1.90 ± 1.06^{b}
19	HDL-C (mmol/l)	1.52 ± 0.52	1.32 ± 0.31^{a}	1.27 ± 0.31^{b}
20	LDL-C (mmol/l)	2.45 ± 0.95	2.84 ± 0.76^{a}	2.83 ± 1.00^{a}
21	hsCRP (mg/l)	1.37 ± 3.36	0.94 ± 0.78	2.31 ± 1.01^{ad}
22	Asprosin (ng/ml)	16.22 ± 9.27	82.40 ± 91.06^{b}	73.25 ± 91.69^{b}

2hPG: 2 h postchallenge plasma glucose; AUC: area under the curve of the first-phase (0–10 min) insulin secretion; AIR: acute insulin response; BMI: body mass index; DBP: diastolic blood pressure; FINS: fasting serum insulin; FPG: fasting plasma glucose; GDI: glucose disposition index; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment for insulin resistance; HOMA-β: homeostasis model assessment for beta-cell function; hsCRP: hypersensitive C-reactive protein, IGR: impaired glucose regulation; LDL-C, low-density lipoprotein cholesterol; NGR: normal glucose regulation; nT2DM: newly diagnosed type 2 diabetes; SBP: systolic blood pressure; TC: total cholesterol; TAG: triacylglycerol; Wc: waist circumference and WHR: waist hip ratio. Data are reported in (14), means ± SD. $^{a}P < 0.05$ in comparable to NGT; $^{b}P<0.01$ in comparable to NGT; $^{c}P < 0.05$ in comparable to pre-DM; $^{d}P<0.01$ in comparable to pre-DM.

Trials to use asprosin for treatment of T2DM

The circulating asprosin might be a predictor of early diagnosis in DM. Moreover, it has a good possibility to be used as a potential therapeutic target for pre-diabetes and T2DM. However, few trials have been done to discuss in this regard. For instance, to make a replenishment of circulating asprosin *in vivo*, a single subcutaneous injection was totally rescued hypophagia in *Fbn1*-NPS/$^{+}$ mice. That demonstrated the binding of NPS-associated hypophagia to the reduction of circulating asprosin (11). Moreover, it was mentioned that a single subcutaneous dose of r-asprosin induced an immediate curve in plasma glucose concentration of fasted mice that was resulted in a compensatory hyperinsulinemia mechanism to normalize blood glucose concentrations within 60 min. Therefore, balancing of asprosin in the plasma with a mAb reduced appetite and body weight in obese mice in order to normalizing the plasma glucose level (16). Remarkably, circulating glucose and insulin were increased within 30 min after a single injection of r-asprosin to wild-type mice (18). Moreover, asprosin can cross the blood–brain barrier after intravenous injection to activate the hypothalamic feeding circuitry and initiate appetite stimulation. In a long term, this therapy will maintain adiposity (16). On the other hand, neutralizing specific mAb against asprosin or genetic deletion of *Fbn1* can block asprosin action through reduced circulating concentrations of insulin and hepatic glucose production *in vivo*. It is important to say that asprosin is a fasting-induced adipokine which controls liver glucose release and insulin sensitivity (11,18). As shown in Table 1, plasma asprosin levels were significantly correlated with several parameters of glucose and lipid metabolic disorders like glucose metabolism, obesity, lipid profiles, insulin resistance, and β-cell function, etc (14). By which, it was reported that plasma asprosin level was positively correlated with waist circumference, FPG, TAG, 2hrs-post-challenge plasma glucose (2hPG), and homeostasis mechanism leaded to insulin resistance. However, it was significantly negatively correlated with homeostasis model to evaluate the β-cell function, AIR, AUC (0–10 min) insulin secretion, and glucose disposition index (GDI), (P<0.05, each). It was mentioned that fasting serum asprosin levels in control humans were significantly lower than those who had T2DM, (P < 0.05). Moreover, in T2DM-patients, reduction of asprosin concentrations after eating meals showed a significant negative correlation with fasting insulin concentrations, (P<0.05). However, there was no significant correlation between asprosin levels and other parameters like FPG (49). In other report, plasma asprosin levels were abundantly increased, (P<0.001) in the T2DM-adult tertiles compared to controls (15), and concluded that fasting glucose and TAG were greatly associated with plasma asprosin in T2DM. On the other side, the remaining asprosin levels among obese of the both sex (19.9±36.7 and 7.1±7.5, male and female respectively) were relatively the same comparable to non-obese (9.2±9.5 and 12.4±10.4). These similarities of asprosin levels resulted from individuals' experiences of sever exercise with the high inter-individual distributions. However, the low intra-individual spreading provided to obese as well as lean subjects (13). Altogether, confirmed the biological importance of asprosin *in vivo*, and its possibility *in vitro* to treat T2DM.

Conclusion

Eventually and after extensive effort done by scientists, asprosin is newly identified hormone would contribute greatly to the improvement of health. Asprosin found in white adipose tissue in mammals. It is positively correlated with T2DM and obesity due to the direct stimulation of hepatic glucose and appetite. However, it is negatively correlated with NPS-patients due to a genetic mutation of *Fbn1*. Our approach provides an overview of asprosin and its possibility to be used as a biomarker for early diagnosis of T2DM. Based on the recent view of literatures, asprosin antibody is a safe and efficient therapy of T2DM in a very short onset. Therefore, it is important to recommend physicians, chemists and also DM-patients to

use antibody of asprosin as a novel and effective therapy of T2DM.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

PREVALENCE OF SHIGA TOXIGENIC AND MULTI DRUG RESISTANT *Escherichia coli* IN READY TO EAT CHICKEN PRODUCTS' SANDWICHES

Alaa Eldin M.A. Morshdy, Mohamed A. Hussein*, Ahmed E. Tharwat, Nafissa A. Moustafa, Osama K. Hussein

Food Control Department, Faculty of Veterinary Medicine Zagazig University, Zagazig 44511, Egypt

*Corresponding author, E-mai: elged2010@yahoo.com

Abstract: Ready to eat (RTE) chicken products are known for their popularity among people from different age groups in Egypt. Two hundred and fifty samples of RTE chicken sandwiches represented by chicken fajitas, shawarma, burger, pane and luncheon (50 for each) were collected and examined for prevalence, virulence and resistance of *Escherichia coli* being one of the most important enteropathogens worldwide. The obtained results declared the presence of *E. coli* in 42, 34, 30, 26 and 14% of the examined samples, respectively. The isolates were found to belong to different *E. coli* pathotypes such as enteropathogenic, enterohemorrhagic, enterotoxigenic and enteroinasive and were positive for serious virulence genes (*Stx*1, *Stx*2 and *eae*A). Moreover, the isolates were tested for their resistance against fourteen commonly used antimicrobials in order to determine their resistance patterns which consequently would reflect their public health significance as well as the degree of drug misuse within the food production chain.

Key words: *E. coli*; antimicrobial resistance; STEC; chicken products; diarrheal diseases

Introduction

Chicken meat is considered as a good source of high quality animal protein, especially in Egypt, where shortages in red meat production are common. Chicken meat is introduced to processing sector and sold as chicken products; further heat treatments are applied in restaurants or at street vending sites, then sold as ready to eat (RTE) chicken meat sandwiches. Ready to eat chicken sandwiches are appreciated for their unique flavors and accessibility. They also provide food security for low income population and livelihood for a significant percentage of the populace in many developing countries (1). The importance of RTE food as a carrier of several microbes has been established, especially street vended food where hygienic standards of preparation are not strictly followed or enforced (2). The fact that very few illnesses can be linked to RTE food with certainty makes it not easy to estimate the problem of foodborne diseases, and these links are usually made only during outbreak situations (3). *Escherichia coli* is one of the most predominant facultative anaerobes of both human and other warm-blooded mammals and often remains harmlessly in the intestinal lumen. Usually, it is considered as an indicator of fecal contamination in food and water;

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however, several *E. coli* clones have the ability to cause diseases within the intestinal tract and in other organs of the host. Pathogenic *E. coli* could be classified into six main pathotypes, including shiga-toxin producing *E. coli*, enteropathogenic *E. coli,* enterotoxigenic *E. coli,* enteroaggregative *E. coli*, enteroinvasive *E. coli,* and diffusively adherent *E. coli* (4). *E. coli* has the ability to transmit, acquire and conserve resistance genes from other bacteria in the environment (5). The rate of antimicrobial resistance in *E. coli* acts as an indicator of resistance transmission in bacterial populations and also as an indicator for the antimicrobials used in the treatment of slaughter animals and humans (6). Therefore, this study was conducted to detect the prevalence of shiga toxin-producing and multi drug resistant *E. coli* in RTE chicken sandwiches collected from different restaurants and street vendors in Zagazig, Sharkia, Egypt.

Material and methods

Samples collection and preparation

Two hundred and fifty RTE chicken sandwiches were collected randomly from different restaurants at Zagazig city, Sharkia Governorate, Egypt, at different sanitation levels during the period from September 2017 till June 2018. The collected samples represented by 50 chicken sandwiches each of fajitas, shawarma, burger, pane and luncheon. Twenty five grams of sandwich core plus 225 ml of sterile peptone water (0.1%) were added and thoroughly homogenized in a sterile blender at 200 rpm for 1-2 min. to provide a homogenate of 1\10 initial dilution (7).

Isolation and identification of E. coli

One ml of the homogenate was added to 9 ml of MacConkey`s broth containing durham's tube then incubated at 44±0.5 °C for 48 hs. A loopfull from each positive tube (acid and gas) of MacConkey`s broth was streaked onto eosin methylene blue (EMB) agar. The inoculated plates were incubated at 37°C for 24 hs. Typical colonies of *E. coli* appear greenish, metallic with dark purple center. Suspected colonies were purified and subcultured onto nutrient agar slopes and incubated for further investigations (8). Morphological and biochemical identification of *E. coli* were then performed as described previously (9-11). The isolates were serologically identified (12) using rapid diagnostic *E. coli* antisera sets (DENKA SEIKEN Co., Japan) for diagnosis of the enteropathogenic types.

Multiplex PCR for detection of toxin producing genes of E. coli

DNA was extracted from *E. coli* isolates using QIAamp DNA Mini kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. Oligonucleotide primers (Pharmacia Biotech, Sweden) used for amplification of *E. coli* virulence genes (*stx*1, *stx*2 and *eae*A) are shown in Table (1). PCR assay was carried out in 1 µl of nucleic acid template (approximately 30 ng of DNA), 10 mM Tris-HCl (pH 8.4), 10 mM KCl, 3 mM MgCl2; 2 mM concentrations of each primer, 0.2 mM concentrations of each 29-deoxynucleoside 59-triphosphate, and 4 U of Ampli*Taq* DNA polymerase (Perkin-Elmer). PCR cycling protocol was performed in a programmable Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany) as following: an initial denaturation at 95°C step for 3 min. followed by 35 cycles of 95°C for 20 sec, 58°C for 40 s, and 72°C for 90 sec then final extension at 72 °C for 5 min (13). *E. coli* O157:H7 Sakai (positive for *stx*1, *stx*2 and *eae*A) and *E. coli* K12DH5α (a nonpathogenic negative control strain) were used as reference strains. Amplified DNA fragments were analyzed by 2% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer stained with Ethedium bromide and captured as well as visualized on UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

Table 1: PCR primers used for multiplex PCR for detection of shiga toxin genes in isolated *E.Coli* from ready to eat chicken sandwitches

Oligonucleotides sequence (5′–′3)	Specificity	Amplicon Size (bp)	Reference
F-**ACACTGGATGATCTCAGTGG** **R-CTGAATCCCCCTCCATTATG**	Shiga toxin type 1	614	(14)
F- **CCATGACAACGGACAGCAGTT** **R-** **CCTGTCAACTGAGCAGCACTTTG**	Shiga toxin Type 2	779	
F- **GTGGCGAATACTGGCGAGACT** **R- CCCCATTCTTTTTCACCGTCG**	Intimin	890	(15)

Antimicrobial susceptibility testing of E. coli isolates

Antimicrobial susceptibility of *E. coli* isolates was performed according to Kirby-Bauer disc-diffusion procedure as described elsewhere (16). The following antimicrobial discs were tested: Penicillin (P), Erythromycin (E), Oxytetracycline (OT), Nalidixic acid (NA), Ampicillin (AM), Sulphamethoxazol (SXT), Cephalothin (KF), Enrofloxacin (ENR), Oxacillin (OX), Neomycin (N), Chloramphenicol (C), Kanamycin (K), Ciprofloxacin (CIP) and Gentamicin (CN) (Oxoid Limited, Basingstoke, Hampshire, UK). The interpretation of the results was done according to Clinical and Laboratory Standards Institute (CLSI) (17). Multiple antibiotic resistance (MAR) index for each isolate was determined according to the following formula (18):

MAR index= No. of antimicrobials to which the isolate is resistant (isolates classified as intermediate were considered sensitive for MAR index) / Total No. of tested antimicrobials.

Results and discussion

Diarrheal diseases are imposing a great hurdle to public health universally, being included in the list of the top ten global causes of death worldwide (19). That fact has attracted our attention to perform a study in order to assess the prevalence, resistance and virulence of *E. coli* in some RTE foods, considering the fact that this microorganism is one of the most important causes of gastrointestinal tract (GIT) disturbance and diarrhea throughout the world (20).

The results presented in Table (2) showed that 42% of chicken fajitas samples harboured *E. coli*. These results were much higher than those obtained previously (21). On the other hand, about 34% of chicken shawarma samples were *E. coli* positive and these results were lower than those recorded by several authors (22,23). Regarding chicken burger samples, the presented study showed that *E. coli* was detected in 30% of the examined samples and this result was higher than that obtained latter (24). Furthermore, about 26% and 14% of chicken pane and chicken luncheon samples, respectively were positive for *E. coli*. These results were higher than those obtained by Samaha et al. (25), while higher results than those of the current study for chicken pane and chicken luncheon samples, respectively were previously detected (23,26).

Although, *E. coli* is readily inactivated above 55°C, the post cooking cross contamination due to contact of raw ingredient with cooked meat may account for prevalence of *E. coli* in the tested RTE chicken sandwiches (27). Also the obtained results showed that chicken fajita samples had the highest prevalence of *E. coli* among the tested samples, and this may be due to inadequate cooking and post processing contamination.

Table 2: Prevalence and serotypes of *E.coli* in ready to eat chicken sandwiches (N=250)

Chicken products		Fajitas	Shawarma	Burger	Pane	Luncheon
	Prevalence	21 (42)	17 (34)	15 (30)	13 (26)	7 (14)
Serotypes of *E. coli*	**O_{111}:H_2**	2 (4)	3 (6)	1 (2)	3 (6)	2 (4)
	O_{91}:H_{21}	1 (2)	ND	2 (4)	1 (2)	ND
	O_{127}:H_6	3 (6)	3 (6)	ND	1 (2)	1 (2)
	O_{119}:H_6	5 (10)	4 (8)	1 (2)	2 (4)	ND
	O_{113}:H_4	ND	2 (4)	3 (6)	1 (2)	1 (2)
	O_{26}:H_{11}	8 (16)	3 (6)	2 (4)	3 (6)	1 (2)
	O_{55}:H7	2 (4)	ND	3 (6)	ND	1(2)
	O_{103}	ND	ND	1 (2)	1(2)	ND
	O_{124}	ND	2 (4)	2 (4)	1(2)	1(2)

Data were represented by No (%); ND, not detected. The percentages in the table were calculated according to number of examined samples per each product type.

Serological identification of *E. coli* is of a great public health importance in order to shed light on the pathotypes included as well as their potential health hazards. In the current study, different serotypes of *E. coli* were identified (Tables 2 and 3). Twenty nine enteropathogenic isolates were obtained from the examined samples represented by four O91:H21 from fajitas, burger and pane samples, twelve O119:H6 isolates from fajitas, burger, shawarma and pane, seven O113:H4 isolates from shawarma, burger, pane and luncheon and six O55:H7 isolates from fajitas, burger and luncheon. Besides, thirty *E. coli* isolates that belonged to enterohemorrhagic pathotype were isolated from the examined samples and represented by five O111:H2 isolates from fajitas, shawarma, burger, pane and luncheon samples, also five O26:H11 isolates from the same samples and only two O103 isolates were obtained from burger and pane. Another pathotype was also identified among the obtained isolates in this study. Eight O127:H6 isolates of the enterotoxigenic pathotype were identified in fajitas, shawarma, pane and luncheon samples. In addition, the study also revealed the isolation of enteroinvasive pathotype from the examined samples. Six enteroinvasive isolates belonging to serotype O124 were isolated from shawarma, burger, pane and luncheon samples. Similar serotypes were also isolated in previous studies performed on RTE meat and meat products. For example, serotypes like O26, O127:H6, O119:H6 and O111 were previously detected in chicken pane and shawerma samples (26). Also, O111, O55, O26 and O91 *E. coli* serotypes were previously documented in RTE chicken meat products (21,28,29). These different pathotypes of *E. coli* had developed the ability to induce diseases in human's GIT, urinary tract and meninges (30). They were reported to cause about 30 to 40 % of all the diarrheal cases in developing countries (31). Furthermore, the current study also referred to the existence of some essential virulence genes in the isolated strains which play a significant role in their pathogenesis. The results presented in Table (2) showed that *Stx*1 was detected in five *E. coli* isolates including O26:H11, O119:H6, O127:H6, O91:H21 and O103, while only two isolates were positive for *Stx*2 (O119:H6 and O55:H7). In addition, six isolates including O26:H11, O55:H7, O127:H6, O113:H4, O103 and O111:H2 were all positive for *eae*A gene. Previous studies had also declared the detection of such virulence genes (*stx*1, *stx*2 and *eae*A) in *E. coli* strains isolated from RTE chicken meats and their products (28,29).

Table 3: Occurrence of virulence genes in enteropathogenic *E. coli* isolated from ready to eat chicken sandwiches

E. coli isolates	Type	*Stx*1	*Stx*2	*eae*A
O26:H11	EHEC	+	_	+
O119:H6	EPEC	+	+	_
O55:H7	EPEC	_	+	+
O127:H6	ETEC	+	_	+
O113:H4	EPEC	_	-	+
O91:H21	EPEC	+	_	_
O103	EHEC	+	_	+
O124	EIEC	_	_	_
O111:H2	EHEC	-	-	+

EHEC, Enterohemorrhagic *E. coli*; EPEC, Enteropathogenic *E. coli*;ETEC, Enterotoxigenic *E. coli*, EIEC, Enteroinvasive *E. coli;* +, Positive; -, negative

The presence of such genes in the *E. coli* isolates contributes greatly to their pathogenicity and virulence (32). Intimin gene (*eae*A) is an essential factor for the microbe to attach strongly to intestinal mucosal cells and initiate disease (4). On the other hand, *stx*1 and *stx*2 have the ability to inhibit protein synthesis in the host cells and subsequently cause them to die (33). That's why the presence of such virulent strains in RTE foods actually sets a great concern in the face of public health. It also worth mentioning that the presence of STEC in RTE chicken meat may be due to cross contamination from the hands of food handlers (28) or also due to the possibility of contaminating RTE chicken by mixing it with contaminated leafy vegetables and salads post-processing (34).

The current study tested the antimicrobial resistance of seventy three *E. coli* isolates against fourteen commonly used antimicrobials in order to detect the patterns of resistance for them which consequently would reflect their public health significance. As shown in Table (4), 100% of the tested isolates were resistant to Penicillin, followed by Erythromycin (94.5%), Oxytetracycline (83.56%), Nalidixic acid (79.45%), Ampicillin (76.7%), Sulphamethoxazol (74%), Cephalotin (72.6%), Enrofloxacin (53.42%), Oxacillin (52.02%), Neomycin (42.5%), Chloramphenicol (38.35%) and Kanamycin (35.6%). The lowest resistance was found against Gentamycin and Ciprofloxacin with percentages of 5.5% and 16.44%, respectively. The obtained results were in same line with that obtained in a previous study (35), they found that tetracycline and Nalidixic acid resistance were 84.4% and 74.1% in retailed chicken from china. Our results disagree with a previously published work (36) in which the highest resistance was against Oxytetracycline (92%) followed by Sulphonamide-trimethoprim (84%), Amoxycillin (76%) and Erythromycin (60%) for *E. coli* isolated from chicken meat. On the other hand, the isolates obtained by several authors (37,38) from ready to eat meat were found to be slightly similar to our study in that 100% of isolates were Penicillin resistant while the lowest resistance percentage (6.7%) was for Gentamycin.

The multi antimicrobial resistance (MAR) index was ranged from 0.071 to 1 with an average of 0.533. Moreover, 4/73 (5.74%) of examined *E. coli* resist all tested antimicrobials, 53/73 (72.6%) resist seven antimicrobials or more as shown in Table (5). Multiple antimicrobial resistance patterns showed that 83.65% of the isolates were resistant to the three or more antimicrobials. Such prevalence of multidrug resistance in *E. coli* have been reported in Bangladesh (39), China (40), Korea (41) and Nepal (42). The proportion of the isolates with MAR index higher than 0.2 was 83.65%, and lower than or equal to 0.2 was 16.35%. MAR index value higher than 0.2 indicated high-risk sources of contamination, where several antimicrobials may often use for the control of diseases (37). The higher resistance of *E. coli* isolates, could be attributed to the misuse of antibiotics for therapeutic or wide use as growth promotors in chicken industry.

Table 4: Antimicrobial susceptibility of *E. coli* isolated from ready to eat chicken sandwiches (N= 73)

Antimicrobial agent	Sensitive	Intermediate	Resistant
Penicillin (P)	0 (0)	0 (0)	73 (100)
Erythromycin (E)	0 (0)	4 (5.5)	69 (94.5)
Oxytetracycline (OT)	9 (12.32)	3 (4.10)	61 (83.56)
Nalidixic acid (NA)	6 (8.21)	9 (12.32)	58 (79.45)
Ampicillin (AM)	0 (0)	17 (23.3)	56 (76.7)
Sulphamethoxazol (SXT)	7 (9.6)	12 (16.44)	54 (74)
Cephalotin (KF)	8 (10.96)	12 (16.44)	53 (72.6)
Enrofloxacin (ENR)	18 (24.65)	16 (21.92)	39 (53.42)
Oxacillin (OX)	26 (35.61)	9 (12.32)	38 (52.05)
Neomycin (N)	32 (43.8)	10 (13.7)	31 (42.5)
Chloramphenicol (C)	38 (52.1)	7 (9.55)	28 (38.35)
Kanamycin (K)	41(56.2)	6 (8.2)	26 (35.6)
Ciprofloxacin (CIP)	55 (75.35)	6 (8.21)	12 (16.44)
Gentamicin (CN)	66 (90.4)	3 (4.1)	4 (5.5)

Data were represented by No (%).

Table 5: Antibiotic resistance pattern and MAR index of *E.coli* isolated from RTE chicken sandwiches (N= 73)

Resistance pattern	Resistance profile	Number of isolates	Number of antibiotics	MAR
I.	P, E, OT, NA, AM, SXT, KF, ENR, OX, N, C, K, CIP, CN	4	14	1
II.	P, E, OT, NA, AM, SXT, KF, ENR, OX, N, C, K, CIP	8	13	0.92
III.	P, E, OT, NA, AM, SXT, KF, ENR, OX, N, C, K	14	12	0.85
IV.	P, E, OT, NA, AM, SXT, KF, ENR, OX, N, C	2	11	0.78
V.	P, E, OT, NA, AM, SXT, KF, ENR, OX, N	3	10	0.714
VI.	P, E, OT, NA, AM, SXT, KF, ENR, OX	7	9	0.642
VII.	P, E, OT, NA, AM, SXT, KF, ENR	1	8	0.571
VIII.	P, E, OT, NA, AM, SXT, KF	14	7	0.5
IX.	P, E,OT, NA, AM, SXT	1	6	0.428
X.	P, E, OT, NA, AM	2	5	0.357
XI.	P, E, OT, NA	2	4	0.285
XII.	P, E, OT	3	3	0.21
XIII.	P, E	8	2	0.142
XIV.	P	4	1	0.071
Average MAR			0.533	

P, Penicillin; E, Erythromycin; OT, Oxytetracycline; NA, Nalidixic acid; AM, Ampicillin; SXT, Sulphamethoxazol; KF, Cephalotin; EN, Enrofloxacin; OX, Oxacillin; N, Neomycin; C, Chloramphenicol; K, Kanamycin; CIP, Ciprofloxacin; CN, Gentamicin; MAR, multiple antibiotic resistance.

Conclusion

In conclusion, RTE chicken sandwiches contaminated with various serotypes of *E. coli* that carry shiga toxin like genes and intimin. The most examined isolates were multiple antimicrobial resistant.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

GROWTH PERFORMANCE, CARCASS TRAITS AND ECONOMIC VALUES OF PEKIN, MUSCOVY, AND MULARD DUCKS

Fardos A.M. Hassan, Elshimaa M. Roushdy, Asmaa W. Zaglool, Mohammed A. Ali, Iman E. El-Araby*

Department of Animal Wealth Development, Faculty of Veterinary Medicine, Zagazig University, El-Zeraa str. 114, 44511 Zagazig, Egypt

*Corresponding author, Email: genetics_engineering@yahoo.com

Abstract: This study aimed to reconnoiter breed variations in productivity, traits of carcass, economic rate, and IGF-1 gene regulation for meat production among Pekin, Muscovy, and Mulard ducks. A 10-week trial was conducted, using 120 ducklings (2-week old) that were divided into three groups based on breed. Each breed was kept in a separate group, divided into four replicates of 10 birds each. Muscovy ducks exhibited superior body weight, weight gain, feed conversion ratio, dressing and breast percentage compared to the other breeds (P<0.001). The highest percentage of crude protein was observed in the meat of Mulard ducks leg (23.17) and breast (50.55), and in Muscovy breast meat (51.04). Pekin ducks yielded a significantly higher (P<0.001) leg and breast fat content (6.27, 6.40 respectively) than Muscovy (4.58, 4.26 respectively) or Mulard ducks (4.13, 3.88 respectively). Notably, Muscovy ducks in comparison to the other breeds yielded the highest gross margin ($1.12) and lowest budget to produce 1kg of live body weight ($2.08) ($P$= 0.004). Furthermore, hepatic IGF-1 and IGF1R expression was higher in the Muscovy breed than in the other breeds. These genes increase the growth and development of muscles. Therefore, the Muscovy ducks are generally superior in terms of performance, carcass traits, and economic values.

Key words: duck breeds; performance; carcass merits; costs; IGF-1; IGF-1R

Introduction

Ducks have been consumed sporadically in the past, but are now reared both intensively and commercially. They are primarily reared for meat and eggs, although their feathers also have economic value. Duck meat is comparable to that of chicken and is an alternative source of protein, minerals, and other nutrients for humans. In comparison to chickens, ducks are better adapted to varying environmental conditions, require less care, and are more resistant to a number of diseases (1). These characteristics likely form the basis for the increasing importance and popularity of the duck industry. Some of the popular duck breeds raised for meat production under Egyptian conditions include the Pekin, Muscovy, and Mulard.

The Pekin duck is commonly bred for meat production in Egypt. Improvements in White Pekin strains take advantage of the duck's natural ability to grow rapidly and its resistance to infections to which other poultry are susceptible. Thus, producers are able to reduce input costs while improving carcass quality and

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feathering. Genetic improvements have now caused the modern domestic White Pekin to surpass the broiler breeds of chicken in feed efficiency and weight gain at a similar living market weight (2). On the other hand, Muscovy ducks are very popular because they adapt well to various rearing conditions and they have high breast meat with unique taste and least calorie content (3) in comparison to Pekin ducks. The Mulard (hybrid of Muscovy and Pekin ducks) has been used for production of fattened liver as well as for meat production (4,5). Their carcasses are characterized by a high proportion of breast and leg muscle and low proportion of subcutaneous fat.

Economic traits such as carcass traits and growth performance are very significant in duck production. These traits are controlled by sets of candidate genes which play an important role in ducks growth and development as the insulin-like growth factors genes (IGF-1 and IGF-2). The IGF-1 has the potential to be a key regulator of growth, body composition and skeletal traits during postnatal development (6), whereas IGF-2 reportedly functions primarily during embryonic growth and development (7). Therefore, this study was carried out to evaluate performance, carcass merits, economic values, and IGF-1 and IGF1R gene regulation in Pekin, Muscovy, and Mulard ducks reared under Egyptian subtropical conditions

Material and methods

Experimental design, diets, and husbandry of duck flock:

A total of 120 male, two weeks old Muscovy, Pekin, and Mulard ducklings (40 each) of uniform body weight were used in this study. Each breed was reared until the age of 12 weeks and maintained as separate groups, divided into four replicates with 10 ducklings each. A wing band was used to label each duck. During the experiment, water and feed were supplied *ad libitum*. Starter diets with 20% crude protein (from 2 to 7 weeks) and

Table 1: Ingredients and chemical composition of experimental diets fed to ducks

Items	Starter (2–7 weeks)	Grower/Finisher (8–12 weeks)
Ingredients (g/kg)		
Yellow corn	570.0	600.0
Soybean meal, 44%	315.0	295.0
Corn gluten, 60%	65.0	30.0
Soybean oil	6.0	30.0
Calcium carbonate	8.0	13.0
Dibasic calcium phosphate	26.0	20.0
Sodium chloride	5.0	5.0
Premix[1]	3.0	3.0
DL- Methionine, 98%	1.0	2.0
L-lysine, 78%	1.0	2.0
Calculated chemical composition[2]		
ME, MJ	12.12	12.56
CP, %	20.34	17.84
EE, %	4.39	6.80
CF, %	3.50	3.37
Ca, %	0.92	0.95
Available Ph, %	0.58	0.45
Lysine, %	1.13	1.18
Methionine, %	0.41	0.50

[1]Supplied per kg of diet: Vitamin A (12000 IU); Vitamin D_3 (2200 IU); Vitamin E (10 mg); Vitamin K_3 (3 mg); Vitamin B_1 (1mg); Vitamin B_2 (5 mg); Vitamin B_6 (1.5 g); Pantothenic acid (10 mg); Vitamin B_{12} (10mg); Niacin (30 mg); Folic acid (1mg); Biotin (50 mg); Fe (30 mg); Mn (60 mg); Cu (4 mg); I (1mg); Co (1mg); Se (1 mg); and Zn (50 mg); Choline chloride (300 mg).

[2] Calculated according to NRC (1994) tables.

ME = Metabolizable energy; CP = Crude protein; EE = Ether extract; CF = Crude fiber.

grower/finisher diets with 18% crude protein (from 8 to 12 weeks) were fed to the ducklings in the form of dry mash. All experimental diets were formulated to ensure an adequate supply of all nutrients according to the National Research Council (8) recommendations for duck breeds (Table 1).

Ducks of all groups were kept under similar management conditions and housed in pens with similar floors (5 birds/m^2) covered with a 5-cm thickness of wood shavings as bedding. The temperature of the houses was maintained at 25°C, and continuous light was provided from the 2nd week until the end of the study. All ducklings were vaccinated by live attenuated vaccines against duck cholera (1 ml/duckling, subcutaneous), duck plague (0.5 ml/duck, intramuscular), and duck virus hepatitis (1 ml/duck, intramuscular) at the age of 28, 46 and 50 days, respectively. The animal experiment protocol was approved by the Institutional Animal care and Use committee at Zagazig University. The experiment was conducted at the research farm of Poultry and Rabbit, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Growth performance

Final live body weight (LBW) was recorded and body weight gain (BWG), average feed intake (AFI), and feed conversion ratios (FCR) were calculated at the end of experiment. The feed was withdrawn before birds weighing for 2h. Feed conversion was calculated as g feed/g gain. LBW and BWG were evaluated based on individual bird data, whereas AFI and FCR were assessed based on the replicate unit.

Sample collection, carcass traits, and meat analysis

Eight ducks from each studied breeds (two from each replicate) were selected according to an average body weight for the respective breed and fasted for 12h before slaughtering. The birds were marked with individual numbers, weighed, and euthanized by cervical dislocation before being manually defeathered and eviscerated. The giblets (liver, gizzard, and heart), eviscerated carcass, breast and thigh muscles were weighed and their percentages relative to live body weight were calculated.

After slaughter, the liver was weighed and two 1-cm sections were immediately resected, gently flushed with PBS, and stored at -80°C until mRNA extraction. Samples of breast and thigh meat of selected individuals were also resected, dried, ground, and subjected to proximate analysis to determine crude protein, moisture, total ash, and fat content. Samples were investigated using standard procedures (9).

IGF-1 and IGF1R gene expression in liver by Real-Time PCR

RNA from the liver samples was extracted using a QIAamp RNeasy Mini kit (Qiagen, Germany) according to the manufacturers' instructions. A GeneQuant spectrophotometer (Pharmacia Biotech, Freiburg, Germany) was used to estimate purity and concentration of RNA. Complementary DNA (cDNA) was obtained by reverse transcriptase of RNA using a RevertAid Reverse Transcription kit (Thermo Fisher). Real-time PCR analysis was performed using QuantiTect® SYBR® Green PCR kit (Qiagen, Germany), with β-actin as the internal control gene. Gene-specific primer sequences F1:CAACGAGCGGTTCAGGTGT, R1:TGGAGTTGAAGGTGGTCTCG, F2: ATCCAGCAGTAGACGCTTACACC, R2: CGTGCAGACTTAGGTGGCTTTA and F3: GGTATTCCACCTCACTCTCCT, R3: AACTTCCTTCACAACTCCATCT were used to amplify 92 bp of Duck β-actin, 117 bp of IGF-1, and160 bp of IGF1R (10).

The qRT-PCR was carried out in 25µl volume of 12.5 µl of 2× QuantiTect SYBR Green PCR Master Mix; 0.5 µl of each primer, 0.25 µl of RevertAid Reverse Transcriptase (200 U/µL); 3 µl of the template and 8.25 µl of nuclease free water. The cycling was programmed as follows: 95°C for 5 min; followed by 40 cycles of 15s at 95°C, 15s at 60°C, and 15s at 72°C. Melt-curve analysis was performed between 65°C to 95°C, using increments in temperature of 0.5°C.

Ct values for the SYBR green RT-PCR were measured using Stratagene MX3005P software

(Stratagene Technical Services, USA). To calculate the variation in gene expression in the RNA of various samples, the Ct of each sample was compared with that of the Pekin breed as a reference (the lowest growth breed) according to the "ΔΔCt" method outlined by Yuan et al. (11).

Economic values of duck breeds

The economic value of the breeds under investigation was evaluated using cost-benefit analysis, by estimating the total variable costs (TVC), gross income for live weight, gross margin, and benefit–cost ratio (BCR). Total variable costs were estimated by considering the cost incurred in obtaining the ducklings, as well as the expenses of feed, litter, labor, veterinary services, electricity, and other miscellaneous expenditure. Fixed costs were not included in the analysis, because they were equal across all breeds. Gross margin analysis was used to determine profitability of the breeds, as described by Olukosi and Erhabor (12). The unit of measurement was USD/kg live weight. The following equation was used to derive gross margin: GM= GI – TVC, where GM= gross margin; GI= gross income/kg live weight; and TVC= total variable cost that represents the total cost of production/kg live weight. The benefit–cost ratio (BCR) was derived by the following formula: GM/TVC.

Statistical analysis

All statistical analyses were performed using SPSS V.16 software (SPSS, IL, USA). Data were analyzed using one-way ANOVA, after normality was verifying using the Kolmogorov–Smirnov test. The Tukey's (HSD) multiple comparison test was used to determine significant differences between mean values. Variability in the data was expressed as the pooled SEM, and the alpha level for determination of significance was set at 0.05.

Results

Growth performance

As shown in Table (2), the Muscovy breed showed the greatest final BW (3903.75) and BWG (3659.65) followed by the Mulard (3518.52 and 3267.42, respectively) and Pekin (3355.00 and 3117.20, respectively). In addition, AFI and FCR were significantly declined in Muscovy breed compared to the other breeds. However, no significant change was detected between the Pekin and the Mulard.

Table 2: Growth performance of Muscovy, Pekin, and Mulard ducks

	Breed				
Parameter	**Muscovy**	**Pekin**	**Mulard**	**SEM**	***P*-value**
Initial BW (g)	244.10	237.80	251.10	2.53	0.098
Final BW (g)	3903.75^{a}	3355.00^{c}	3518.52^{b}	27.05	< 0.001
BWG (g)	3659.65^{a}	3117.20^{c}	3267.42^{b}	28.36	< 0.001
AFI (g)	10744.90^{b}	11336.00^{a}	11240.61^{a}	84.16	< 0.001
FCR (g feed: g gain)	2.94^{b}	3.64^{a}	3.44^{a}	0.09	< 0.001

BW: Body weight, BWG: Body weight gain, AFI: Average feed intake, FCR: Feed conversion ratio
$^{a–c}$Means bearing different superscript letters within the same row are significantly different (P<0.05).
SEM: Standard error of means.

Carcass traits and meat composition

Carcass traits of the various breeds are summarized in table 3. The results revealed that the dressing percentage of Muscovy ducks (75.20) was highly significant (P< 0.001) than that of Mulard or Pekin ducks (73.73, 72.41 respectively). Both Muscovy and Mulard ducks possess a higher relative breast weight (51.04, 50.55 respectively) compared to Pekin ducks (49.39). The relative thigh weight differed significantly among the breeds, with the Muscovy breed yielding the highest values (P = 0.001). Differences in the percentages observed

Table 3: Carcass traits and meat composition of Muscovy, Pekin, and Mulard ducks

Parameter	Breed			SEM	*P*-value
	Muscovy	Pekin	Mulard		
Carcass characteristics					
Dressing %	75.20[a]	72.41[c]	73.73[b]	0.30	< 0.001
Breast %	51.04[a]	49.39[b]	50.55[a]	0.20	< 0.001
Thigh %	24.16[a]	23.02[b]	23.17[b]	0.16	0.001
Heart %	0.66	0.65	0.68	0.01	0.163
Liver %	2.36[b]	2.26[b]	3.34[a]	0.15	< 0.001
Gizzard %	2.35	2.39	2.07	0.08	0.179
Breast meat composition					
Moisture %	74.32[a]	71.53[b]	74.73[a]	0.38	< 0.001
Protein %	19.21[a]	17.41[b]	19.64[a]	0.26	< 0.001
Fat %	4.26[b]	6.40[a]	3.88[b]	0.29	< 0.001
Ash %	1.31	1.26	1.21	0.02	0.125
Thigh meat composition					
Moisture %	73.46	72.04	73.14	0.33	0.188
Protein %	17.52[b]	17.26[b]	19.08[a]	0.22	< 0.001
Fat %	4.58[b]	6.27[a]	4.13[b]	0.22	< 0.001
Ash %	1.12	1.04	1.09	0.02	0.253

[a–c]Means bearing different superscripts within the same row are significantly different (P<0.05). SEM: Standard error of means.

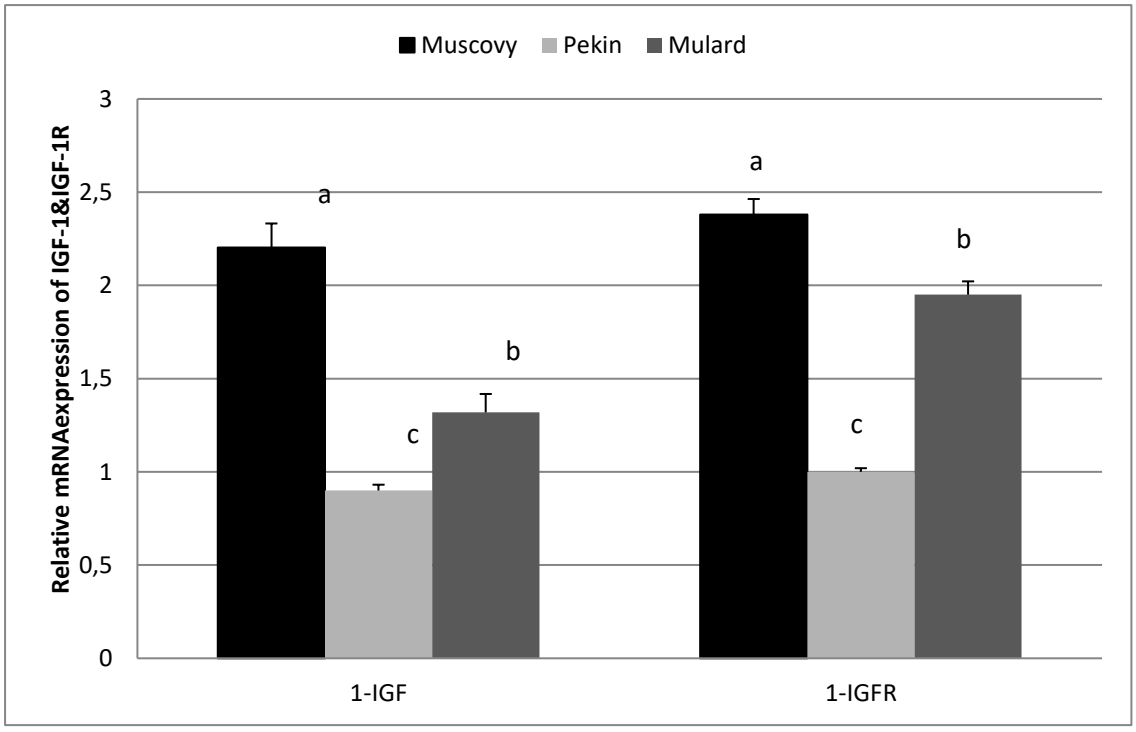

Figure 1: Quantitative expression of IGF-1 and IGF1R extracted from the liver of various duck breeds (mean ± SEM) after 10 weeks. Groups with different letters are significantly different (P< 0.05, one-way ANOVA)

Table 4: Economic values for Muscovy, Pekin, and Mulard ducks

Parameter	Breed			SEM	*P*-value
	Muscovy	Pekin	Mulard		
Feed cost / duck ($)	4.59[b]	4.85[a]	4.83[a]	0.04	0.005
Feed cost / kg weight gain ($)	1.26[b]	1.67[a]	1.58[a]	0.04	< 0.001
TVC / kg live weight ($)	2.08[b]	2.15[b]	2.30[a]	0.03	0.004
Gross income / duck ($)	12.49[a]	8.15[c]	10.34[b]	0.21	< 0.001
GM / kg live weight ($)	1.12[a]	0.28[c]	0.64[b]	0.11	< 0.001
Benefit–cost ratio (BCR)	0.54[a]	0.13[c]	0.28[b]	0.05	< 0.001

Costs per kg feed=$0.45 and $0.41 for starter and grower ration, respectively.
Purchasing price per duck= $2.56, $1.41, $2.30 for Muscovy, Pekin, and Mulard, respectively.
Selling price (Gross income)/kg live weight= $3.20, $2.43, $2.94 for Muscovy, Pekin and Mulard, respectively.
TVC = Total variable costs; GM = Gross margin.
[a-c] Means bearing different superscripts within the same row are significantly different ($P< 0.05$).
SEM: Standard error of means.

for heart and gizzard were not significant ($P>0.05$) among the breeds; however, the liver percentage of Mulard ducks (3.34%) was considerably higher than that of Muscovy and Pekin ducks (2.36 and 2.26%, respectively).

As shown in Table 3, the breast and thigh meat composition differed significantly among the various breeds. The moisture content was highly significant ($P< 0.001$) in the breast meat of Mulard and Muscovy ducks compared to that of Pekin ducks. The highest percentage of crude protein was observed in the leg and breast meat of Mulard ones, and in Muscovy breast meat. Pekin ducks yielded a significantly higher ($P<0.001$) content of fat in both leg and breast meat than Muscovy and Mulard ducks, whereas the carcass fatness of Muscovy and Mulard ducks was similar. No significant differences ($P>0.05$) were detected in the ash content of breast and thigh muscles among the various breeds.

IGF-1 and IGF1Rgenes expression

The relative changes in IGF-1 mRNA transcript levels are presented in Figure (1). These results clearly demonstrate that the Muscovy ducks showed higher significant IGF-1 gene expression, followed by Mulard and Pekin ducks in that order.

Economic values of duck breeds

Economic calculations revealed that Muscovy breed had a significantly lower ($P<0.001$) feed cost and feed cost/kg gain compared to the other breeds, whereas there is no significant difference between Pekin and Mulard breeds (Table 4). However, the total variable costs of Muscovy and Pekin were significantly lesser than those of Mulard ducks ($P = 0.004$). Muscovy ducks showed the highest values for gross income ($12.49), gross margin ($1.12), and benefit–cost ratio (0.54), followed by Mulard and Pekin ducks in that order.

Discussion

It is important to note that the three breeds under investigation (Muscovy, Pekin, and Mulard) differ considerably in terms of growth rate and the characteristics of valuable body parts, but all have the ability to grow continuously until the 12th week of life (13). As shown in our results, Muscovy ducks yielded superior values for body weight, weight gain, average feed intake, and feed conversion ratio (3903.75, 3659.65, 10744.90 and 2.94, respectively), which is consistent with previous studies (14-16). However, Bhuiyan et al. (17) claimed that the Pekin breed is superior to both Muscovy and Deshi white ducks. The highest weight in Mulard hybrid ducks during the 12th week of life, in comparison to Muscovy and Pekin ducks (13).

Numerous studies have shown that the carcass's composition and the meat yield of ducks varied by breed. In the present study, Muscovy ducks yielded a significantly higher dressing percentage of 75.20 compared to Pekin (72.41%) and Mulard ducks (73.73%). Moreover, the highest breast and thigh percentages

were observed in Muscovy ducks. The high dressing percentage observed in the Muscovy could be attributed to its heaviness. In addition, two possible reasons for the higher dressing percentage of Muscovy ducks are reduced plumage and smaller internal organs in comparison to other breeds (14). Similarly, El-Soukkary et al. (18) reported that the Muscovy duck had a significantly higher commercial cut yield (including the breast and drumstick) than Pekin and Sudani ducks. Also, Wawro et al. (19) reported that the highest values for breast and leg muscle weight were observed in the carcasses of Muscovy males. However, Bhuiyan et al. (17) reported that the highest dressing yield was observed in Pekin ducks (70%) as compared to Muscovy and Deshi White ducks.

The present study clarified that the moisture content in the breast meat of Mulard and Muscovy was highly significant than that of Pekin breed. The highest percentage of crude protein was observed in the meat of leg and breast of Mulard ducks, and in Muscovy breast meat. Pekin ducks yielded a significantly higher in both leg and breast meat fat content than either Muscovy or Mulard ducks. No significant variances ($P > 0.05$) were detected in ash content of breast and thigh muscles among the various breeds. These results are consistent with those of another study conducted by Wawro et al. (19), who reported the highest crude protein values ($\overline{X}$ = 19.5%) in Muscovy and Mulard breast muscles, and low significant value in the Pekin breast muscle ($\overline{X}$ = 19.0%). According to Isguzar et al. (20), the fat content of the Pekin leg and breast meat is significantly higher than that of local Turkish breeds. The moisture percentage in the Muscovy leg and breast meat was higher than Sudani ducks (21). In contrast, Bons et al. (22) noted greater content of breast protein (21.5%) and muscles of leg (22.5%) of Pekin breed. The thigh and breast muscle water content were significantly higher, and the ash content was significantly lower in the Pekin than in the Muscovy (15).

The IGF system has been considered as an essential regulatory system for controlling development and growth in mammals and chickens. IGF-1, as a member of the IGF family, is a growth, metabolism, body composition, skeletal characteristics, fat deposition and growth of adipose tissue candidate gene in chickens (23). Moreover, IGFIR is a membrane glycoprotein mediating the biological actions of IGF-1 and IGF-2, which have a great effect on chicken growth and quality traits of meat and carcass (24). IGF1R played important roles on the developmental and adult stages such as the cell cycle, transplantation, subsistence, metabolism, propagation and differentiation (25) In previous studies, higher significant of hepatic IGF-1 expression has shown breed specificity in ducks (26), and chickens (27). Similarly, the present results showed significant differences in IGF-1 expression among the various breeds. The highest expression was observed in the Muscovy, a finding that might be responsible for its superior muscle growth and carcass merit.

Assessment of the three breeds indicated that the Muscovy was the most economical (followed by the Mulard and Pekin), both in terms of the cost to produce 1kg live weight and the feed cost per unit gain. In addition, the highest profit margin was realized from the Muscovy. The main contributing factors to the comparatively higher profit margin of Muscovy ducks include the higher market price of the meat, and to a lesser extent, the superior biological efficiency of Muscovy ducks in comparison to Mulard and Pekin ducks (28). In comparison to other breeds, the Muscovy yields the best values for net income, net income margin, and gross return attributed to the total variable costs per 100 parent ducks (29).

Conclusion

In conclusion, Muscovy ducks showed higher performance, dressing percentage, and IGF-1 expression than Mulard or Pekin ducks. The Muscovy and Mulard breeds yielded better quality than the Pekin, owing to higher protein content and lower body fat.

Conflict of interest

The authors declare that they have no conflict of interest.

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Original Research Article

PREVALENCE OF ENTEROTOXIGENIC AND MULTI-DRUG-RESISTANT *Staphylococcus aureus* IN READY TO EAT MEAT SANDWICHES

Alaa Eldin M.A. Morshdy, Mohamed A. Hussein*, Ahmed E. Tharwat, Basma A. Fakhry

Food Control Department, Faculty of Veterinary Medicine Zagazig University, El-Zeraa Str. 114, Zagazig 44511, Egypt

*Corresponding author, E-mail: elged2010@yahoo.com

Abstract: Due to recent spread of multiple drug resistant pathogens, this study was peformed to investigate the presence of multi-drug resistant enterotoxigenic *Staphylococcus aureus* (*S. aureus*) in some ready to eat meat products (RTE). For this, one hundred and forty samples of ready to eat meat sandwiches were collected from restaurants and street vendors in Zagazig city, Egypt. *Staphylococcus aureus* is one of the most important food poisoning bacteria in RTE. The counts were 3.31 ± 0.49, 2.86 ± 0.36, 3.28 ± 0.24, 3.92 ± 0.41, 2.52 ± 0.11, 3.64 ± 0.39 and 3.12 ± 0.35 $\log_{10}$ CFU/g in examined kofta, luncheon, burger, shawarma, hawawshi, liver and sausage sandwiches, respectively. The examined sandwiches were categorized into good (32.1%), acceptable (32.9%), unsatisfactory (26.4%) and potentially hazard (8.6%). About 72.7 % of examined *S. aureus* strains carry one or more staphylococcal enterotoxin (*se*) genes and *mec*A gene detected in 81.8% of coagulase positive *S. aureus*. The antibiogram showed that 100% of *S. aureus* isolates were resistant to kanamycin, 92% for penicillin and neomycin, 84% for oxacillin and erythromycin and 68% for ampicillin and nalidixic acid. The average of multi-antibiotic resistant (MAR) index of isolated *S. aureus* was 0.59. Moreover, five isolates were resistant to all tested antibiotic.

Key words: *Staphylococcus aureus*; methicillin resistant; enterotoxin; ready to eat

Introduction

Foodborne illness is increased of worldwide. This may in part related to a change in a system of food production such as least processing as well as changing consumer needs for ready-to-eat (RTE) sandwiches (1). Ready to eat meat linked with foodborne disease outbreaks have been associated with various foodborne micro-organisms (2,3). The number of microorganisms in raw ingredients introduced in RTE meat sandwiches is important, however, many factors such as processing, handling, storage and display may impact the microbio-logical load of final RTE meat sandwiches at the point of sale (4). Delicatessen foods such as vegetable salads and sauces have also been incriminated in foodborne illness outbreaks. These foods are usually prepared by hand and this through contact may lead to an increased prevalence of contamination with potential foodborne bacteria, such as *S. aureus* (5). Antimicrobial resistance is a significant public health concern all over the world. The virulent and antibiotic-resistant bacteria cloud be trans-mitted to

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humans by the consumption of RTE meat sandwiches and the presence of these bacteria such as *S. aureus*, indicating poor hygienic measures which may produce a health risk for consumers (6) especially, multi antibiotic resistant (MAR) strains which are more risky and of greater food safety attention (7). Treatment of infectious disease caused by MAR bacteria needs alternatives to conservative antibiotics and the search for recent antibiotics is becoming critical (8). Methicillin-resistant *S. aureus* (MRSA) has newly emerged as a health anxiety and currently causes about 94,000 invasive infections annually in the United States of America, leading to an estimated 18,650 deaths (9). Moreover, MRSA has been found in several species of meat-producing animals, poultry (10) and cattle (11). Detection of MRSA in these animal products, illustrates the importance of exploring meat products as potential sources for transmission of MRSA from the farm animals for the meat product consumer. Therefore, the present study was conducted for determination of Enterotoxigenic *S. aureus* in addition to antibiogram for antibiotic sensitivity in most popular RTE meat sandwiches in Egypt.

Material and methods

One hundred and forty samples of ready to eat sandwiches were collected randomly from different restaurants in Zagazig city, Sharkia Governorate, Egypt, at different sanitation levels. The samples were collected from May 2017 to April 2018. The collected samples represented by 20 each of kofta, luncheon, burger, shawarma, hawawshi, liver and sausage sandwiches. Each collected sample was kept in a separate sterile plastic bag and preserved in an ice box then transferred to the laboratory under an aseptic condition without undue delay.

Isolation and identification of Staphylococcus aureus

Staphylococcus aureus was isolated as follows: 25 g of each sandwich core (meat, vegetables and sauce) were mixed with 225 ml of peptone water (Oxoid, Basingstoke, Hampshire, CM0009B, UK) and homogenized in a stomacher (Lab-Blender 400, PBI, Milano, Italy). The samples were then inoculated onto Baird-Parker agar (Oxoid CM 275) and incubated at 35°C for 24 and 48 h. The samples characteristic colonies (gray-black, surrounded by a dull halo) were considered to contain coagulase positive *S. aureus* (CPS) (12). Five colonies from each Petri of presumed *S. aureus* isolates were examined by Gram-staining and catalase test. Gram and catalase-positive isolates were further identified according to American Public Health Association (APHA) (13).

Determination of Staphylococcal enterotoxin and mecA genes

After overnight culture on nutrient agar plates, two colonies were suspended in 20 ml of sterile distilled water and the suspension was then heated at 100 ºC for 20 min. Accurately, 50-200 µl of the culture were placed in Eppendorf tube for DNA extraction using QIA amp kit according to Shah et al., (14).

Table 1: Primer sequences of *S. aureus* used for PCR identification system

Oligonucleotide sequence (5′ → 3′)	Product size (bp)	Target gene	References
F- TTGGAAACGGTTAAAACGAA **R- GAACCTTCCCATCAAAAACA**	120	*sea* (F)	(15)
F- TCGCATCAAACTGACAAACG **R- GCGGTACTCTATAAGTGCC**	478	*seb* (F)	
F- GACATAAAAGCTAGGAATTT **R- AAATCGGATTAACATTATCC**	257	*sec* (F)	
F- CTAGTTTGGTAATATCTCCT **R- TAATGCTATATCTTATAGGG**	317	*sed* (F)	
F- GTA GAA ATG ACT GAA CGT CCG ATA A **R- CCA ATT CCA CAT TGT TTC GGT CTA A**	310	*mecA*(F)	(16)

Amplification of enterotoxin genes of S. aureus

Ten µl of DNA sample was diluted in 990 µl of nuclease free water for PCR. The genomic DNA samples were amplified in a reaction mixture (25µl) containing 13.25 sterile dH_2O, 2.5 ml 10 x buffer, 0.63 ml 10mMNTPs, 1ml 25Mm $Mgcl_2$, 1.25µl primer F(20pmol/ml), 1.25µl primer R (20pmol/ml) and fill up to 25µl PCR grade water. Concerning the primers used for demonstration of *S. aureus* enterotoxins (sea, seb, secand sed) (Table 1), the multiplex amplification was performed on a thermal cycler (Master cycler, Eppendorf, Hamburg, Germany). The initial denaturation for 5 min at 95°C followed by 30 cycles of denaturation (94°C for 2 min), annealing (55°C for 1 min.), and extension (72°C for 2 min). A final extension step (72°C for 5 min) was performed after the completion of the cycles (17).

Amplification of mecA genes of S. aureus

The Preparation of uniplex PCR Master mix according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit. The 12.5µl Emerald Amp GT PCR master mix (2x premix), 4.5µl PCR grade water, 1µl forward primer (20 pmol), 1µl reverse primer (20 pmol) and 6µl template DNA (Table 1). The primary denaturation was at 94˚C for 5 min, secondary denaturation was at 94˚C for 30 min, annealing was at 50˚C for 30 sec, extension was at 72˚C for 30 sec, the cycle number was 35 and final extension was at72˚C for 7 min. Amplified products were analyzed by 3% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer stained with Ethedium bromide and captured as well as visualized on UV transilluminator at 254nm. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes (16).

Antibiogram for antibiotic sensitivity of isolated S. aureus

Antimicrobial susceptibility was tested by the single diffusion method according to Deresse et al. (18). Sensitivity discs with variable concentrations were used to determine the susceptibility of the isolated strains (Oxoid Limited, Basingstoke, Hampshire, UK). Agar plate method was applied by using of nutrient agar as a substrate for growth of the tested bacterium for its antibiotic sensitivity. The bacterial culture was uniformly spread on the surface of nutrient agar. Then the antibiotic discs were placed over the surface of inoculated plate. Moreover, the plate was then incubated at suitable temperature (25°C) for 2-7 days and checked for the growth of the bacterium around the antibiotic discs. The maximal inhibition zone for the growth of microbe is said to that antibiotic had a maximum effect on the microbe growth. Therefore, the antimicrobial susceptibility testing was applied according to the guidelines stipulated by National Committee for Clinical Laboratory Standards (NCCLS) (19). Multiple Antibiotic Resistances (MAR) index for each strain was determined according to the formula stipulated by Singh et al., (20) as follow:

MAR index = Number of antibiotics with resistantce profile / the number of used antibiotics.

Isolates classified as intermediate were considered sensitive for MAR index.

Results and discussion

Staphylococcal food poisoning usually occurs in foods that require human handling during preparation and left at room temperature for long periods before consumption. The data illustrated in Table (2) declared that *S. aureus* was detected in 90, 50, 75, 80, 65, 55and 60% with mean counts 3.31±0.49, 2.86±0.36, 3.28±0.24, 3.92±0.41, 2.52±0.11, 3.64±0.39 and 3.12±0.35log_{10} CFU/g in examined kofta, luncheon, burger, shawarma, hawawshi, liver and sausage sandwiches, respectively. The high prevalence of *S. aureus* attributed to the poor personal hygiene during processing of sandwiches, which resulted in higher contamination (21). There were significant differences between the examined sandwiches ($p<0.05$). The lowest mean value detected in hawawshi, which attributed to preparation of hawawshi sandwiches without the addition of tahini sauce or green salads that not previously heat-treated

during stages of preparation. Moreover, the variation could be related to the difference of the cooking system (frying, roasting, grilling, etc.), the microbiological quality of raw ingredient, the shape and size of the meat piece(s), the utensils used for cooking (oven, crock pot, stew pot, grill, etc.), the presence of seasoning ingredients (sauces, spices, vegetables, etc.) (22). *S. aureus* previously isolated from 17.9 % of RTE and count ranged from 2.30 to 3.60 $\log_{10}$ CFU/g in Taiwan (23). Enterotoxigenic *S. aureus* detected in 60% of coriander sauce, 58% of coconut slices and in 86% samples of RTE salads collected from New Delhi and Patiala City, India (24). There is no available regulation for *S. aureus* count in Egyptian RTE food, so the examined sandwiches were categorized according to the Compendium of Microbiological Criteria for Food (25) into good (32.1%), acceptable (32.9%), unsatis-factory (26.4%) and potentially hazard (8.6%). The unsatisfactory plus potentially hazard samples were 49 (45%) carry a significant risk if applied under certain conditions as excessive handling, bad hygienic conditions, time and temperature abuse that help *S. aureus* to proliferate and produce different staphylo-coccal enterotoxin (SEs) that may cause food poisoning when ingested (26). *S. aureus* produces a number (SEs) that responsible for inflammation and hyperemia of the gastro-intestinal mucosa. Moreover, symptoms as nausea, vomiting, and abdominal cramps, and diarrhea rarely occur. These usually start within one to eight hours after ingestion of food contaminated with *S. aureus* and symptoms last one or two days. The illness seldom is fatal (27).

The results declared in Table (3) showed DNA expression of *S. aureus* enterotoxins genes by using multiplex PCR of *sea*, *seb*, *sec* and *sed* enterotoxin genes for characterization of *S. aureus*. It was found 8/11 isolates were enterotoxin producing strains, 4 strains of *S. aureus* carry *sea* gene, 5 strains carry *sec* gene, 2 strains carry *seb* gene, one strain carry *sea* plus *sec* gene and one strain carry *sea* plus *seb* plus *sec*. It is obvious from the results that *S. aureus* strains carry more than enterotoxin genes, and presence of these strains in ready to eat meat imposes potential hazards and may induce food poisoning if the conditions are suitable for staphylococcal enterotoxin production.

These results agree to some extent with those obtained in different studied that used multiplex PCR for detection of *S. aureus* enterotoxin genes (28-30). The amount of SEs required for typical symptoms of food poisoning is very low, ranging from 20 ng to 1µg, which equal 10^5 Staphylococci CFU/g of food (31).

Table 2: Prevalence and count of *S. aureus* in comparison to standard of ready to eat meat (n= 140)

Sandwiches	prevalence	Mean± SD range	Categories			
			good	acceptable	unsatisfactory	Potentially hazard
Kofta	90%	3.31 ± 0.49^{ab} 2.64-4.41	2	4	10	4
Luncheon	50%	2.86 ± 0.36^{bc} 2.12-3.15	10	8	2	0
Burger	75%	3.28 ± 0.24^{ab} 2.32-4.17	5	5	7	3
Shawarma	80%	3.92 ± 0.41^{a} 3.2-4.58	4	5	8	3
Hawawshi	65%	2.52 ± 0.11^{c} 2-3.2	7	8	5	0
Liver	55%	3.64 ± 0.39^{a} 2.99-4.2	9	7	3	1
Sausage	60%	3.12 ± 0.35^{b} 2.80-4.12	8	9	2	1
Total			45 (32.1%)	46(32.9%)	37(26.4%)	12(8.6%)

Means of the same column carrying small superscript letters were significantly different ($P < 0.05$).

Table 3: Enterotoxin genes in examined *S. aureus* isolates from ready to eat meat sandwiches

Staphylococcus aureus	*sea* (120 bp)	*seb* (478 bp)	*sec* (257 bp)	*sed* (317 bp)
1	-	-	-	-
2	-	-	+	-
3	+	+	+	-
4	+	-	-	-
5	+	-	+	-
6	-	-	-	-
7	+	-	-	-
8	-	-	+	-
9	-	-	-	-
10	-	-	+	-
11	-	+	-	-

Table 4: Antibiotic susceptibility of *S. aureus* isolated from ready to eat meat sandwiches

Antibiotic class	Antibiotic	Sensitive	Intermediate	Resistant
Penicillin	Penicillin (P)	-	2 (8%)	23 (92%)
	Ampicillin (AM)	5 (20%)	3 (12%)	17 (68%)
	Oxacillin (OX)	3 (12%)	1 (4%)	21(84%)
Cephalosporin	Cephalotin (CN)	18 (72%)	2 (8%)	5 (20%)
Fluroquinolones	Ciprofloxacin (CP)	11 (44%)	2 (8%)	12 (48%)
	Enrofloxacin (EN)	13 (52%)	4 (16%)	8 (32%)
Quinolones	Nalidixic acid (NA)	2 (8%)	6 (24%)	17 (68%)
Aminoglycosides	Gentamicin (G)	10 (40%)	2 (8%)	13 (52%)
	Neomycin (N)	-	2 (8%)	23 (92%)
	Kanamycin (K)	-	-	25 (100%)
Tetracycline	Oxytetracycline (T)	6 (24%)	3 (12%)	16 (64 %)
Macrolides	Erythromycin (E)	1 (4%)	3 (12%)	21 (84%)
Sulfonamides	Sulphamethoxazol (SXT)	7 (28%)	2 (8%)	16 (64%)
Phenicols	Chloramphenicol (C)	3 (12%)	4 (16%)	18 (72 %)

Table 5: Antibiotic resistance pattern and MAR index of *S. aureus* isolated from ready to eat meat sandwiches

Resistance pattern	Resistance profile	Number of isolates	Number of antibiotics	MAR
I.	K ,P, N, OX, E, C, AM, NA, T, SXT, G, CP, EN, CN	5	14	1
II.	K ,P, N, OX, E, C, AM, NA, T, SXT, G, CP, EN	3	13	0.92
III.	K ,P, N, OX, E, C, AM, NA, T, SXT, G, CP	4	12	0.85
IV.	K ,P, N, OX, E, C, AM, NA, T, SXT, G	1	11	0.78
V.	K ,P, N, OX, E, C, AM, NA, T, SXT	3	10	0.71
VI.	K ,P, N, OX, E, C, AM, NA	1	8	0.57
VII.	K ,P, N, OX, E, C	1	6	0.42
VIII.	K ,P, N, OX, E	3	5	0.35
IX.	K ,P, N	2	3	0.21
X.	K	2	1	0.071
Average				0.59

Methicillin-resistant *S. aureus* (MRSA) has recently appeared as a health anxiety and causes approximately 94,000 invasive infections annually in the USA, leading to an estimated 18,650 deaths (9). In this study *S. aureus* isolates were tested for the presence of the *mec*A gene, the *mec*A gene detected in 81.8% of coagulase positive *S. aureus* samples.

The results of this study showed a higher prevalence than that recorded by Kitai et al. (32) they detected *mec*A gene in 0.5% from 444 raw chicken meat products sampled from

supermarkets in Japan. Also a study conducted in Korea, including 930 retail meat samples, showed the presence of MRSA in 0.2% detected at chicken meat samples, but not detected in any pork or beef sample (33). Van Loo et al., (34) found two (2.5%) MRSA strains in 79 samples of raw pork and beef.

Regarding to the detection of staphylococcal enterotoxin genes and *mec*A genes, Sergelidis et al. (35) found that MRSA strains isolated from meat and poultry had the ability to produce enterotoxins and produce SEs genes.

The MRSA is transmitted to cooked products due to temperature abuse during storage may result in the multiplication of MRSA. The higher MRSA contamination of various examined RTE sandwiches possible related with the use of antibiotics in animal husbandry (36).

The results in Table (4) shows that 100% of the isolates were resistant to kanamycin, 92% for penicillin and neomycin, 84% for oxacillin and erythromycin and 68% for ampicillin and nalidixic acid, 64% for oxytetracycline and sulphamethoxazol. On contrary sensitivity observed for cephalotin and enrofloxacin by 72% and 52%, respectively. Results were partially in agreement with other studies (37-38).

The multiple antibiotic resistance MAR index of isolated *S. aureus* ranged from 0.071 to 1 with an average 0.59. Moreover, 5 (20%) strains were multi-resistant to all tested antibiotic and 23 (92%) of *S. aureus* isolates are considered as multi antibiotic resistant (Table 5). The variation in the MAR index could be attributed to differences in the sources of samples; geographic distribution, which has differential selective pressures for the antibiotic resistance levels; and test methodologies (39).

The an average of MAR results has shown that MAR higher than 0.2 could be due to contamination from high-risk sources (40), such as humans and farm animals frequently exposed to antibiotics in animals raised for food for different purposes such as prophylaxis, and growth promotion, or therapeutics and these resistant bacteria can be transmitted to human through foods (41), resulting in potential risk to consumers. The high MAR in the current study indicated that the isolates originated from high-risk source samples, therefore monitoring of antimicrobial resistance is essential to identify the effectiveness of new generations of antibiotics. *S. aureus* strains are known to be frequently resistant to antibiotic therapy due to their capacity to produce an exopolysaccharide barrier (42) and carry a wide variety of multi–drug resistant genes on plasmids, which can be exchanged and spread among different species of Staphylococci (43).

Conclusion

In conclusion, RTE meat sandwiches contaminated with high numbers of multi drug resistant and enterotoxin producing *S. aureus*. These findings reflect poor personnel hygiene during processing, which may lead to health problems to consumers.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

POSSIBLE ANALGESIC, 1ANTI-INFLAMMATORY AND ANTI-ULCEROGENIC EFFECTS OF *Alhagi maurorum* METHANOLIC EXTRACT IN RATS AND MICE

Hesham A. Khalifa[1], Asmaa M. Elattar[2]*, Naglaa Z.H. Eleiwa[1]

[1]Department of Pharmacology, Faculty of Veterinary Medicine, Zagazig University, [2]The Medical Center Pharmacy, Health administration, Belbeis, Sharkia, Egypt

*Corresponding author, E-mail: ph.asmaa.magdi.92@gmail.com

Abstract: *Alhagi maruroum*, a species of family *leguminosae*, has high contents of flavonoids, triterpenes, sterols and glycosides. It is a woody perennial herb that was used in the folk medicine for gastric disturbance, rheumatic pains, liver disease and urinary tract infection. This study was designed to spot the light on some pharmacological activities of *Alhagi maurorum*. The fresh aerial parts of the plant were washed, dried at room temperature, crushed into fine powder, extracted with 80% methanol for three days with frequent agitation, filtrated and dried by rotatory evaporator under reduced pressure at 45°C. The concentrated filtrate then was lyophilized in lyophilizer. The extract was tested for some pharmacological activities (analgesic, anti-inflammatory and antiulcerogenic) in rats and mice. The current results proved that administration of *Alhagi maurorum* aqueous methanolic extract in mice at dose of 200 and 400 mg/kg BW exhibited analgesic effect that was noticed from a significant elevation in the reaction time of mice in a dose dependent manner when compared to the control mice by using hot plate method. Moreover, *Alhagi maurorum* aqueous methanolic extract demonstrated anti-inflammatory effect by using hind paw edema method in rats as confirmed by a significant dose dependent reduction in the thickness of the hind paw edema compared with the control group. Additionally, administration of *Alhagi* extract elicited a significant improvement in gastric damage induced by indomethacin in rats where mean ulcer score in *alhagi* 200 and *alhagi* 400 groups was 1.25±0.045 and 0.25±0.004, respectively compared with 1.75±0.054 of indomethacin group. It could be deduced from those results that *Alhagi maurorum* aqueous methanolic extract owns analgesic, anti-inflammatory, and antiulcerogenic activities.

Key words: *Alhagi maurorum*; aqueous methanolic extract; analgesic; anti-inflammatory; anti-ulcerogenic

Introduction

Natural products own valuable and advantageous biological activities (1). Researchers are designing several studies on natural products to find solutions toward tumor, viral and microbial infections (2). *Alhagi marorum* belongs to the family *Leguminosae* commonly known as camelthorn (3). The family *leguminosae* contains about 550 genera and more than 13000 species (4). It supports us with

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many edible plants. *Alhagi* species contain several compounds such as fatty acids and sterols (5,6), flavonoids (7-9), alkaloids (10), triterpenes resins, tannins and antipyretic, antioxidant and diuretic (11). *Alhagi marorum* is a foliage deciduous shrub. It grows up to 2 m and produces flowers in July. The flowers are small in size, bright pink to maroon in color and hermaphrodite. The plant grows in sandy and Loamy soils (12).

Alhagi maurorum has been used traditionally to treat polyps in nasal cavities, certain tumors (3) and kidney stones (13). It has been mentioned that *Alhagi maurorum* has many therapeutic properties as; laxative, gastroprotective, expectorant, antiseptic and antidiarroheal properties. Oil extracted from its leaves has been used for treatment of hemorrhoids and rheumatism, while its flowers have been reported to treat piles (12,14).

Alhagi was used as diuretic, antimicrobial and in treating of hemorrhoids and uterine diseases, moreover, its root was used as aphrodisiac (4,15,16). *Alhagi* is national to North Africa, Middle East and South East Europe. It is originating in wide areas including Asia (Bahrain, Kuwait, Afghanistan, India, Palestine, Jordan, Lebanon, Syria, Saudi Arabia, Iraq and Yemen); Africa (Egypt, Algeria, Libya, Niger, Sudan and South Africa); North America (USA); Europe and Australia (16).

Ethanolic extract of *Alhagi maurorum* gives gastroprotective effects against ulcers caused by phenylbutazone, indomethacin and ethanol (16). Antioxidant effect of the aqueous extract of *Alhagi maurorum* was assessed by measuring the concentration of lipid peroxidation marker malondialdehyde (MDA) and total antioxidant capacity (TAC). The test extract has shown a significant reduction in MDA level (17). *Alhagi maurorum* has been recorded to exert anti-inflammatory effect by inhibition the release of histamine and prostaglandin (pro-inflammatory mediators) of acute inflammation (18). The extract of *Alhagi maurorum* given to rats orally at dose of 100mg/kg body weight (BW) showed hepatoprotective and antioxidant activities, beside inhibition of fucosidase tumor marker and risky lipid ratio (19,20). Tamarixtin 3-o-dirhamnoside, isorhamnetin 3-o-rotinoside, isorhamnetin 3-o-robinoside, quercetin 3-o-rhamnoside, isorhamnetin 3-o-glucosylneohesperidoside and quercetin 3,7-diglycoside are examples of flavonoids isolated from *Alhagi maurorum* (21). Proceeding from the above, the current work was contrived to pore over the analgesic, antiinflammatory and antiulcerogenic activities of *Alhagi maurorum* aqueous methanolic extract.

Material and methods

Plant material

The parts above the ground (leaves, stems and flowers) were gathered during 2017 from Elsalihia village, Faqous, Sharkia Governorate, Egypt.

Animals

Forty-five adult male wister albino rats and 20 mice were obtained from Faculty of Veterinary Medicine, Zagazig University. Animals were kept under observation for two weeks for acclimatization to the laboratory conditions before starting the experiment. They were held under sanitary condition in metal cages and they had free access to standard laboratory diet. Housing and management of animals were conducted as stipulated in the guide for the care and the use of laboratory animal's guidelines of the National Institute of Health (NIH) and approved by local authorities of Zagazig University, Zagazig, Egypt.

Plant extraction

Three kilo grams of the fresh parts above the ground were washed completely with fresh water at room temperature to eliminate the dirt before the drying process and exposed to dryness for a week at room temperature. The plant was subjected to grinding into fine powder. Two hundred grams of the powder were put with 800 ml methanol and 200 ml water in a clean flask for 3 days with frequent agitation then filtration was done and the residue was taken for further maceration. The filtrate was dried by rotatory evaporator under reduced pressure at 45°C. The concentrated filtrate was lyophilized by using a lyophilizer

(Klee Lyophilizer, German) to obtain a stable cake dissolved in water when prepared to administration. The extract was placed in a refrigerator at 4°C in a glass container to protect it from the light until use. The extract was dissolved in sterile saline solution to be used in the experiment.

Drugs and chemicals

Indomethacin (Indomethacin100^{R}, MISR, Egypt), Ranitidine (Rnitidine150^{R}, MUP, Egypt), Carragenen (SIGMA-ALDRICH, USA) and Diclofenac sodium, voltaren 75mg/3mL^{R} (NOVARTIS, Switzerland) were dissolved in sterile saline solution to be used in this study.

Analgesic activity evaluation

Evaluation of the analgesic activity of the plant was performed using the hot plate method as described by Jacob and Bosovski (22). Twenty mature male albino mice weighing 20-25 g were allocated into 4 groups; each of five. The 1st group (control) was injected intra-peritoneally (IP) with the solvent (sterile saline solution). The 2nd group (diclofenac) was injected IP by diclofenac sodium at a dose of 10 mg/kg BW (23). The 3rd group (Alhagi 200) received *Alhagi maurorum* methanolic extract at a dose of 200 mg/kg BW, per os (PO) (24). The 4th group (Alhagi 400) received *Alhagi maurorum* methanolic extract at a dose of 400 mg/kg BW, PO (24). Ten minutes later, each mouse was put in a two litres-beaker immersed in water bath thermostatically controlled at 56°C. The time passed away till the mouse licked its paw or jumped which was considered as the reaction time and was taken to evaluate the analgesic effect. Readings were recorded at 10, 20, 30, 60, 90 and 120 minutes after treatment.

Anti-inflammatory activity evaluation

Anti-inflammatory activity was assessed by using the method of rat hind paws edema (25) using diclofenac sodium as a standard. Twenty mature male albino rats weighing 150-200g were used. The animals were distributed into 4 equal groups; each of five. The 1st group (control) was treated IP with the solvent (sterile saline solution). The 2nd group (diclofenac) was given diclofenac sodium IP at a dose of 10 mg/kg BW (23). The 3rd group (*Alhagi* 200) received *Alhagi maurorum* methanolic extract at a dose of 200 mg/kg BW, PO (24). The 4th group (*Alhagi* 400) received *Alhagi maurorum* methanolic extract at a dose of 400 mg/kg BW, PO (24). After an hour, carrageenan was injected in the right hind paw by dose of 0.1 ml of 10% to induce edema. The thickness of the paw was measured using a skin caliber at 1, 2, 3 and 4 hrs after the carragenan injection to detect the anti-inflammatory activity of the tested extract.

Antiulcerogenic activity

Alhagi maurorum was tested for its antiulcerogenic activity using rantidine as a reference drug at a dose of 100 mg /kg BW, PO once daily for rats (19) and indomethacin for induction of ulcer at a single dose of 100 mg/kg BW, PO (19). Twenty-five male albino rats of average weight 150-200g were allocated into five groups; each of five. The first group (control) received distilled water (0.5ml PO /animal) once daily for 10 days. The second group (ranitidine) received ranitidine (100 mg/kg BW, PO) once daily for 10 days then treated with a single dose of indomethacin (100 mg/kg BW, PO) one hour after the last dosing. The third group (indomethacin) received a single dose of indomethacin (100 mg/kg BW, PO) on the10th day from the beginning of the experiment. The fourth group (*Alhagi* 200) received *Alhagi marorum* methanolic extract at dose of 200 mg /kg BW, PO once daily for 10 days then treated with a single dose of indomethacin (100 mg/kg BW, PO) one hour after last extract dosing. The fifth group (*Alhagi* 400) received *Alhagi marorum* methanolic extract at dose of 400 mg /kg BW, PO once daily for 10 days then treated with a single dose of indomethacin (100 mg/kg BW p.o) one hour after last extract dosing. Four hours after indomethacin administration. All rats were sacrificed, the stomachs were rapidly removed, opened along their curvature and examined for ulceration. The number and severity of discrete areas of damage in the glandular mucosa were scored. The ulcer score was calculated accor-

ding to the 1 to 5 scoring system devised by Wilhelmi and Menasse (26).

Ulcer index (U.I.) = Mean ulcer score of a group of animals similarly treated × % of ulcerated animals of this group (27).

Statistical Analysis

Repeated measure analysis of variance (ANOVA) test was done to determine the effect of time and different treatments on rats' hind-paw thickness and reaction time that represents the anti-inflammatory activity and the analgesic activity, respectively. Data were statistically described using mean ±SE. The results were considered significant with P value less than 0.05 ($P < 0.05$). All statistical analyses were done using IBM SPSS version 25.

Results

Analgesic activity

Mice treated orally with aqueous methanolic extract of *Alhagi maurorum* (200 and 400 mg/kg BW) revealed a significant ($P < 0.05$) dose dependent increase in the reaction time (till the mouse licked its paw or jumped) compared with the control group. However mice treated with diclofenac sodium (standard analgesic drug) demonstrated a significant ($P < 0.05$) rise in the reaction time compared with the control and *Alhagi maurorum* treated groups (Figure 1).

Anti-inflammatory activity

Oral administration of aqueous methanolic extract of *alhagi maurorum* in rats at dose of 200 and 400 mg/kg BW revealed a significant ($P<0.05$) dose dependent decrease in the thickness of hind paw edema at 1^{st} ,2^{nd} ,3^{rd} and 4^{th} h after carragenan injection compared to the control group. Meanwhile, administration of diclofenac sodium (standard anti-inflammmatory drug) in rats significantly ($P<0.05$) reduced the thickness of hind paw edema at 1^{st} ,2^{nd} ,3^{rd} and 4^{th} h after carragenan injection compared with the control and *Alhagi maurorum* groups (Table 1).

Antiulcerogenic activity

Oral indomethacin administration in rats by a single dose of 100 mg/kg BW resulted in gastric mucosal damage confirmed by a significant ($P<0.05$) elevation in mean ulcer score and ulcer index compared with the control group. Meanwhile, the aqueous methanolic extract of *Alhagi maurorum* at a dose of 200 and 400 mg/kg BW per os significantly ($P<0.05$) improved the gastric damage induced by indomethacin in a dose dependent manner. However, rats treated with ranitidine orally at dose of 100 mg/kg BW for 10 days then were administered with a single dose of indomethacin after last extract dosing exhibited a significant ($P<0.05$) reduction in mean ulcer score and ulcer index compared with indomethacin and *Alhagi maurorum* 200 treated groups (Table 2).

Table 1: Anti-inflammatory effect of *Alhagi maurorum* aqueous methanolic extract and diclofenac sodium against carragenan induced hind paw edema in rats. Mean±SE N=5

Group	Thickness of rat hind-paw (mm)					P-value
	Zero time	1 h	2 h	3 h	4 h	
Control	0.16 ± 0.01^{j}	0.65 ± 0.010^{de}	0.80 ± 0.08^{bc}	0.90 ± 0.03^{ab}	0.97 ± 0.05^{a}	0.000
Diclofenace	0.17 ± 0.02^{j}	0.24 ± 0.04^{ij}	0.34 ± 0.05^{hi}	0.38 ± 0.04^{h}	0.44 ± 0.04^{gh}	0.000
Alhagi200	0.18 ± 0.01^{j}	0.43 ± 0.02^{h}	0.55 ± 0.03^{ef}	0.63 ± 0.03^{ef}	0.74 ± 0.03^{cd}	0.000
Alhagi400	0.17 ± 0.02^{j}	0.37 ± 0.02^{h}	0.44 ± 0.02^{gh}	0.54 ± 0.03^{fg}	0.62 ± 0.02^{ef}	0.000

Means with different superscripts were significant at ($P<0.05$).

Table 2: Antiulcerogenic activity of *Alhagi maurorum* methanolic extract and ranitidine against indomethacin induced gastric ulcer in rats. Means ±SE, N=5

Group	Mean ulcer score	Incidence of gastric ulceration (%)	Ulcer index
Control	0.00±0.00^{d}	0.00	0.00
Ranitidine	0.25±0.005^{c}	25	6.25
Indomethacin	1.75±0.054^{a}	100	175
Alhagi 200	1.25±0.045^{b}	75	93.75
Alhagi 400	0.25±0.004^{c}	25	6.25

Means with different superscripts were significant at (P <0.05)

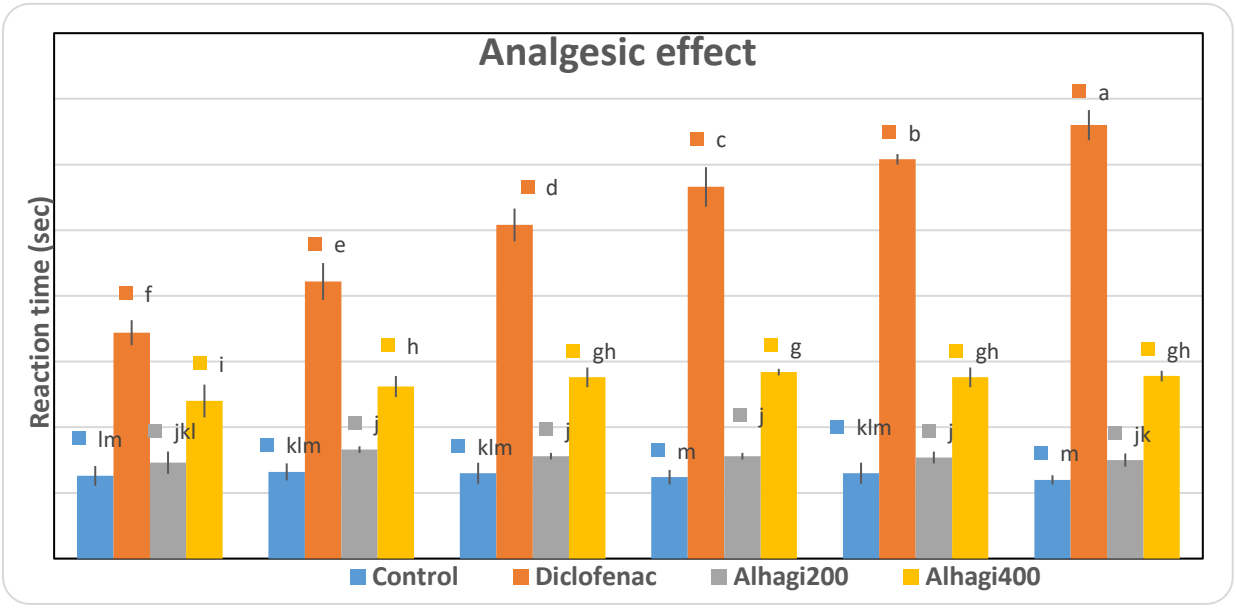

Figure 1: Analgesic activity of *Alhagi maurorum* aqueous methanolic extract and declofenac by using hot plate method in mice. Mean±SE, N=5. Columns carrying different superscripts are significant at ($p < 0.05$)

Discussion

In the present work, administration of *Alhagi maurorum* aqueous methanolic extract in rats at dose of 200 and 400 mg/kg BW, PO revealed anti-inflammatory activity as confirmed by a significant reduction in the thickness of hind paw edema in a dose dependent manner compared with the control group. The anti-inflammatory effect of *Alhagi maurorum* may be due to presence of lupeol (bioactive anti-inflammtory component) (28). It has been reported that *Alhagi maurorum* had anti-inflammatory activity by inhibition the release of inflammatory mediators (histamine and prostaglandin) (19,29). The cause of pain inhibition of *Alhagi marurorum* extract is like to anti-inflammatory drugs especially nonsteroidal (30). The anti-inflammatory activity of the extract would be explained by their antioxidant properties particularly radical scavenging activity toward diphenyl picryl hydrazyl (DPPH) radical (31). Our results are similar to previous studies which revealed that *Alhagi* has anti-inflammatory effect which examined by reducing the paw edema thickness (31-33).

In the present study, Diclofenac administration evoked a significant anti-inflammatory activity visible from the reduction in the thickness of paw edema in rats. It has been reported that the ability of diclofenac to show

a high degree of analgesic, anti-inflammatory, and antipyretic effects in various pharmacological studies. It has the ability to suppress prostaglandin biosynthesis *in vitro* and *in vivo* (34).

The current study showed that rats treated with aqueous methanolic extract of *Alhagi maurorum* at dose of 200 and 400 mg/kg BW PO demonstrated a significant analgesic effect compared with the control group. The analgesic effect could be imputed to presence of the high content of phenolics and flavonoids (35). *Alhagi maurorum* has been recorded to inhibit histamine, serotonin and prostaglandins that enable it to possess analgesic activity (19,29). Our data are consistent with a former study which showed that the aqueous methanolic extract of *Alhagi maurorum* showed analgesic effect after formalin administration (18).

The present study also demonstrated that *Alhagi maurorum* aqueous methanolic extract showed a significant dose dependent improvement in the gastric damage induced by indomethacin as confirmed by significant improvement in mean ulcer score and ulcer index. Quercetin and catechin (flavonoids) isolated from *Alhagi* have antioxidant effect by inhibiting lipid peroxidation and scavenging free radicals (36). This effect demonstrated the ulcer prevention (37). Alhagit in and alhagidin, flavanone glycosides, have been isolated from *Alhagi pseudoalhag* may contribute to the antiulcerogenic effect of the aqeous methanolic extract of *Alhagi maurorum* (38). *Alhagi maurorum* has been reported to contain terpenes (24,39). Terpenes have been illustrated to have antiulcerogenic activity in other studies (40,41). The mechanism of action of triterpenes is the decline of mucosal prostaglandins metabolism, cytoprotective effect and decrease gastric vascular permeability (42). It has been shown that the methanolic extract of *Alhagi* has calcium channel blocking activity on the gastrointestinal tract smooth muscle (24). Similarly, formers studies have revealed the ability of *Alhagi maurorum* to protect gastric mucosa against phenylbutazone, indomethacin, ethanol (16), aspirin (19) and water immersion restraint stress (17) induced ulcer.

Conclusion

It might be concluded that, *Alhagi maurorum* aqueous methanolic extract possesses prophylactic anti-inflammatory, analgesic and antiulcerogenic activities in a dose dependent manner and further studies should be conducted to assess the phytochemical components of the extract.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

VIRULENCE AND ANTIMICROBIAL RESISTANCE GENES OF *Escherichia coli* IN READY TO EAT SANDWICHES IN SHARKIA GOVERNORATE

Mohamed A. Hussein[1], Elsaid A. Eldaly[1], Hanan G. Seadawy[2], Elham F. El-Nagar[2]*

[1]Department of Food Hygiene, Faculty of Veterinary Medicine, Zagazig University, [2]Animal Health Research Institute, Zagazig Provincial Lab., Egypt

*Corresponding author, Email: elnagarvet_2005@yahoo.com

Abstract: Ready to eat food has been associated with some epidemics of foodborne illness all over the world. This study was designed to evaluate the microbial quality of ready to eat (RTE) sandwiches in Sharkia Governorate with special reference to virulence and antibiotic resistance genes of *Escherichia coli* (*E. coli*). One hundred and eighty RTE sandwiches containing meat (shawerma, kofta, sausage and hawawshi), chicken (chicken shawerma, pane, shish tawook, grilled chicken) and fish (grilled Tilapia nilotica, grilled *Mugel cephalus*, fried shrimp and fried fillet), fifteen of each were randomly collected from restaurants in Sharkia Governorate. Bacteriological examination revealed that the mean values of aerobic plate count (APC) ranged from 1.23×10^4 to 8.4×10^5 CFU/g and of total coliform count indicated by most probable number (MPN/g) ranged from 0.09×10^2 to 6.95×10^2. *E. coli* percentages ranged from 6.7% to 26.7%. Serotyping of *E. coli* strains revealed five serotypes (O_{153}: H_2, O_{78}, O_{127}: H_6, O_{91}: H_{21} and O_{26}: H_{11}) by different percentages, O_{26}: H_{11} was the most common one (31.6%) and O_{153}: H_2 was the lowest one (5.3%). All recorded isolates showed 100% antibiotic resistant to both erythromycin and amoxicillin – clavulanic acid. It was further found that *eae*A gene present in all isolated serotypes except O_{153}: H_2 while Shiga toxin (*Stx*1 and *Stx*2) were not detected at all, also found that resistant *E. coli* isolates to amoxicillin-clavulanic acid and erythromycin possessed bla_{TEM}, mph_A resistance genes (100%). Current results point to that RTE sandwiches in Sharkia Governorate are potential vehicles of antimicrobial-resistant *E. coli* among other possible foodborne pathogens with public health significance.

Key words: ready-to-eat; aerobic plate count; *E. coli*; coliform; resistance genes

Introduction

Ready-to-eat foods which prepared and sold on restaurants and public places act as a source of available, inexpensive and nutritional meals, also consider a source of income for the vendors (1). In spite of the commercial and nutritive value of this foods, the consumption of these foods suggested to increase the risk of foodborne illnesses as these foods may be contaminated from different sources (2). The aerobic plate count (APC) is an important factor for evaluation of microbial quality assessment in food products and is an indicator of the overall degree of microbial contamination of foods (3). Although most of *E. coli* are nonpat-

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hogenic but they are considered as indicator of faecal contamination in food and about 10 to 15% of intestinal coliforms are opportunistic and pathogenic serotypes (4) and cause a variety of lesions in immunocompro-mised hosts. The presence of specific microorganisms such as *E. coli* and or coliform in ready to eat food indicates that there are degrees of ignorance of the handlers to the proper hygienic practices (5)**.** Also the major sources of microbial contamination are the place of ready to eat (RTE) food preparation, utensils which used in cooking , raw materials, time and temperature of cooking in addition to the personal hygiene of workers (6)**.** Shiga toxin-producing *E. coli*. (STEC) are a rare but potentially fatal cause of gastroenteritis. They are associated with a wide spectrum of disease ranging from mild to bloody diarrhea, through to hemorrhagic colitis and hemolytic uremic syndrome (HUS) (7) but the *eae*A gene produces a 94-kDa outer membrane protein called intimin, which has been shown to be necessary but not sufficient to produce the attaching-and-effacing lesion. Improper handling considers one of the causes of foodborne diseases and that inadequate hand hygiene is a risk factor for food contamination (8)**.** Overuse of antibiotics act as a major factor for the development of antibiotic resistant bacteria which affect both the environment and human health (9). Antibiotics generally used for treatment of both human and animal diseases (10) and are used as prophylactic during animal growth. Foodborne transmission of pathogenic microorganisms has been recognized as a risk in the past few years and bacterial pathogens as *E. coli*, particularly antibiotic resistant strains, have long been dangerous zoonotic hazards (11). The resistance of Enterobacteriaceae to third-generation cephalosporin is mediated by TEM- and SHV-type L-lactamases. While, the most incriminated plasmid-borne beta-lactamases in amoxicillin-clavulanic acid resistance *E. coli* isolates is TEM type and OXA-1enzymes (12). Therefore, this study was conducted to evaluate the microbial quality of RTE sandwiches in El- Sharkia Governorate with special reference to virulence and antibiotic resistance genes of *E.* coli.

Material and methods

Collection of samples

A total 180 random samples of ready-to-eat (well done) sandwiches containing meat (shawerma, kofta, sausage and hawawshi), chicken (chicken shawerma, pane, shish tawook, grilled chicken) and fish (grilled Tilapia nilotica, grilled *Mugel cephalus*, fried shrimp and fried fillet) (15 of each) were collected from different restaurants in Sharkia Governorate. Each sample was kept in a separate sterile plastic bag, put in an icebox then transferred to the laboratory under complete aseptic conditions without undue delay for bacteriological examination (Examined within four hours from collection).

Preparation of samples

Twenty five grams of the meat products (sandwich core and in case of fish from dorsal muscle after removal of skin) samples were taken under aseptic condition to sterile stomacher bag then 225ml sterile peptone water were added, the contents were homogenized at stomacher for 2 minutes, the mixture was allowed to settled, for 5 minutes at room temperature. The contents were transferred into sterile flask, thoroughly mixed, 1ml was transferred into separate sterile test tube containing 9ml sterile peptone water, from which tenth- fold serial dilutions were prepared (13). The prepared samples were subjected to the following bacteriological examination:

Determination of total aerobic plate count CFU/g and coliform count MPN/g

The total aerobic plate count CFU/g and coliform count MPN/g were determined according to International Commission of Microbiological Specification for Foods (ICMSF) (14) and Food and Drug Administration (FDA) (15), respectively.

Isolation and identification of E. coli

It was applied by using MacConkey broth as enriched broth and eosin methylene blue (EMB) as plating Media (16).

Antimicrobial susceptibility testing for E. coli

Antimicrobial susceptibility testing for *E. coli* was determined by a standard disk diffusion method using the Bauer et al. (17) disk diffusion technique on Mueller-Hinton agar according to guidelines of Clinical and Laboratory Standards Institute guidelines (18).

Serological identification of E. coli

Serological identification of *E. coli* was measured according to Kok et al. (19) by using rapid diagnostic *E. coli* antisera sets (DENKA SEIKEN Co., Japan) for diagnosis of the enteropathogenic types.

Molecular identification of E. coli virulence and antibiotic resistance genes

DNA extraction was carried out using QIAamp DNA Mini Kit. The screening for the presence of shiga toxins virulence genes were applied according to Dipineto et al. (20) by using the following primer F: 5'- ACA CTG GAT GAT CTC AGT GG -3' and R: 5'- CTG AAT CCC CCT CCA TTA TG -3', which amplify a fragment of 614 bp (*stx*1) and F: 5'- CCA TGA CAA CGG ACA GCA GTT -3', and R: 5'- CCT GTC AAC TGA GCA GCA CTT TG -3', which amplify a fragment of 779 bp (*stx*2). The *eae*A gene was screened according to Wang et al. (21) using the primers F: 5'- ATG CTT AGT GCT GGT TTA GG -3', and R: 5'- GCC TTC ATC ATT TCG CTT TC -3', which amplify a fragment of 248 bp. The antibiotic resistance genes as *mph*A was examined according to Nguyen et al. (22) using the primers F: 5'- GTG AGG AGG AGC TTC GCG AG -3', and R: 5'- TGC CGC AGG ACT CGG AGG TC -3', which amplify a fragment of 403 bp. The *bla*TEM was determined according to Mabilat and Courvalin (23) using the primers F: 5'- ATC AGC AAT AAA CCA GC -3', and R: 5'- CCC CGA AGA ACG TTT TC -3', which amplify a fragment of 516 bp. The PCR products were tested for positive amplification by agarose gel electrophoresis (24). For each PCR experiment, appropriate positive and negative controls.

Statistical analysis

The obtained data were statistically analyzed using analysis of variance (ANOVA) test and comparisons of means were performed according to Duncan Multiple Range test for comparison of Means using SPSS14.

Results

Data presented in Table (1) revealed that the mean values of total APC CFU/g of sausage, kofta, shawerma, and hawawshi meals were 1.5×10^5, 2.2×10^5, 3.7×10^5 and 8.4×10^5, respectively. While for shish tawook, chicken shawerma, pane and grilled chicken was 7×10^4, 8.9×10^4, 3.7×10^5 and 5.8×10^5, respectively and for fried shrimp, fried fillet, grilled T. nilotica and grilled *M. cephalus* was 1.23×10^4, 3.3×10^4, 5.9×10^4 and 1.1×10^5, respectively. As reveled in Table (1) the mean value of total coliform count (MPN/g) in kofta, shawerma, sausage and hawawshi meals were 0.39×10^2, 0.71×10^2, 0.75×10^2 and 6.95×10^2, respectively. While in shish tawook, chicken shawerma, pane and grilled chicken was 0.15×10^2, 0.20×10^2, 0.148×10^3, and 0.20×10^3, respectively. Also in fried shrimp, fried fillet, grilled T. nilotica and grilled *M. cephalus* was $0.0\ 9\times10^2$, 0.65×10^2, 0.39×10^3 and 0.39×10^3, respectively. Coliform incidence in Table (2) was 26.7%, 33.3% 33.3% and 40% in shish tawook, pane, chicken shawerma and grilled chicken, respectively; 13.3%, 20%, 33.3%, 40%, in sausage, shawerma, kofta and hawawshi, respectively and 20%, 33.3%, 40% and 40% in fried shrimp, fried fillet, grilled T. nilotica and grilled *M. cephalus*, respectively. While *E. coli* incidence was 6.7 %, 13.3 %, 20% and 26.7% in shish tawook, pane, chicken shawerma and grilled chicken, respectively 13.3%, 13.3% and 20% in kofta, shawerma and hawawshi, respectively and 6.7% in both grilled T. nilotica and *grilled M. cephalus*, while not detected in sausage, fried shrimp and fried fillet.

E. coli isolates revealed five different serotypes (Table 3), the most common one was O_{26} : H_{11} (31.6%) which isolated from two samples of both shawerma and grilled chicken and one sample in both hawawshi and chicken shawerma, followed by five O_{91}: H_{21} isolates

(26.3%) recorded from two samples of both pane and chicken shawerma and one sample of grilled *M. cephalus* ,then four O_{127}:H_6 isolates (21%) obtained from two samples of kofta and two samples of hawawshi, three O_{78} isolates (15.8%) reported from one sample of shish tawook and two samples of grilled chicken, and finally O_{153} : H_2 (5.3%) isolated from only one sample of grilled T. nilotica.

Data in Table (4) showed that all isolates were 100% sensitive to streptomycin, doxycycline, chloramphenicol, sulphamethe-xazol–trimethoprim, 89.5% were sensitive to both ciprofloxacin and gentamycin and 84.2% were sensitive to cefotaxime while 100% of isolated samples were resistant to both erythromycin and amoxicillin–clavulanic acid. Using further investigation (PCR) in Figure (1) for five representative serotypes isolates (kofta, chicken shawerma, hawashi, grilled chicken and grilled Tilapia nilotica) (A) showed that *eae*A gene was present in all isolated serotypes except O_{153}:H_2 (grilled Tilapia nilotica) while Shiga toxin genes (*stx1* and *stx*2) were absent from all. (B) showed that all resistant *E. coli* isolates to amoxicillin-clavulanic acid possessed bla_{TEM} resistance genes (100%), (C) showed that all resistant *E. coli* isolates to erythromycin possessed mph_A resistance genes (100%).

Table 1: Mean values of APC (CFU/g) and Coliform count (MPN/g) in the examined samples of ready to eat sandwiches (n=15)

Item		APC Mean ± SE	Coliform count Mean ± SE
Meat meals	Sausage	$1.5\times10^5 \pm 8.3\times10^{4ab}$	$0.75\times10^2 \pm 0.24\times10^{2b}$
	Kofta	$2.2\times10^5 \pm 1.1\times10^{5ab}$	$0.39\times10^2 \pm 0.09\times10^{2b}$
	Shawerma	$3.7\times10^5 \pm 1.3\times10^{5ab}$	$0.71\times10^2 \pm 0.21\times10^{2b}$
	Hawawshi	$8.4\times10^5 \pm 2.2\times10^{5a}$	$6.95\times10^2 \pm 1.48\times10^{2a}$
Chicken meals	Shish tawook	$7\times10^4 \pm 2.8\times10^{4b}$	$0.15\times10^2 \pm 0.04\times10^{2b}$
	Chicken shawerma	$8.9\times10^4 \pm 3.7\times10^{4b}$	$0.20\times10^2 \pm 0.09\times10^{2b}$
	Pane	$3.7\times10^5 \pm 1.8\times10^{5ab}$	$0.148\times10^3 \pm 0.32\times10^{2ab}$
	Grilled chicken	$5.8\times10^5 \pm 2.4\times10^{5ab}$	$0.20\times10^3 \pm 0.113\times10^{2ab}$
Fish meals	Fried shrimp	$1.2\times10^5 \pm 4.2\times10^{4ab}$	$0.09\times10^2 \pm 0.029\times10^{2b}$
	Fried fillet	$3.3\times10^4 \pm 1.4\times10^{4b}$	$0.65\times10^2 \pm 0.32\times10^{2ab}$
	Grilled T. nilotica	$5.9\times10^4 \pm 1.2\times10^{4b}$	$0.39\times10^3 \pm 0.14\times10^{3ab}$
	Grilled *M. cephalus*	$1.1\times10^5 \pm 6.6\times10^{4ab}$	$0.39\times10^3 \pm 0.14\times10^{3ab}$

SE= stander error. Means of the same column carrying different superscript letters were significantly different ($p<0.05$).

Table 2: Incidence of isolated bacteria from the examined samples of ready to eat sandwiches (n=15)

Item		Coliform		*E.coli*	
		NO	%	NO	%
Chicken meals	Shish tawook	4	26.7	1	6.7
	Pane	5	33.3	2	13.3
	Chicken shawerma	5	33.3	3	20
	Grilled chicken	6	40	4	26.7
Meat meals	Sausage	2	13.3	ND	ND
	Shawerma	3	20	2	13.3
	Kofta	5	33.3	2	13.3
	Hawawshi	6	40	3	20
Fish meals	Fried shrimp	3	20	ND	ND
	Fried fillet	5	33.3	ND	ND
	Grilled T. nilotica	6	40	1	6.7
	Grilled *M. cephalus*	6	40	1	6.7

ND = not detected.

Table 3: Incidence of serotypes of *E. coli* strains isolated from examined isolates: (n=19)

***E. coli* strains**		**O_{127}:H_6**		**O_{91}: H_{21}**		**O_{26} : H_{11}**		**O_{78}**		**O_{153} : H_2**		
		No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**Total**
Meat meals	Shawerma	-	-	-	-	2	10.5	-	-	-	-	2
	Kofta	2	10.5	-	-	-	-	-	-	-	-	2
	Hawawshi	2	10.5	-	--	1	5.3	-	-	-	-	3
Chicken meals	Shawerma	-	-	2	10.5	1	5.3	-	-	-	-	3
	Pane	-	-	2	10.5	-		-	-	-	-	2
	Shish tawook	-	-	-	-	-		1	5.3	-	-	1
	Grilled chicken	-	-	-	-	2	10.5	2	10.5	-	-	4
Fish meals	Grilled T. nilotica	-	-	-	-	-	-	-	-	1	5.3	1
	Grilled *M. cephalus*	-	-	1	5.3	-	-	-	-	-	-	1
Total		4	21	5	26.3	6	31.6	3	15.8	1	5.3	19

Table 4: Percentages of antimicrobial susceptibility of *E. coli* strains isolated from the examined isolates of ready-to-eat sandwiches (n = 19)

Antimicrobial agents	**Susceptible**		**Intermediate**		**Resistant**	
	No.	**%**	**No.**	**%**	**No.**	**%**
Streptomycin (S)	19	100%	-	-	-	-
Ciprofloxacin (CIP)	17	89.5%	2	10.5%	-	-
Gentamycin (CN)	17	89.5%	2	10.5%	-	-
Erythromycin (E)	-	-	-	-	19	100%
Amoxicillin – clavulanic Acid(AMC)	-	-	-	-	19	100%
Doxycycline(DO)	19	100%	-	-	-	-
Chloramphenicol (C)	19	100%	-	-	-	-
Sulphamethexazol – trimethoprim(SXT)	19	100%	-	-	-	-
Cefotaxime (CTX)	16	84.2%	3	15.8%	-	-

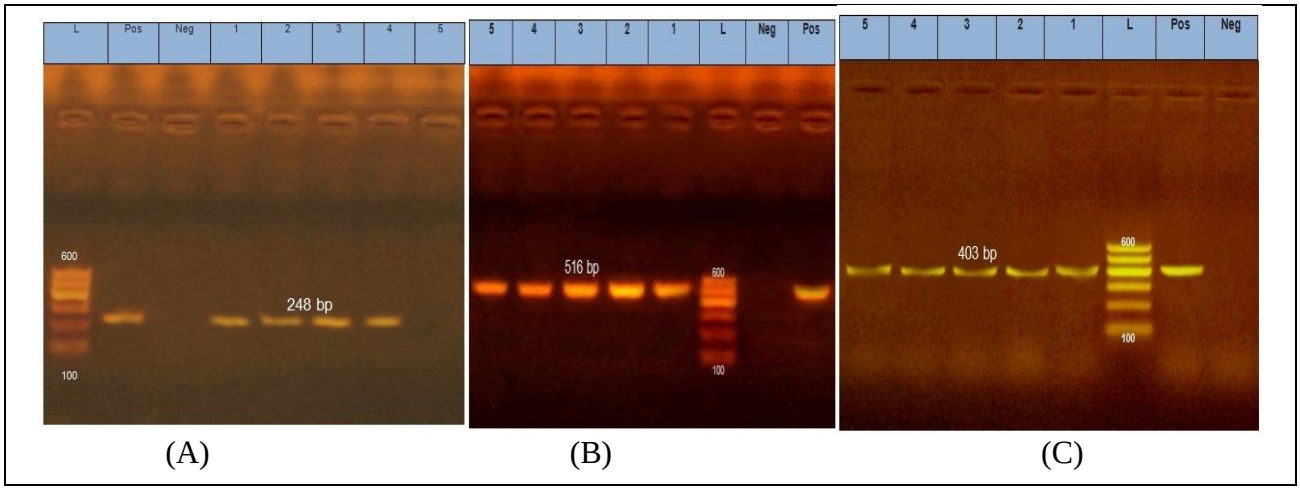

Figure 1: Agarose gel electrophoresis of PCR (A) *E. coli* virulence genes PCR in 1.5% agarose gel [L:100pb Ladder, 1: kofta (O_{127}:H_6); 2: chicken shawerma (O_{91}: H_{21}); 3: hawashi (O_{26} : H_{11}); 4: grilled chicken (O_{78}); 5: grilled Tilapia nilotica (O_{153}: H_2)] *eae*A (248 bp) (intimin gene) was +ve for sample number 1, 2, 3 and for 4, while negative in sample number 5. (B) bla_{TEM} (516 bp) resistance gene PCR in 1.5% agarose gel [L:100 bp Ladder, 1: kofta (O_{127}:H_6); 2: chicken shawerma (O_{91}: H_{21}); 3: hawashi (O_{26} : H_{11}); 4: grilled chicken (O_{78}); 5: grilled Tilapia nilotica (O_{153}: H_2)] +ve in all samples. (C) mph_A (403bp) resistance gene PCR in 1.5% agarose gel [L:100pb Ladder, 1: kofta (O_{127}:H_6); 2: chicken shawerma (O_{91}: H_{21}); 3: hawashi (O_{26} : H_{11}); 4: grilled chicken (O_{78}); 5: grilled Tilapia nilotica (O_{153}: H_2)] +ve in all samples

Discussion

This study revealed that, ready-to-eat sandwiches containing meat, chicken and fish had significant growth of microorganisms. But the microbial load of samples of certain products was higher than others to the extent that it may pose a threat to the health of regular consumers as some sandwiches are seasoned with peppers and with tahini (sesame seeds paste and oil) and served in a pita bread wrap as hawawshi. In addition, some restaurants use local mayonnaise to season the sandwiches, all this additives affect microbial quality of sandwiches. Aerobic plate count (APC) considered as important indicator for evaluation of sanitary condition of RTE sandwiches. Hawawshi was significantly higher contaminated ($P<0.05$) with APC, which attributed to the bad quality of ingredient introduced in processing or due to its coating with aluminum foil, which may consider as a source of contamination

The APC in hawawshi was lower than a study applied by Ibrahim (25) which was 21.2×10^6 CFU/g. The APC of kofta was $2.2x10^5$ CFU/g which is lower than Fahim et al. (26) who found that it was 8.51×10^5 CFU/g, while higher than a study applied by Sobieh (27) which was 1.83×10^4 CFU/g. The APC of chicken shawerma was lower than a study applied by Rafaie (28) who found that it was 2.46×10^7 CFU/g. Also APC of sausage was lower than that reported by Tudor et al. (29) who found that it was $1.2x10^4$ - $4.8x10^4$ CFU/g in meat products including sausage, while higher than El-Mossalami (30) which was $3.2x10^4$ CFU/g. Regarding to fish meals APC of grilled T. nilotica and grilled *M. cephalus* was lower than a study applied by Salim (31) which was 6.3×10^4 CFU/g in grilled T. nilotica and 3.7×10^5 CFU/g in grilled *M. cephalus*. The APC in fried shrimp was lower than Hassanin et al. (32) recorded 5.4×10^3 CFU/g in fried shrimp, also lower than Edris et al. (33) which was 2.78 CFU/g in breaded shrimp. APC in pane was higher than Ibrahim et al (34) who found that it was 7.35×10^4 CFU/g. As the high incidences of bacterial contamination are mainly due to the unsanitary and largely unhygienic nature of the food preparations and services areas as foods are good indicators of the state of environment in which they are prepared or served (35)**.** Total coliform count findings were lower than obtained by Rafaie et al. (28) who recorded $33.9x10^5$ CFU/g and $1.8x10^5$ CFU/g in shawerma and kofta samples, respectively. Also lower than that reported by Fahim et al. (26) which was 1.12×10^4 CFU/g in hawawshi sandwiches and Ibrahim et al. (34) who found that it was 1.18×10^3 CFU/g, 4.32×10^3 CFU/g, 9.97×10^3 CFU/g in RTE pane, shish tawook and shawerma, respectively. Also Hassanin et al. (32) recorded $2.4x10^2$ CFU/g in fried shrimp. On the other hand, it was higher than Edris et al. (33) who found that it was 1.59 CFU/g in breaded shrimp and Khater et al. (36) which was 2.92 CFU/g in RTE grilled kofta. Coliform bacteria were significant organisms in meat as indicator of fecal contamination and had the ability to grow well over wide range of temperature below 10˚C up to 46˚C (37). In the current study, coliform % was higher than that reported by Moustafa et al. (38) recorded in 20% of hawawshi samples and 10% of both shawerma and sausage. Lower than Salem et al. (39) isolate it from 76% of RTE chicken samples and 52% of RTE meat samples. *E. coli* % in this study was higher than that mentioned by EL-Shater et al. (40) record *E. coli* in 8% of shawerma samples and El-Dosoky et al. (41) isolate it from 10% of shawerma samples and Ismail (42) detected it in 17.3% of hawawshi. While, it was lower than resultes recorded by Khater et al. (36) which was 30% in kofta and Nimri et al. (43) which was 28.3% in shawerma. Similar results obtained by Hassanien et al. (32) isolate it from 8.6% of grilled *M. cebhalus* and Hosein et al. (44) reported that, all examined shrimp were free from *E. coli*. Absence of *E. coli* from fillet sandwiches, shrimp sandwiches and sausage sandwiches may be attributed to efficient heat treatment during cooking, high quality of ingredient introduced in processing, absence of other additives as salads or mayonnaise and application of good hygienic measures. Although, *E. coli* is readily inactivated above 55°C, the post cooking cross contamination

may occur as a result of contact of raw ingredient with cooked meat (45) or the hands of food handlers as mentioned by Awadallah et al. (46) or also due to eat chicken by mixing it with contaminated leafy vegetables and salads post-processing (47). In general, heat-treated foods must be free from *E. coli*. The presence of *E.coli* in the food is considered as indicator of fecal contamination beside they induce severe diarrhea in infants and young children as well as cases of food poisoning and gastroenteritis among consumers. Although most of *E.coli* are harmless, several are known to produce toxins that may cause diarrhea. The pathogenic *E. coli* can be grouped into enteropathogenic (EPEC), enterotoxogenic (ETEC), enterohaemorrhagic (EHEC), entero-aggregative (EAEC), enteroinvasive (EIEC) and Diffusely adherent *E. coli* (DAEC) (48). The pathogenic strains of *E. coli* were previously isolated from different ready-to-eat meat products by Soliman and El–Tabiy (49) who isolate O_{26} (13.3%) and O_{127} (6.7%) from kofta, Fahim et al. (26) isolate O_{111}: H_4 and O_{127}:H_6 strains from 10% and 5% of kofta respectively, O_{26}:H_{11}(5%), O_{55} : H_7(5%), O_{111} : H_4(5%) and O_{119}: H_4(10%) from sausage sandwiches. While O_{26}: H_{11} (10%), O_{55}:H_7 (5%), O_{86} (5%), O_{111}: H_4(5%) and O_{124} (5%) in hawawshi.

Results of antibiotic sensitivity test agree with Rao et al. (50) and Hemeg (51) who found that all isolates from ready to eat meat were 100% resistant to amoxicillin–clavulanic acid, but disagree with Nimri et al. (43) found 50% of *E. coli* isolates were resistant to tetracycline and streptomycin. The rate of antibiotic resistance in *E. coli* acts as an indicator of resistance transmission in bacterial populations and also as an indicator for the antimicrobials used in the treatment of slaughter animals and humans (52). *Eae*A gene was detected in 80% of examined isolates mean while shiga like toxin genes were not detected. Intimin gene (*eae*A) is an essential factor for the microbe to attach strongly to intestinal mucosal cells and initiate disease (48). On contrary, the finding of Nimri et al. (43) was 30% of *E. coli* isolates were positive for the *stx*1 gene. The results of *bla*$_{TEM}$ gene were nearly similar to findings of Paula et al. (53) who declared that *bla*$_{TEM}$ gene was detected in (34.3%) of deli meats. Zongo et al. (54) reported that *E.coli* isolates had low resistance with antibiotics of beta lactamin family in RTE food Yoon et al. (55) found that cephalosporin-resistant *E. coli*, was 8.7% in meat samples and 31.8% in chicken samples. The most common mechanism of resistance among enterobacteriaceae is the production of b-lactamases, chromosomally or plasmid encoded, which inactivate certain b-lactam antibiotics by hydrolyzing the b-lactam ring (56).

Conclusion

Our results indicate that RTE sandwiches in Sharkia serve as potential vehicles for the transmission of antimicrobial-resistant *E. coli* among other possible emerging foodborne pathogens, and are therefore a potential public health hazard. Therefore, using of high quality raw materials, efficient heat treatment, adequate cleaning and sanitization of utensils, day-by- day observance of proper personal, food handling of cooked food and lastly adequate education of food hygiene should be done. In addition, strict hygienic measures should be applied during preparation of ready to eat food to improve the quality of the product and to safeguard the consumers.

Conflict of interest

None of the authors have any conflict of interest to declare

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Original Research Article

PIGEON AND FOWL HEARTS: A COMPARATIVE MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL (TROPONIN T) STUDY

Yaser H.A. Elewa[1,2], Sherif Kh.A. Mohamed[3]*, Attia A.A. Moselhy[3], Dina M.M. AlSadek[2], Haitham A. Badr[4]

[1]Faculty of Veterinary Medicine, Basic Veterinary Sciences, Hokkaido University, Sapporo 060-0818, Japan, [2]Department of Histology and Cytology, [3]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, [4]Department of Biochemistry, Faculty of Agriculture, Zagazig University, Zagazig 44511, Egypt

*Corresponding author, E-mail: sherifanatomy81@gmail.com

Abstract: Similar to mammals, the avian heart is a muscular pump responsible for distributing blood throughout the body. However, a larger relative heart weight to body weight (RHW/BW) was reported in birds than mammals. Furthermore, it has been revealed that smaller birds specially flying one have relatively larger hearts (relative to body mass) than larger birds (non-flying). Although this fact, there are no literatures regarding comparative descriptive variations of the heart in flying and non-flying birds that could adapt various functions, such as flying and running. Therefore, this study aimed to conduct a comparative anatomical features and morphometrical measurments between the hearts of pigeon and fowl. The present study also analyzed the immunohistochemical expression of Troponin T (TnT) as one of the major regulator of striated muscle contraction. The study revealed significant larger RHW/BW in pigeon heart. Furthermore, the hearts of both fowl and pigeon are directed caudoventrally in the cranial part of thoracoabdominal cavity, however, a more oblique position nearly parallel to the sternum was observed in pigeon`s hearts. Interestingly, the mean morphometric values (length of both cranial and caudal borders, diameter of the base, and thickness of both right and left ventricular wall and interventricular septum) of the pigeon hearts showed significant higher differences than that of fowl. Additionally, more expression of TnT was observed in pigeon hearts. Therefore, our findings suggest that such morphological and immunological varities are possibly essential factors for providing intense ventricular contraction of the pigeon`s heart to cover the high metabolic requirments for flight.

Key words: fowl; heart; pigeon; immunohistochemistry; troponin T

Introduction

The birds have an efficient cardiovascular system that permit them to adapt the metabolic demands of different physiological processes such as flight, swimming, or diving and running. It has been reported that the birds, unlike their crocodile relatives can maintain their core temperature at 37°C by the increasing the basal metabolic rate, cardiac output and blood pressure (1). The major organ involved in this system is the heart. The heart of birds, like mammals, is a muscular pump consisting of four chambers (two atria and two ventricles),

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and showed a double circulatory system with a complete separation of oxygenated and de-oxygenated blood.

Interestingly, the avian heart is significantly heavier in proportion to the body weight when compared to other vertebrates and mammales and represents 0.8-1.2% of the body weight (1). Furthermore, it was previously reported that the bird`s heart show a larger stroke volume, and higher cardiac output than do mammals of similar body mass (2,3). Additionally, a larger heart and an increase in cardiac output are required to enable the avian heart and cardiovascular system for providing enough oxygen to body tissues during flight (4). Despite the previous facts, little was so far reported concerning the hearts of birds (5-8). Furthermore, comparative morphological variations of the avian heart among flying and non flyng birds remains unclarified.

The heart is composed of contractile cardiac muscle cells (cardiomyocytes) that differs slightly from the skeletal and smooth muscle cells whereas cardiomyocyte contracts in coordination with its neighbouring cells to maintain a regular pumping rhythm. Any disturbances in such coordination leads to abnormal heart rhythms such as ventricular fibrillation (4). The contractility of cardiac muscle differs from bird to bird and from one age to another.

Troponin T is the tropomyosin-binding subunit of the troponin complex, which plays a great role in the regulation of striated muscle contraction (9). In vertebrates, three Troponin T (TnT) genes were reported; cardiac TnT (cTnT), skeletal muscle fast-twitch TnT (fTnT) and slow-twitch TnT (sTnT) (10). The cTnT is one of the regulatory proteins that control the calcium mediated interaction between actin and myosin. In avian heart, cTnT was reported to be expressed in the chicken heart (11), however, no reports about its expression among flying and non-flying birds. The present work aimed to investigate some morphological variations in pigeon and fowl hearts in relation to the presence and release of cardiac Troponin T in active flying birds rather than non-flying or resting ones.

Material and methods

Birds of study and ethics statement

This study was conducted on healthy adult pigeons and fowls (n= 16/ species) of both sexes of pure baladi breeds, obtained from commercial farms in Zagzaig, Egypt. The fowl was 3-5 months-old and 1.4±0.5 kg body weight, however, the pigeon was 6-11 months-old and 0.450±0.250 kg body weight. This experiment was carried out according to the institutional ethical committee of the Zagazig University, Egypt. The macromorphological study of this work was performed at the Anatomy and Embryology Department, Zagazig University, Egypt. The micromorphology and immunohistochemistry were done at Faculty of Veterinary Medicine, Basic Veterinary Sciences, Hokkaido University, Sapporo, Japan.

Macromorphology

Six fresh hearts from each species were dissected for further macro-morphological studies. Before removing their hearts, the anatomical positions of fowl and pigeons hearts and their relations were studied inside the thorax. Each heart was washed with normal saline and the pericardium was investigated. The pericardium was removed and the weight of the hearts was measured after cutting open in the ventricles and atria for removal of any blood residues and thrombi in its chambers. Also, the shape and surface topography of the fresh hearts were studied.

Morphometric study

Both avian and pigeon hearts were weighed and related to body mass, also a comparative gross morphometry, between fowl and pigeon hearts, were measured such as (average body weight, length of cranial and caudal border, transverse diameter, thickness of right and left ventricular wall and interventricular septum). Varnier caliper was used in current investigations.

Micromorphology

The walls of the other ten heart ventricles were opened, the blood was washed and then

the specimens were immediately fixed at 10% neutral buffered formalin and samples were taken from same areas of ventricular wall in both strains. Some of the specimens were subjected to the routine histological technique for preparations of parafine blocks. Sections of 5-7 µm thickness were obtained and stained with different histological stains such as Harris's Hematoxylin and Eosin (H&E), Periodic Acid Schiff (PAS) and Masson´s trichrome (12).

For detection of cardiac Troponin T, immunohistochemical staining of 3 µm paraffin sections with primary antibody (mouse anti-cTnT monoclonal antibody (11-13), catalog No. MA5-12960, Thermo Fisher Scientific thermofisher, Rockford, USA) at dilution of 1:1000 in 1.5% bovine serum albumin/PBS (pH 7.2). For the negative control sections, 0·01 m PBS was used instead of the primary antibody. The immunohistochemical procedures were performed according to Elewa et al. (13).

Terminology

The nomenclatures used along the course of this work were adopted using Nomina Anatomica Avium (14) and Nomina Anatomica Veterinaria (15).

Results

Macromorphology

The heart in both fowl and pigeon is situated in the cranial part of the thoracoabdominal cavity, ventral to the esophagus and tracheal bifurcation of the two lungs and just cranial to the liver (Figure 1A and B). It is directed caudoventrally, and is observed to be in a slight oblique orientation to the sternum in fowl (Figure 1C), however, a more oblique orientation, nearly parallel and resting on the sternum in pigeon (Figure 1D).

The base of the fowl heart, begins from the first intercostal space and the apex ends at the third intercostal space (Figure 1A and C). However, in pigeon, the base extends from second intercostal space and the apex reaches the sixth intercostal space, (or at the end of costal arch) (Figure 1B and D). In both fowl and pigeon, the apex is attached to the keel bone by the fibrous pericardium (sternopericardial ligaments) (Figure 1C and D). In pigeon, most of the heart is embedded between the rostral part of the right and left lobes of the liver, while, only the apex of the heart in fowl is done.

The fowl heart has an elongated cone shape with a pointed apex (Figure 1E and F). However that of pigeon, showed a flattened conical shape with a blunt apex (Figure 1G). The heart of both birds is consisted of four-chambers (two atria and two ventricles). In both studied species, the wall of the left ventricle is clearly thicker (by about five times) than the right one. The interventricular septum in both species is thicker than both right and left ventricular wall (Figure 1F and H)

Morphometric measurments:

The average heart weight in pigeon (5.02±0.07 gm) is significantly lower than that of fowl (10.97±0.22 gm), while, a higher significant difference in the average relative heart weight to body weight (RHW/BW) in pigeon (1.01±0.016) when compared to that of fowl (0.49±0.009) (Figure 2A).

The length of the cranial and caudal borders, and the transverse diameter of the fowl heart were significantly higher than that of pigeon (Figure 2B and C). These measurments were 3.21±0.05, 3.38±0.07, and 1.48±0.06 in fowl heart and were 1.7±0.02, 1.69±0.05 and 1.04±0.06 in pigeon heart, respectively. Additionally, the thickness of the right and left ventricular wall, and the interventricular septum of the fowl heart were also notably higher than that of pigeon (Figure 2B and D). These measurments were 0.59±0.04, 2.32±0.05, and 5.11±0.05 in fowl heart and were 0.36±0.02, 1.42±0.07 and 2.95±0.05 in pigeon heart, respectively.

Micromorphology

The wall of the heart in fowl and pigeon is composed of three basic layers; endocardium (tunica intima), myocardium (tunica media) and epicardium (tunica adventitia or visceral pericardium). The main thickness of the wall, particularly of the ventricle, is composed of the myocardium (Figure 3A and B). The endocardium supported by a delicate layer of collage-

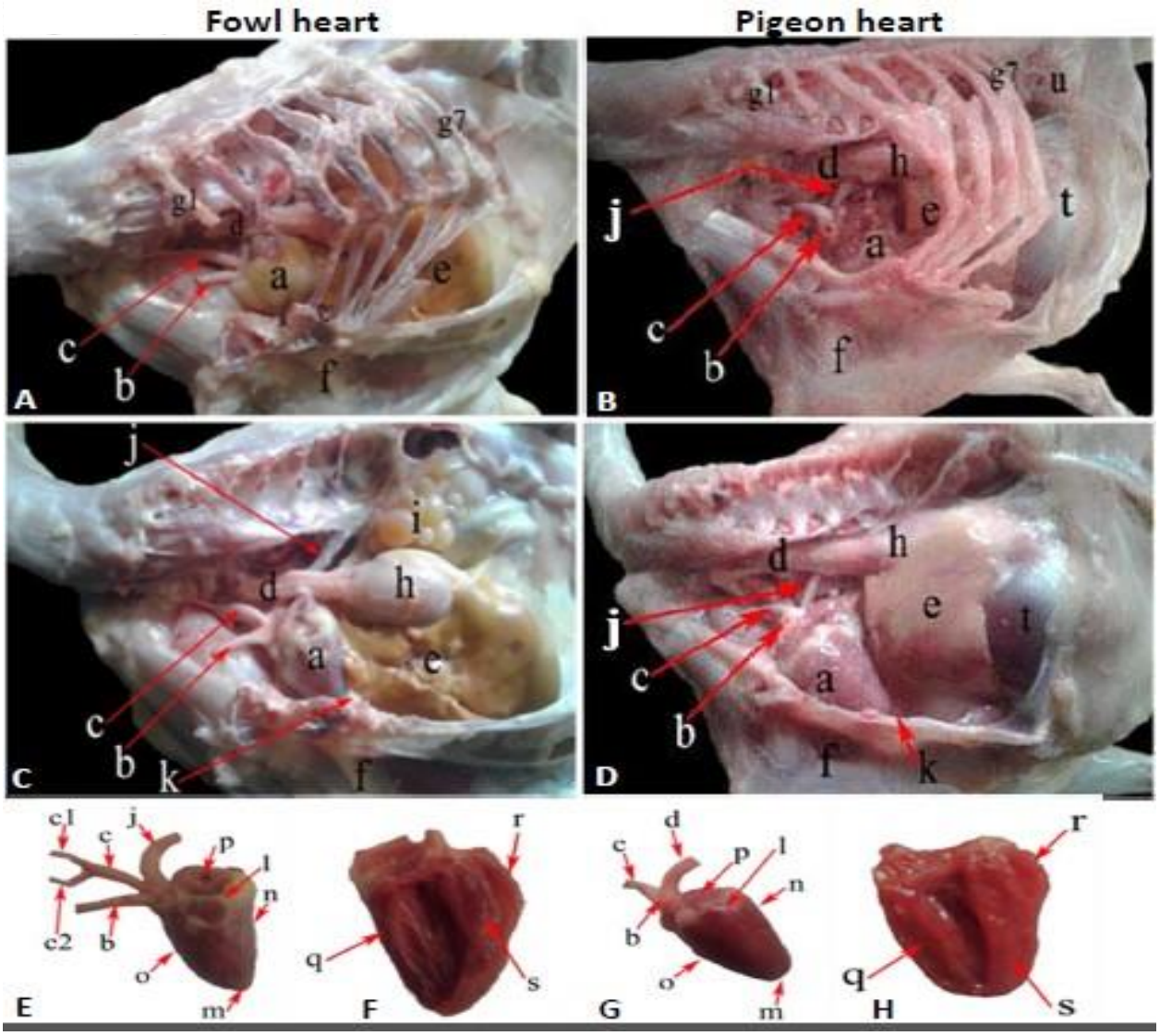

Figure 1: Photomacrographs of the fowl and pigeon hearts showing; Cor (a), Truncus pulmonalis (b), Arteria carotis communis (c), Arteria carotis communis dexter (c1) Arteria carotis communis sinister (c2) Esophagus (d), Hepar (e), Sternum (f), Costae (g1-g7), Proventriculus (h), Ovarium sinistrum (i), Aorta descendens (j) Ligamentum sternopericardiaca (K), Basis cordis (l), Apex cordis (m), Margo sinister (n), Margo dexter (o), Atrium sinistrum (p), Ventriculus sinister (q), Ventriculus dexter (r), Septum interventricular (s), Ventriculus (t), Testis (u)

nic fibres (Figure 3C), followed by a dense fibroelastic layer (subendocardial layer). The deepest layer of endocardium contains loose connective tissue and has blood vessels and nerves (Figure 3D). This latter layer is intercalated and more difficult to differentiate from the myocardial bundles in fowl, as opposed to (Figure 3E), in pigeon (Figure 3F). When compared to the fowl (Figures 3E and 4A), the deepest layer is observed to be thicker and contains numerous bundles of Purkinje fibres in the pigeon (Figure 4B). Purkinje fibres spread throughout the whole ventricular wall which are denser and thicker toward the lining side of the left ventricle when compared to the right one (Figure 4C). The myocardial fibers consist of cardiac muscle fibres running in longitudinal, circular and oblique orientations. Interestingly, the subepicardial and sub-endocardial myofibers in fowl ventricle showed longitudinal orientations (Figure 4A and C) but oblique ones were observed in pigeon (Figure 4B and D). However, the myocardial fibers in the intermediat areas revealed circular orientations in both fowl and pigeons throughout all levels (Figure 4).

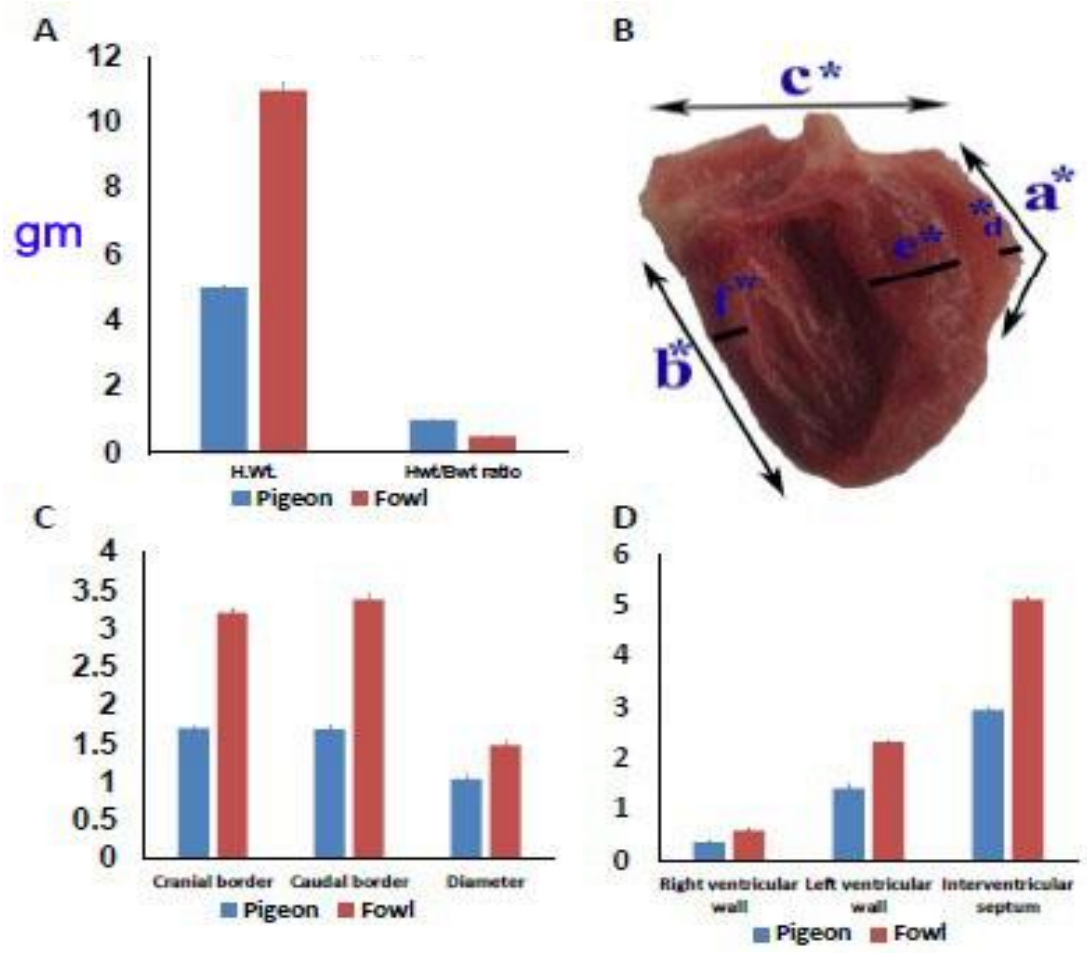

Figure 2: Morphometric measurements of fowl and pigeon hearts showing, (A) The ratio between the heart and body weight. (B) Length of cranial border (a*), Length of caudal border (b*), Diameter of heart base (c*), Thickness of right ventricles (d*), Thickness of interventricular septum (e*) and Thickness of left ventricles (f*). (C) Length of cranial and caudal borders and Diameter of heart base. (D) Thickness of right and left ventricles and thickness of interventricular septum

Masson's trichrome stained heart sections of both fowl and pigeon revealed a delicate layer of collagenic fibres surrounding the epicardium with the presence of a trivial amount of collagen fibers just around each individual myocyte, the wall of the blood vessels and Purkinje fibres (Figures 3C and D, 4E and 5A and B). The coronary blood vessels are well supplied by the myocardium present in-between its bundles, as seen most clearly in the pigeon (Figures 4E and 5A) than in the fowl (Figure 3D). H and E stained fowl heart declaired branched and symmetrically myocardial striated fibres with each other having elongated oval nuclei that run parallel with its longitudinal axis (Figure 4E and F).

Immunohistochemical staining of cTnT is located mainly in between the subendocardial layer and myocardium but it can be found in scanty amounts inside myocardial fibres in the fowl heart (Figure 5C and D). When compared to the fowl, Troponin T is more abundantly found and has a stronger reaction in the whole core of the myocardial layer of the pigeon than that of the fowl, (Figure 5E and F). The present study revealed that cardiac Troponin T found by large amounts in the heart of flying active birds, as in pigeons, and notable few amount in less active stable one as fowl. The latter indicates that there is a positive relationship between the activity of the flying birds and the amount of cardiac Troponin T in their hearts.

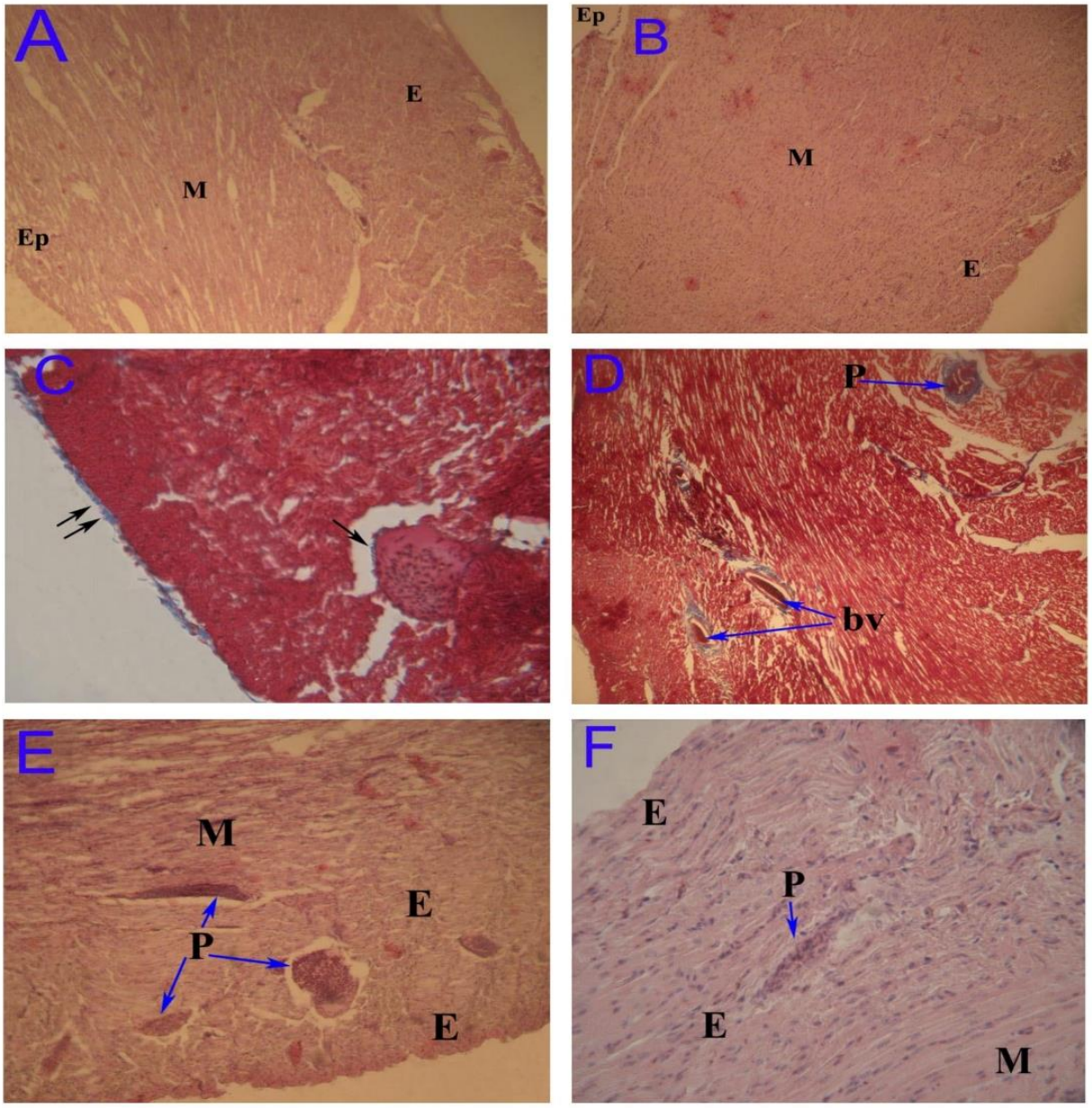

Figure 3: (A). A photomicrograph of the fowl heart (ventricular wall) showing, endocardium (E), myocardium (M) and epicardium (EP). H&E stain. X4. (B). A photomicrograph of the pigeon heart (ventricular wall) showing, endocardium (E), myocardium (M) and epicardium (EP). H&E stain. X10. (C). A photomicrograph of the fowl heart (ventricular wall) showing, a delicate layer of collagenic fibres supporting endocardium (double arrows) and mild reaction surrounding Purkinje fibres (arrow). Masson´s trichrome stain. X10. (D). A photomicrograph of the fowl heart (ventricular wall) showing, a positive reaction surrounding the wall of blood vessels (bv) and Purkinje fibers (P) in the deepest layer of endocardium. Masson´s trichrome stain. X10. (E). A photomicrograph of the fowl heart (ventricular wall) showing, endocardium (E) which intercalated and difficult to differentiate with the myocardial bundles (M). Purkinje fibers (P). H&E stain. X10. (F). A photomicrograph of the pigeon heart (ventricular wall) showing, a clear demarcation between endocardium (E) and myocardium (M). Purkinje fibres (P). H&E stain. X20

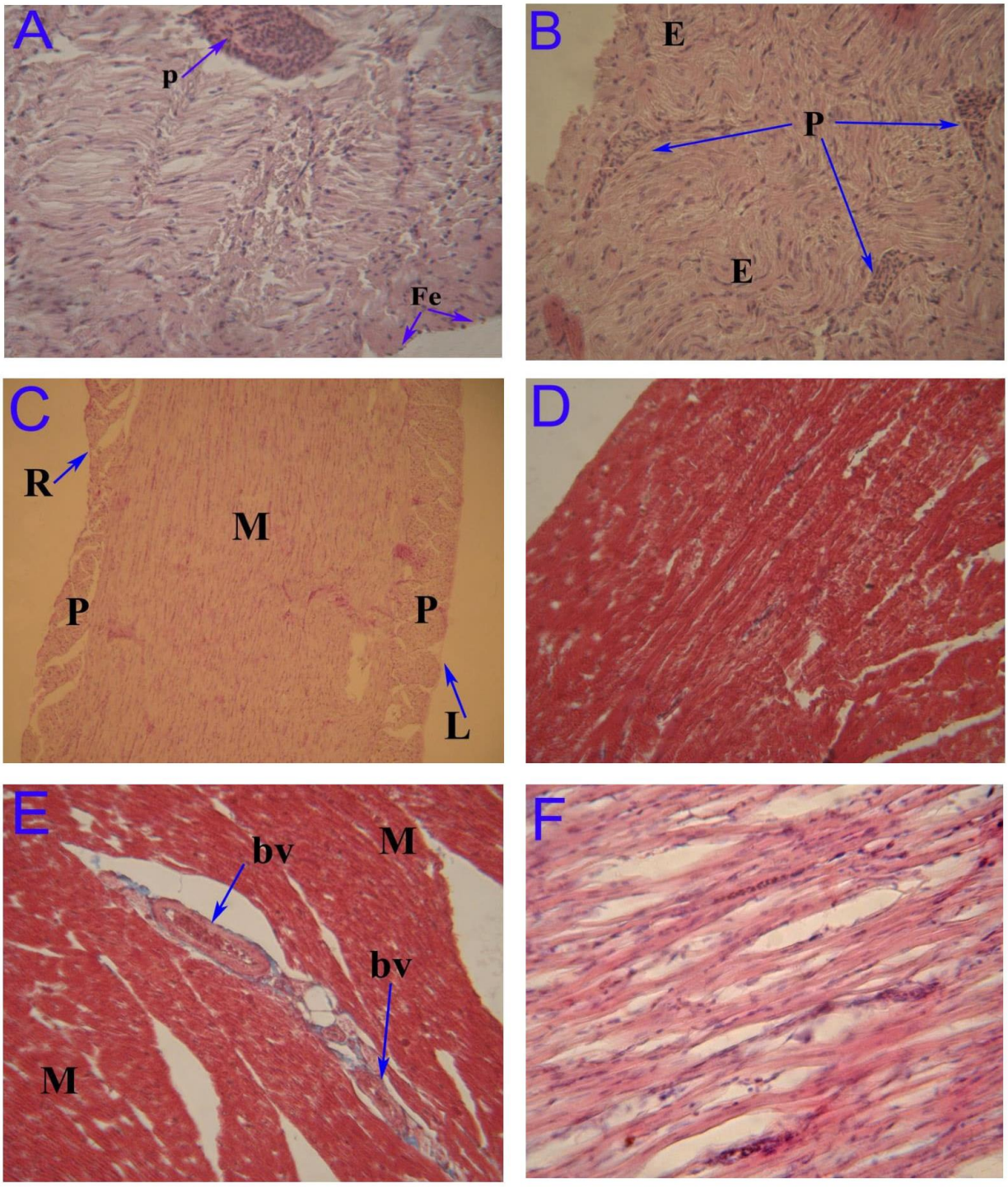

Figure 4: (A). A photomicrograph of the fowl heart (ventricular wall) showing, flattened endothelial cells (Fe) and Purkinje fibers (P). H&E stain. X20. (B). A photomicrograph of the pigeon heart (ventricular wall) showing, numerous bundles of Purkinje fibres (P) in endocardium (E). H&E stain. X20. (C). A photomicrograph of the fowl heart (interventricular septum) showing, a positive PAS reaction in the myocardial layer (M). Purkinje fibres (P), endocardium lining the left ventricle (L) and endocardium lining the right ventricle (R). PAS stain. X4. (D). A photomicrograph of the pigeon heart (ventricular wall) showing, a weak reaction between myocardial fibers. Masson's trichrome stain. X20. (E). A photomicrograph of the pigeon heart (ventricular wall) showing, numerous distributions of blood vessels having a positive trichrome reaction around its wall (bv) in myocardium (M). Masson´s trichrome stain. X40. (F). A photomicrograph of the fowl striated myocardial muscle fibers having elongated oval nuclei. H&E stain. X40

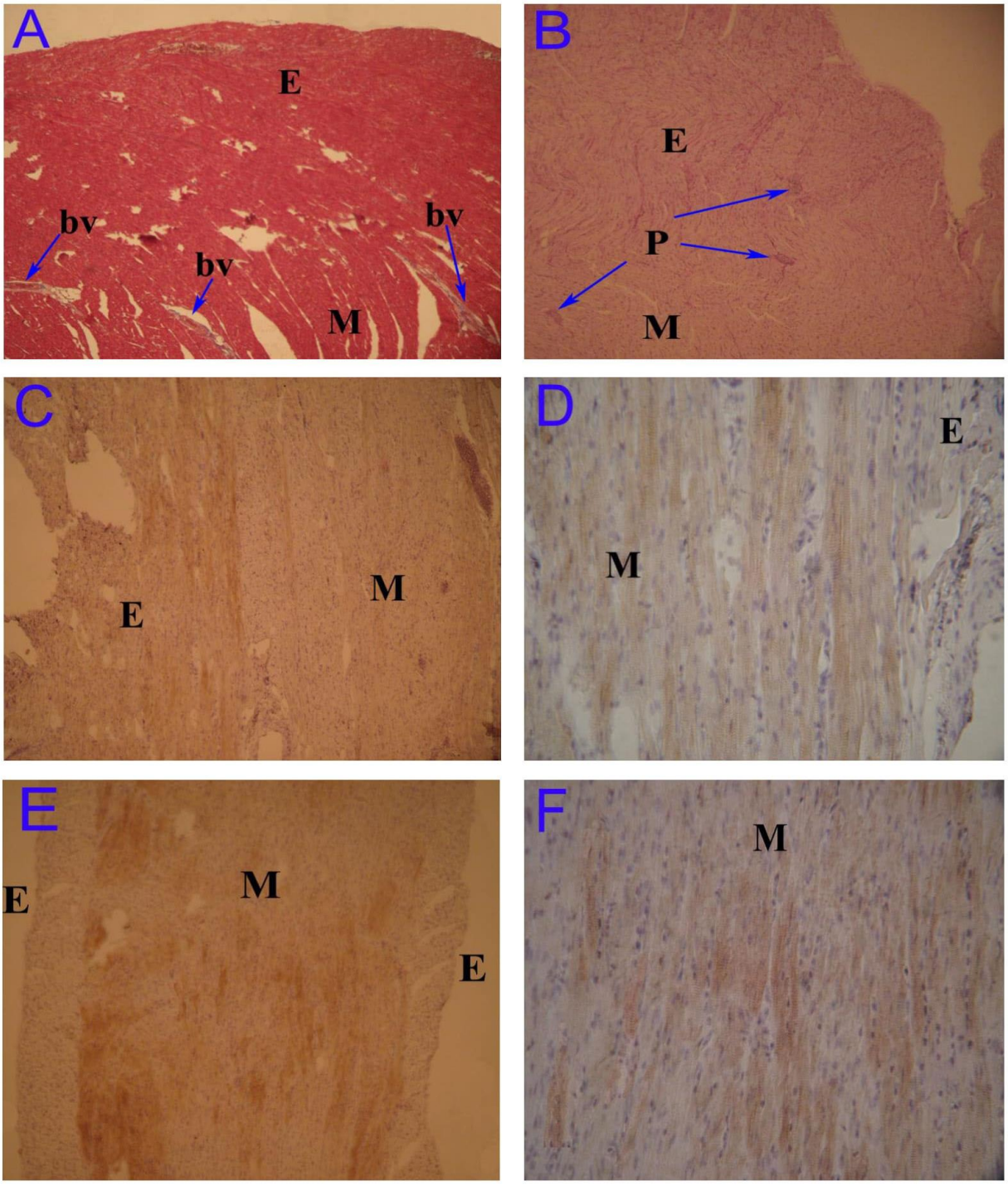

Figure 5: (A). A photomicrograph of the pigeon heart (ventricular wall) showing, numerous distributions of blood vessels (bv) in myocardium (M). Endocardium (E). Masson´s trichrome stain. X10. (B). A photomicrograph of the pigeon heart (ventricular wall) showing, distribution of Purkinje fibres (P) in endocardium (E) and myocardium (M) . PAS stain. X10. (C). A photomicrograph of the fowl heart (ventricular wall) showing, a positive troponin T reaction between the subendocardial layer (E) and myocardium (M) but scanty amount inside myocardial fibres. Immunohistochemistry. X10. (D). A photomicrograph of the fowl heart (ventricular wall) showing, a positive troponin T reaction between the subendocardial layer (E) and myocardium (M). Immunohistochemistry. X20. (E). A photomicrograph of the pigeon heart (interventricular septum) showing, a strong troponin T reaction in the whole core of myocardial layer (M). Immunohistochemistry X4. (F). A photomicrograph of the pigeon heart (ventricular wall) showing, a strong troponin T reaction in the whole core of myocardial layer. Immunohistochemistry. X20

Discussion

An efficient cardiovascular system (heart and vessels) have been reported in birds to adapt different metabolic demand including running, swimming, diving and flight. This requires that their cardiovascular system (CVS) be able to meet the demands of providing adequate delivery of oxygen to vascular beds that are taxed by extreme metabolic demands (16).

Interestingly, it has been revealed that the avian have larger hearts and a greater cardiac output when compared to body mass than mammals. This cardiac output was directly proportional to the rate of O2 consumption through pumping more blood (3). Additionally, a higher O2 consumption was reported during flying than resting condition (17,18). Although these facts, the literature regarding comparative descriptive variations of the heart among flying and non- flying birds are rare. Therefore, to clarify the possible adaptations in flying birds that allows more blood to be pumped to meet the high metabolic need associated with flight, this study characterized the gross morpho-metrical variations and cTnT expresion in pigeon and fowl as a representative of among flying and non-flying birds.

In correlation with others (8,19) bird's heart has an elongated cone like shape. Moreover, the present investigation showed that some difference do exist between fowl and pigeon hearts, as can be witnessed with the pointed apex in the fowl's heart versus the blunt one of the pigeon's. Parallel to the current study other researchers stated that birds' hearts are situated in the cranial part of thoracoabdominal cavity and are embedded between the right and left lobes of the liver (8,19,20). The conical heart of fowl and pigeons are enclosed by the pericardium whose fibrous pericardium is continued ventrally to the sternum as sternopericardial ligament. This is similar to hearts of ostrich, duck, goose and turkey (21). Minor differences exist between the position of the fowl and pigeon hearts in present work and with the research conducted by other (8). The heart base lies at around against the level of the second rib, while the apex of the heart is pointed toward the sternum and situated between the fifth and sixth ribs. Concerning the weight of the heart, our results agreed with others (22) that recorded weight of chicken and pigeon heart`s is about 0.44% and 1.0% of the body weight respectively. The fowl heart accounts for 0.5 to 1.42% of the body weight, while pigeon's heart accounts for 1.1 to 1.4% of its weight (8). The results revealed that the pigeon heart has a correspondingly greater relative weight than that of the fowl's in relation to the total body weight, which is needed for basal and energy metabolism in a decent flying bird. The thickness of the left ventricular wall is about five times greater than that of the right one which is similar to findings in the hearts of fowl and turkeys (8,19), ducks (6) and ostriches (21). This proved that the left ventricle provides the power for the high pressure systemic circulation. In agreement with Hodges (23) the wall of the heart in fowl and pigeon is distinguished into three basic layers; endocardium, myocardium and epicardium. In the present study, endocardium consists of flattened endothelial cells which is supported by collagenic fibres and dense fibrelastic layer which was correlated with that described by Hodges (23) in fowl. Meanwhile, our research clarified that, the deepest layer of pigeon endocardium is thicker and contains numerous bundles of Purkinje fibres than in fowl. Notably, the myocardium structure in current investigation is closely similar to that mentioned by Hodges (23) in fowl. In this regard, the obtained result revealed that the coronary blood vessels and Purkinje fibres are shown intensively supplied the myocardium in pigeon than fowl. Cardiac troponins (cTn) are proteins that control the calcium-mediated interaction between actin and myosin, allowing contraction at the sarcomere level. Undhad et al. (24) stated that, however the concentration of the cTn can be correlated microscopic lesion and loss of immune labeling in human myocardium damage, our study confirmed that cTnT release in healthy, active flying heart of pigeon and decrease in non active (resting) one of the fowl. In the same line with Koller (25) and Anversa et al. (26), the release of cTn is possible in irreversible damage that has been

proposed either as pathognomic of human cardiac necrosis or might reflect part of a remodeling process (physiologic substrate). The present work in complete agreement with Hessel et al. (27), which demonstrated that viable cardiomyocytes release cTn as an intact protein by a stretch related mechanism. The latter author added that, metabolic inhibition of cardiomyocytes induces parallel release of intact cTnT and their degradation products, starting only after the onset of irreversible cardiomyocyt damage (28). The cTnT is less sensitive and has less utility when used as a sole marker for detecting human myocardial necrosis (29,30). In contrast to current work, the cTnT used as cardiac biomarker for the detection of myocardial injury as acute myocardial infarction and cardiac necrosis (30,31). The current work is parallel with the findings of Middleton et al. (32) who demonstrated that the elevation and release of cardiac Troponin approved a physiological not a pathological substance. It is interesting to note that, this work observed that cTnT increase in healthy myocardium of flying birds as pigeon rather than resting one as fowl.

Conclusions

In conclusion, the difference in activity (flying) between fowl and pigeon hearts affects the expression of cardiac Troponin T. Furthermore, the anatomical and histological studies of the two birds clarified some variation of the layers of their hearts. The immunohistochemical distribution of Troponin T is intensively found and has strong reaction in the whole core of myocardial layer of flying active birds as in pigeon than that of stable one as fowl. This indicates that there is a positive relationship between flying birds (pigeons) and the presence of Troponin T in their hearts.

Conflicts of interest

The authors declare no conflict of interest

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Original Research Article

EFFECT OF SANGUINARINE PHYTOBIOTIC, SODIUM BUTYRATE COMPARED TO AMPICILLIN ON CONTROLLING NECROTIC ENTERITIS IN BROILER CHICKENS

Sawsan M. El-Sheikh[1], Mohamed H. Khairy[1], Naglaa Z. Eleiwa[1], Osama E. Abdalla[2], Asmaa G. Abd El-Monsef[2]*

[1]Department of Pharmacology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, [2]Department of Pharmacology, Animal Health Research Institute, Zagazig, Egypt

*Corresponding author, E-mail: asmaagamal_1982@yahoo.com

Abstract: This study aimed to investigate the efficacy of Sanguinarine phytobiotic, Sodium butyrate compared to ampicillin in treatment of *Clostridium perfringens* infection in broiler chickens with a special reference to their effects on growth performance, hematobiochemical and immunological profile. A total of 150 one-day old Cobb broiler chicks were used in this study. On day 14th of age all chicks were divided into 5 equal groups (30 each). Group (G1) non-infected, non-treated (control), G2 infected with *C. perfringens*, non treated, G3 infected, treated with Sodium butyrate, G4 infected and treated with Sanguinarine phytobiotic and G5 infected and treated with ampicillin. Administration of drugs in drinking water was continued for 5 days from 18-23 day of age. The results revealed that the infected broiler chickens with *C. perfringens* and non treated(G2) showed clinical signs of necrotic enteritis represented by loss of appetite, diarrhea, dehydration, anorexia in addition to a recorded mortality rate 30% and lesion score 80%. Moreover, significant reduction ($P<0.05$) in body weight, weight gain, erythrogram, phagocytosis and phagocytic index, Nitric oxide, HI titers, total protein, albumin and total globulin beside significant increase ($P<0.05$) in feed conversion rate (FCR), leukogram, liver enzymes, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase (AST-ALT and ALP), uric acid and creatinine associated with non-significant increase ($P<0.05$) in globulin fractions were observed. Treatment of the infected chickens with these drugs led to improvement in clinical signs, mortality rate, lesion score, growth performance, hematobiochemical and immunological profile compared with infected non treated birds (G2), but ampicillin had superior effect against *Costridium perfringes* infection. It could be concluded that *C. perfringens* infection in broiler chickens induced adverse effects on growth performance, hemato-biochemical and immumological profile of birds which could be reversed or ameliorated by using Sanguinarine phytobiotic, Sodium butyrate or ampicillin. So, the study recommended the use of such treatments hand in hand with antibiotics for controlling necrotic enteritis in broilers.

Key words: necrotic enteritis; Sodium butyrate; phytobiotic; ampicillin

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Introduction

Chicken meat is considered one of a high quality meat due to low its fat content (1). Therefore, poultry industry is one of the most important tool providing high quality protein for human consumption all over the world (2).

*Clostridium perfringens*is a spore-forming gram positive anaerobic rod which is a common inhabitant in the intestine of healthy broiler chickens (3). This microorganism with some predisposing factors as dietary ingredients or changes in the diet, severe stress, coccidiosis, or immunosuppressive affections can help in the overgrowth of *C. perfringens* , subsequent toxin production and development of necrotic enteritis (NE) (4). NE is an enterotoxemia of poultry which causes an important economic loss, about of two billions dollars/year in USA (5). *C. perfringens* toxogenic types (A and C) are the etiological agent associated with necrotic enteritis (6). It occurs in broilers aging between 2-6 weeks (7). The incidence of *C. perfringens* causing NE has increased since the ban of in-feed antibiotic growth promoters (AGP) (8).

Sodium butyrate is an organic acid added to poultry diet to enhance the performance and the immune response of birds (9). It is known as acidifiers and used a valuable tool in maintaining the gut health (10). It has the potential to minimize the enteric pathogens load and minimizing the pathogenic microorganism by reducing the intestinal pH (11). Also, organic acids do not require withdrawal period, increase shelf-life of poultry products they can make a valuable contribution to flock health and safety of food that might provide a significant tool for the poultry industry in combating occurrence of intestinal diseases and in reduction of food borne pathogens (12).

Phytobiotics are defined as natural feed additives that are safe to animal and poultry (13). Phytobiotics are different substances, mainly plant materials extracts as leaves, flowers, seeds, buds, fruits, twigs, root, bark, wood, and herbs (14). The active materials have many various secondary plant metabolites with wide range of physiological effects (15). Those were reported to improve broiler performance (16).

Ampicillin is semi-synthetic, broad-spectrum penicillin; it belongs to β-lactame group of antibiotic. Its only difference from penicillin is the existence of an amino group which enhances its penetration via the outer membrane of bacteria. Its effect is either bactericidal or bacteriolytic and its action is mostly marked during the active growth phase (17). It is stable in acid and well absorbed from the gastrointestinal tract, used as oral medication against systemic infections (18). However, the excessive use of antibiotics growth promotes (AGP) has resulted in the development of drug-resistant bacteria, antibiotic residues in the body of the birds and imbalance of normal microbiota (19). So, there was an increasing interest in searching for growth-promoting and immune system-strengthening alternatives.

The aim of this study was to evaluate the efficacy of Sanguinarine phytobiotic, Sodium butyrate compared to ampicillin in treatment of *C. perfringens* infection in broiler chickens, with regard to their effects on growth performance, heamatobiochemical and immunological profile.

Material and methods

Drugs and treatment

Sodium butyrate (Admix)® 30: It is water soluble formulation. It is produced by EGAVET Company (Reg. No: 1/9995-22/9/2014) and it was added in drinking water for 5 successive days. Sanguinarine (San-grovit)® It is Phytobiotics powder, natural herbal extract anti-Clostridial growth promoter, extracted from Macleaya Cordata. Obtained from Delta Vet Center, Egypt (Batch No: 1703a031) and it was added in drinking water for 5 successive days. Ampicillin trihydrate (Ampicure)®: It is water soluble powder antibiotic. It is a broad-spectrum semisynthetic antibiotic inactivated by beta-lactamases. It is relatively stable in gastric acid. It was obtained from Pharma Sweed–Egypt Company (Reg. No: 3618/2014) and was added in drinking water for 5 successive days.

Chickens

A total of 150, one-day old commercial mixed sex Cobb broiler chicks were purshased from El-Kahera poultry Company and were kept in wire floor batteries under hygienic measures. Chicks were fed commertial ready made ration obtained from Feed Mix Company.

Vaccines and vaccination

All chicks were vaccinated with Newcastle disease virus (NDV) (HitchnerB1 on 7 days and LaSota on 18 days of age) and Infectious bursal disease (IBD) vaccine on 14 days.

- ND vaccines: Hitchner B1 and LaSota live virus vaccines were obtained from Intervet Boxmeer Company. Vial contain 10^6 EID_{50} Newcastle disease virus, dissolved in physiological saline (30 mL per 1000 doses) as eye drops.
- Holland. Gumboro vaccine was obtained from Rhone-Merieau Company, France Vial dissolved in 50 ml physiological salin / 1000 bird as eye drops.

Clostridium infection

Toxigenic *C. perferingens* type A strain was obtained from Animal Health Research Institute, Dokki,Giza.On 14th day of age chicks were orally inoculated with 0.5mL of *C. perfringens* type A broth culture ($1x10^9$CFU /mL) (20).

Experimental design

On day 14 of age all chicks were randomly divided to 5 groups (30 each). Group (G)1 non-infected,non-treated (control), G2 infected orally with *C. perfringens*, non-treated, G3 infected and treated with Na-butyrate (2 ml/liter), G4 infected and treated with Phytobiotics (Sangrovit) (1 gm/10 litre) and G5 infected and treated with ampicillin trihydrate (Ampicure) (1.25 gm/litre). Treatment was started in all groups after appearance of clinical signs and was given in drinking water for 5 successive days. Chicks were weekly weighed to determine body weight, weight gain, feed consumption and feed conversion rate (FCR).

Sampling

Two blood samples from each bird at one day post treatment were collected. 1st sample was taken and divided into 2 parts, the first part was put in a tube contain EDTA as anticoagulant for the haematological studies to determine erythrogram and leukogam (total erythrocytic, leukocytic and differential leucocytic count) (21). (PCV) (22), haemoglobin content (23). The second part was put in a tube containing heparin for determination of phagocytic activity (24).

The 2nd blood sample was collected without anticoaggulant to obtain clear serum for measuring transaminases aspartate aminotransferase and alanine aminotransferase (AST-ALT) (25), Alkaline phosphatase (ALP) (26), serum total protein (27). Serum protein fractions were performed using cellulose acetate electropheresis test (28), serum uric acid (29) and creatinine (30). Determination of nitric oxide (31) and hemagglutination inhibition (HI) test for estimation titer of ND (32).

Statistical analysis

The obtained data was analyzed by using computerized SPSS program version 16 using one way ANOVA (33).

Results

The broiler chickens infected with *C. perfringens* showed clinical signs represented by loss of appetite, depression, polydipsia, ruffled feathers, diarrhea, dehydr-ation, emaciation, in addition to, a recorded mortality rate was 30% and lesion score 80%. The broiler chickens infected with *C. perfri-ngens* (G2) showed a significant reduction ($P<0.05$) in body weight, weight gain, feed consumption beside increase in feed conversion rate. Growth performance of infected broiler chicks was improved with sanguinarine phytobiotic, sodium butyrate and ampicillin treatment (Table1).

G2 showed a significant ($P<0.05$) reduction in erythrocytic count, haemoglobin content, PCV, beside a significant increase in total leukocytic count, heterophile, lymphocyte, monocyte, eosinophile, basophile compared with G1 (Table 2).

Table 1: Effect of Sodium butyrate, Sanguinarine Phytobiotic and Ampicillin on growth performance at 1st, 7th and 14th post treatment of broiler chickens infected with *C. Perfringens*

Groups	Initial body Weight (14 day of age, g)	1st day post treatment			FCR	7th day post treatment			FCR	14th day post treatment			FCR
		Body weight (g)	Weight gain (g)	FC		Body weight (g)	Weight gain (g)	FC		Body weight (g)	Weight gain (g)	FC	
G1	455.68±0.22^{a}	791.87±2.44^{a}	336.19±1.55^{a}	487.48	1.45	1146.68±2.67^{a}	354.81±2.78^{a}	592.89	1.67	1799.67±5.22^{a}	652.99±2.98^{a}	1142.73	1.75
G2	459.96±3.86a	680.89±2.53^{c}	220.90±4.98^{c}	386.58	1.75	994.80±3.56^{c}	313.91±3.86^{c}	574.54	1.83	1563.24±4.10^{c}	568.44±4.76^{c}	1080.72	1.90
G3	459.47±0.69^{a}	740.98±2.44^{b}	281.51±1.57^{b}	470.12	1.67	1076.48±0.22^{b}	335.50±3.13^{b}	580.42	1.73	1689.76±2.32^{b}	613.28±3.89^{b}	1103.90	1.80
G4	457.98±0.49^{a}	738.79±2.44^{b}	280.81±1.69^{b}	474.57	1.69	1079.40±0.22^{b}	340.61±3.37^{b}	579.04	1.70	1680.24±2.22^{b}	600.84±1.65^{b}	1093.53	1.82
G5	461.47±0.38^{a}	780.98±0.22^{a}	319.59±1.47^{a}	485.78	1.52	1134.86±1.67^{a}	353.88±6.36^{a}	583.90	1.65	1781.87±3.52^{a}	647.01±3.67^{a}	1138.37	1.76

G1: Control; G2: Infected non treated, G3: Infected+Na But; G4: Infected +Phyto; G5: Infected + Amp. Values were means ± standard error. Means within the same column with different superscripts were significantly different (P<0.05) FCR: feed conversion rate, FC: feed consumption.

Table 2: Effect of Sodium butyrate, Sanguinarine Phytobiotic and Ampicillin on erythrogram and leukogram in broiler chickens infected with C. perfingens

Groups	RBCs (106/ mm3)	Hb (gm/dl)	PCV (%)	Total WBCs X10^{3}/µl	Deferential leukoctic count X10^{3}/µl				
					heterophils	lymphocyte	monocyte	eosinophils	Basophils
G1	4.27±0.22^{a}	10.08±0.35^{a}	30.15±0.30a	11.13±0.42^{c}	2.67±0.15^{c}	3.61±0.29^{c}	1.38±0.15^{c}	1.73±0.13^{c}	1.74±0.07^{c}
G2	2.79±0.22^{c}	7.06±0.36^{c}	26.21±0.51^{c}	15.22±0.90^{a}	3.73±0.12^{a}	4.99±0.25^{a}	2.08±0.11^{a}	2.43±0.10^{a}	1.99±0.05^{a}
G3	3.41±0.16^{b}	8.17±0.57^{b}	28.79±0.64^{b}	13.28±0.99^{b}	3.21±0.20^{b}	4.42±0.30^{b}	1.74±0.13^{b}	2.05±0.10^{b}	1.86±0.06^{c}
G4	3.51±0.24^{b}	8.19±0.54^{b}	28.80±0.52^{b}	13.30±0.91^{b}	3.29±0.18^{b}	4.36±0.19^{b}	1.73±0.15^{b}	2.07±0.11^{b}	1.85±0.08^{c}
G5	3.94±0.36^{a}	9.81±0.45^{a}	29.87±0.71^{a}	11.59±0.91^{c}	2.89±0.15^{c}	3.72±0.48^{c}	1.44±0.17^{c}	1.79±0.10^{c}	1.75±0.06^{c}

G1: Control; G2: Infected non treated, G3: Infected+Na But; G4: Infected +Phyto; G5: Infected + Amp. Values were means ± standard error. Means within the same column with different superscripts were significantly different (P<0.05).

Phagocytosis and phagocytic index were significantly decreased in infected broiler chickens compared with non-infected broiler chickens (Table 3). Similarly, nitric oxide and HI titer against Newcastle were inhibited. In contrast, the treated broiler chickens with Sanguinarine phytobiotic, Sodium butyrate and ampicillin showed enhancing in phagocytosis and phagocytic index, Nitric oxide and HI titers against Newcastle (Table 3).

Total protein and albumin contents were significantly decreased (P<0.05) in infected broiler chickens compared with G1 (Table 4). Infected broiler chickens treated with ampicillin showed increasing in total protein and albumin contents compared with other treatments (Table 4). While α, β, and γ globulin contents were non-significantly inhibited under ampicillin treatment. Liver enzymes and kidney function were increased in broiler chickens infected with *C. perfringens* compared with non-infected non treated group (Table 5).

Table 3: Effect of Sodium butyrate, Sanguinarine Phytobiotic and Ampicillin on phagocytic activity %, phagocytuc index, Nitric oxide and HI titers against ND in broiler chickens infected with *C. perfringens*

Groups	Phagocytosis	Phagocytic index	Nitric oxide	HI titers against Newcastle
G1	58.68±2.81^{a}	2.80±1.30^{a}	19.05±1.32^{a}	2.43±1.32^{a}
G2	54.32±2.92^{c}	2.00±0.32^{c}	14.67±0.17^{c}	2.00±0.39^{b}
G3	56.36±3.84^{b}	2.61±0.31^{b}	16.85±0.33^{b}	2.25±0.18^{ab}
G4	56.42±2.85^{b}	2.62±0.25^{b}	16.99±0.52^{b}	2.40±0.31^{ab}
G5	57.75±1.90^{a}	2.60±0.37^{b}	19.65±0.46^{a}	2.25±0.18^{ab}

G1: Control; G2: Infected non treated, G3: Infected+Na But; G4: Infected +Phyto; G5: Infected + Amp. Values were means ± standard error. Means within the same column with different superscripts were significantly different (*P*<0.05).

Table 4: Effect of Sodium butyrate, Sanguinarine Phytobiotic and Ampicillin on serum total protein, albumin and globulin fraction in broiler chickens infected with *C. perfringens*

Group	T.P	Alb	Globulin(Gm/dl)				A/G ratio
			α	β	γ	Total	
G1	5.67±0.10^{a}	2.95±0.15^{a}	0.75±0.10^{a}	0.93±0.18^{a}	1.04±0.14^{a}	2.72±0.05^{a}	1.08±0.19^{a}
G2	4.07±0.11^{b}	2.05±0.10^{b}	0.84±0.12^{a}	0.92±0.14^{a}	0.92±0.16^{a}	2.02±0.11^{c}	1.01±0.14^{a}
G3	5.03±0.25^{ab}	2.53±0.12^{ab}	0.80±0.16^{a}	0.90±0.12^{a}	0.90±0.11^{a}	2.60±0.10^{ab}	1.01±0.10^{a}
G4	5.24±0.16^{a}	2.55±0.15^{a}	0.79±0.13^{a}	0.91±0.14^{a}	0.89±0.11^{a}	2.59±0.02^{ab}	1.02±0.09^{a}
G5	5.32±0.25^{a}	2.75±0.06^{a}	0.74±0.16^{a}	0.85±0.14^{a}	0.88±0.15^{a}	2.47±0.09^{b}	1.07±0.06^{a}

G1: Control; G2: Infected non treated, G3: Infected+Na But; G4: Infected +Phyto; G5: Infected + Amp. Values were means ± standard error. Means within the same column with different superscripts were significantly different (*P*<0.05). TP: total protein; Alb: albumin and A/G ratio: albumin/globulin ratio.

Table 5: Effect of Sodium butyrate, Sanguinarine Phytobiotic and Ampicillin on liver enzymes and kidney function in broiler chickens infected with *C. Perfringens*

Group	Liver enzymes (U/L)			Kidney function(mg/dl)	
	AST	ALT	ALP	Uric acid	Creatinine
G1	39.35±0.74^{c}	48.96±1.25^{c}	235.32±1.31^{c}	4.20±0.39^{c}	1.04±0.11^{c}
G2	55.59±0.66^{a}	55.29±0.93^{a}	350.10±1.42^{a}	7.58±0.48^{a}	2.21±0.18^{a}
G3	47.92±0.71^{b}	52.44±1.11^{b}	243.60±1.80^{b}	6.15±0.28^{b}	1.40±0.09^{b}
G4	46.66±0.48^{b}	52.53±1.22^{b}	245.02±1.81^{b}	6.25±0.27^{b}	1.35±0.04^{b}
G5	41.42±0.61^{c}	50.07±1.20^{c}	244.41±1.72^{b}	5.85±0.19^{b}	1.33±0.02^{b}

G1: Control; G2: Infected non treated, G3: Infected+Na But; G4: Infected +Phyto; G5: Infected + Amp. Values were means ± standard error. Means within the same column with different superscripts were significantly different (*P*<0.05). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase and ALP: Alkaline phosphatase.

Discussion

The current work was designed to investigate the efficacy of Sanguinarine phytobiotic, Sodium butyrate compared with ampicillin in treatment of *C. perfringens* infection in broilers with a special reference to their effects on growth performance, hemato-biochemical and immunological parameters. The clinical signs of NE in G1 may be due to toxin excreted by *C. perfringens* which caused high mortality rate (34). Also, Olkowski et al. (35) stated that, *C. perfringens* infections in poultry induced clinical signs as loss of appetite, depression, diarrhea, emaciation and high mortality rate. Broiler chickens infected with *C. perfringens* showed typical clinical signs, mortality rate and reduction in growth performance of chickens (36,37).

Medication of broilers infected with *C. perfringens* using sodium butyrate, sanguinarine phytobiotic or ampicillin was effective and resulted in disappearance of clin-ical symptoms and improved growth performa-nce and health status of infected birds. Lee et al. (13) reported that, infected chicks received phytobiotic showed improved body weight, weight gain and feed conversion due to phytobiotic had good effect on *C. perfringens* colonization in intestines of broiler chickens (38). Cross et al. (16) stated that, phytobiotic in ration probably prevent necrotic clinical signs and improved body performance by their direct antimicrobial effect on *C. perfringens***.** Good efficacy of a phytobiotic for improving clinical signs, lesion scores and body performance in experimental necrotic enteritis may be due could be attributed to the presence of essential oils in phytobiotic that could improve nutrient digestibility due to enhance the activities of trypsin and amylase (39,40).

Lesson et al. (41) recorded that, butyric acid play a role in controlling the proliferation of *C. perfringens* in broiler chicken**s** leading to improve body performance by increasing villus height lead to increase absorption of nutrients**.** Sodium butyrate has beneficial effects in controling NE in chickens by disappearing clinical signs and improving body weight (42). Furthermore, sodium butyrate have dose-dependent activity against necrotic enteritis (43,44).

Ampicillin was very effective in treatment of necrotic enteritis in broilers as it improved body weight, weight gain, feed consumption and FCR. These findings were attributed to that, *C. perfringens* was highly sensitive to ampicillin and antimicrobial compounds in poultry industry as improved poultry health and the health status of the infected chickens as evidenced by disappearance of clinical signs of the disease and improved body performance (45). While, broilers chickens infected with *C. perfringens* showed significant reduction in weight gain coupled with elevation in feed conversion rate (46). Moreover, another beta lactam (amoxicillin) have good potency in treatment of NE in broiler chickens and induced disappearance of clinical signs and improved body performance (37).

The current work revealed that, broiler chickens infected with *C. perfringens* showed significant decrease in total erythrocytic count, hemoglobin content and PCV, associated with significant increase in leukocyte, heterophil, lymphocyte eosinophil's basophils and monocyte. The changes in haematological parameters in broiler suffering from NE might be due to bacterial toxin (47)**.** The decrease in erythrogram might be due to intravascular hemolysis induced by *C. perfringens* (48). Clostridial toxins caused breakdown of phospholipids of erythrocytes membrane and cause hemolysis by damaging circulating erythrocytes (49). Similar findings were previously reported (50-52).

Our results revealed that, oral administration of sodium butyrate, sanguinarine phytobiotic or ampicillin for treatment of clostridial infection in broiler chickens induced significant impro-vement in both erythrogram and leukogram.

The essential oils which present in phytobiotics reduced *C. perfringens* count in broiler and stimulating the absorption of essential nutrients as minerals and protein induced increase in erythrocyte and hemo-globin content when compared with infec-ted non-treated group (53). Also, phytobiotics suppress intestinal bacteria due to its

antibacterial effect leading to enhancement of both erythrogram and leukogram (54,55).

Sodium butyrate had bacteriostatic or bactericidal effect by penetrating the bacterial cell wall and dissociate to H^+ and anions inside the cell, lowering pH and resulting in energy deficiency and osmotic problems in the organism. This led to improvement of biological function of host as blood picture (56).

Hematological parameters in chickens suffering from NE were improved towards the normal level after treatment with ampicillin, this improvement might be due to antimicrobials effects which suppress the invasion of *C. perfringens* so improved absorption of essential substance for erythropoiesis (57).

Our study revealed that broiler chickens infected with *C. perfringens* revealed significant reduction in phagocytosis, Phagocytic index, Nitric oxide and HI titers of ND compared with control group. According to Coles (58), Grilli et al. (59), Yitbarek et al. (60) and Chake et al. (61), *C. perfringens* infection in broiler revealed reduction in phagocytosis, phagocytic index, Nitric oxide and HI titers of ND.

Infected broiler chickens with *C. perfringens* and treated with sodium butyrate, sanguiranine phytobiotic or ampicillin showed improvement in phagocytosis and Phagocytic index, Nitric oxide and HI titers of ND..This in accordance with Wati et al. (62) that broiler chickens received ration contain phytobiotic prevent necrotic enteritis and improved phagocytosis and Phagocytic index and improved immunological parameters as Nitric oxide and HI titers of ND.

Sodium butyrate could stimulate host defense in chicken, by increase the activity of chicken phagocytosis, phagocytic index and nitric oxide production (63-65). Butyric regulates the macrophage activities in the intestine and the macrophage regulates the function of T cells and dendritic cells in the gut which have role in host immunity (66).

Elevation in liver enzymes (AST, ALT, and ALP), uric acid and creatinine in broiler chickens infected with *C. perfringens* might be due to liver and kidney damage by closterdial toxins (47). Reduction in proteinogram in infected chicks might be attributed to a state of anorexia and mal absorption of nutrients from inflamed intestine leading to inability of liver to synthesis proteins (67). Similarly, Mabrouk, (68) stated that broiler chicken infected with *C. perfringens* showed significant decrease in serum total protein, albumin and globulin associated with significant increase in liver enzymes (AST, ALT and ALP), Uric acid and creatinine. Medication of *C. perfringens* infected broiler chickens using sodium butyrate, phytobiotic or ampicillin showed improvement in serum total protein, albumin, globulin, liver enzymes (AST, ALT and ALP), uric acid and creatinine when compared with G2.

Phytobiotics suppressed *C. perfringens* infection in broiler chickens and improved protein picture. They act on the intestinal walls, promoting the absorption of more nutrients and secretion of digestive enzymes which enhance the nutrient digestibility leading to improved protein profile (69,70). The same results were reported by Lesson et al. (41) who stated that, sodium butyrate improved liver enzymes and protein picture in broiler suffering from NE. Ampicillin is very important in treatment of NE in chickens and improved both liver and kidney function and showed improvement in protein picture (44,50).

Conclusion

It could be concluded that experimental oral infection with *C. perfringens* in broiler chickens induced adverse effects on health state, growth performance, hemato-biochemical and immumological profile of birds which could be reversed or ameliorated by using Sanguinarine phytobiotic, Sodium butyrate or ampicillin. So, the study recommended the use of such tratments hand in hand with antibiotics for controlling of necrotic enteritis in broilers, but ampicillin had superior action aginst *Clostridium perfringens* infection.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

THE AMELIORATIVE EFFECT OF *ALKANNA ORIENTALIS* EXTRACT AGAINST *CERASTES CERASTES* VENOM HEPATIC AND HEMATOLOGICAL TOXICITY

Mahmoud M. Nasser[1]*, Abd El-Aziz A. Diab[2], Soliman S.A. Ibrahim[2], Ahmed L. El-Said[1], Eman M.E. Mohallal[1], Samih I. El-Dahmy[3]

[1]Desert Research Center, Cairo, 11753, [2]Zoology Department, Faculty of Science, Zagazig University, 44519, [3]Pharmacognosy Department, Faculty of Pharmacy, Zagazig University, 44519, Egypt

*Corresponding author: nasser_mahmoud55@yahoo.com

Abstract:*Cerastes cerastes* is the most abundant venomous snakes of the North Africa, envenoming by it inflicts local tissue damage, hemorrhage and causing severe systemic toxicity that usually lead to victim death. Therefore, the present study aimed to evaluate the toxicity of *Cerastes cerastes* venom on male albino rats and the ameliorative and prophylaxis role of *Alkanna orientalis* extract against venom toxicity. Forty-eight adult male albino rats (180±200g) were divided into 6 groups. Group I injected intraperitonial with physiological saline (100µl). Group II with *Alkanna orientalis* extract (250mg/kg/BW). Group III was injected with 1/10 LD_{50} of *Cerastes cerastes* venom (0.435mg/kg/BW). Group IV injected with *Cerastes Cerastes* venom (0.435mg/kg/BW) then immediately with *Alkanna orientalis* extract. Group V was given *Alkanna orientalis* extract orally (250mg/kg/b.wt) then after 2 hours injected with *Cerastes cerastes* venom (0.435 mg/kg/BW) Group VI was injected with *Cerastes cerastes* venom then was injected immediately with antivenom immunoglobulin (300µl). Group III showed a significant increase (p<0.001) in serum aspartate transaminase, alanine transaminase, alkaline phosphatase and total &direct bilirubin as well as a significant decrease(p<0.001) in plasma prothrombin and partial thromboplastin concentration, plasma albumin, total protein, serum cholinesterase, hemoglobin, red blood cells and platelets count as compared with control group. In addition, it resulted in internal abdominal cavity hemorrhage. While, group II did not show any significant change in all previous parameters. As well as, *Alkanna orientalis* extract when given intraperitonial immediately or orally 2 hours before *Cerastes cerastes* venom as antidote, it minimize the alterations of hematological, biochemical parameters and the internal hemorrhage. By comparing groups IV and V with group VI, we found out that *Alkanna orientalis* extract could be considered more effective antidote than antivenom immunoglobulin for *Cerastes cerastes* venom toxicity. In conclusion, *Alkanna orientalis* extract showed a new therapeutic and prophylaxis agents against *Cerastes cerastes* venom toxicity.

Key words: *Cerastes cerastes*; *Alkanna orientalis*; *Boraginaceae* family; venom toxicity; haematological effect

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Introduction

Snake's envenomation has been reported as an important social and medical serious problems in the snakes natural habitat, up to 1.8–2.5 million victims occurred per a year with about 100,000–125,000 mortality occurred annually and more than 100,000 of victims are suffering from severe complications, which can lead to amputation of the attacked part (1). In Egypt, 1000–10,000 incidences of snake's envenomation per year with about 11–100 death cases annually (2). *Cerastes cerastes* (the Egyptian desert horned viper) showed a widely distribution also, it can be considered as the most dangerous vipers of the Middle East and North Africa (3).

Cerastes cerastes vipers envenoming was documented to cause prominent local tissue damage, that results in the disruption of the extra cellular matrix and the basement membrane covering the body vessels and blood capillaries which responsible for rapid discharge of target-specific toxins (4). Kebir-Chelghoum and Laraba-Djebari (5) reported that the venom able to induce cell toxicity that change the membrane permeability resulting in discharging of the internal cell contents, oxidative stress as well as, it cause an indirect inflammatory response. Therefore, the usage of anti-inflammatory and antioxidant drugs may be important in the treatment of bitted victims. In addition, cerebrovascular complications and neuromuscular paralysis previously documented as a neurological toxic effect of snakebites (6).

Although the antivenom can neutralize the venom and prevent further damage it cannot restore the damage that was already occurred **(**7). Therefore, it is very important to find out new natural or artificial venom inhibitors, which able to complement the antivenom action especially, in restoring the local tissue damage (8). Proteins, antioxidants, glycosaminoglycans, polysaccharides, flavonoids and alkaloids considered as venom spreading factors inhibitors (9). The usage of medicinal plants was the policy of the World Health Organization (WHO) since 1970. Plants and herbs considered as pharmacological ingredient sources that provide about 25% of drugs that recently used (10). Because of the low cost of traditional medicinal plants, it is widely used for diseases treatment in countries with low-income populations (11).

Boraginaceae family consists of several shrub plants which abundant in mild and tropical regions. It is classified into 200 genus and 2000 species, most of these plants have important medicinal effects. Flowers, stems, roots and leaves of them are used for medicinal purposes (12). *Boraginaceae* was reported as an antidote for some snakebite such as, *Argusia Argentea* methanolic extract is effective in *Trimeresurus Flavoviridis* bites (13). *Carmona retusa*, whole of the plant was used as antidote paste for *Dabois russelii* bites (14). Rosmarinic acid from methanolic extract of *cordial verbenacea* is effective against *B. Jararacussu* (15).

Boraginaceae, especially *Alkanna orientalis and Echium humil*, contain valuable amounts of gamma-linolenic acid (GLA), alpha-linolenic acid (ALA) and stearidonic acid (SDA) in addition to rosmarinic acid (RA). These substances have a potent antioxidant and anti-inflammatory effect thus; they can restore the toxic effect of *Cerastes cerastes* venom (16-18).

Alkanna orientalis (Boraginaceae) is considered a rich source of RA that acts as antioxidant, anti inflammatory, anti mutagen, antibacterial, and antiviral activities (19). The inhibitory activity of RA against snake venom phospholipases A2 from *Cordia verbenacea*. as well as, the interaction between RA and phospholipases A2, by using crude venoms and purified phospholipases A2 of *B. jararaca* was reported by Ticli et al. (20). The hemorrhagic snake venoms are known to be metalloproteinases, and their mechanism of action would be different from that of phospholipases A2. Accordingly, it is interesting that RA inhibits both metalloproteinase and phospholipases A2 (20). On the same pattern, El Sohly et al. (21) documented the first report on flavonols from aerial parts of *Alkanna orientalis*. Flavanoids are one of the foremost plant components that work against PLA_2 and lipooxygenase, they possess anti inflammatory, anti hepatotoxic, anti allergic, antitumor and enzyme inhibiting properties. Flavanoid weakly inhibits the group I PLA_2 from *Naja naja* and strongly inhibits the

group II PLA2 from *Vipera* (22). Hence, this study was designed to clarify the ameliorative impact of *Alkanna orientalis* extract in comparison with antivenom immunoglobulin against *Cerastes cerastes* toxicity.

Material and methods

Experimental animals

Male albino rats (n=88), weighing 180–200 gm, were obtained from the Animal house, Faculty of Science, Zagazig University. The animals settled in plastic cages, fed a standard diet and water. Exposed to a 12 h light/dark cycle, and maintained at a laboratory temperature of 22±2ºC. The rats quarantined for 15 days before the experiments. All Institutional and National Guidelines for the care and use of animals were followed.

Cerastes cerastes venom

Twenty-five *Cerastes cerastes* vipers collected from New Valley (El Kharga), settled in plastic cages at physiology lab in the desert research centre. They were milked by allowing these vipers to inject its venom through a rubber into a small beaker, and then the venom was rapidly frozen in liquid nitrogen until it was used in the study.

Cerastes cerastes venom LD_{50}

LD_{50} of *Cerastes cerastes* venom was determined according to the method described by Ramakrishnan (23). A total of 40 adult male albino rats were divided into 5 groups (gp I-V), injected intraperitonial (i.p.) with *Cerastes cerastes* venom at dose of 2.0, 3.0, 4.0, 5.0, 6.0 mg/kg/BW for each group, respectively. Dead and alive rats in each group 24 hours post injection were recorded. The calculated LD_{50} was 4.35 mg / kg/BW according to Log LD_{50} = log LD next below 50% + log increasing factor X proportionate distance. The proportionate distance = [(mortality at dilution next above 50%)-50%] / [(mortality next above 50%)-(mortality next below 50%)].

Alkanna Orientalis extraction

Alkanna Orientalis extract was prepared according to Sukhdev et al. (24) method for intraperitonial administration. *Alkanna Orientalis* plant obtained from Saint Katherine, South Sinai, Egypt, where it grow, and then it was left in air for drying without direct sun heat. After drying, the plant was grinded then immersed in 70% ethyl alcohol for 4 days. The extract filtered and concentrated at room temperature. The dried extract was stored at 4ºC until using.

Antivenom

Antivenom immunoglobulin was obtained from VACSERA CO.,Agouza .Giza, Egypt.

Animal treatment schedule

The period of this study is 2 hours after *Cerastes cerastes* venom injection, as acute dose and in group II rats were sacrificed after 2 hours of *Alkanna orientalis* extract injection.

A total of 48 Male albino rats were divided into six groups (n=8/group), as the following:

Group I (control group): rats were injected (i.p.) with 100µl of 0.09% physiological saline Group II (*Alkanna orientalis* treated group): rats were injected (i.p.) with *Alkanna orientalis* extract (250 mg/kg/BW) dissolved in 100µl distilled water. Group III (*Cerastes cerastes* venom treated group): rats injected (i.p.) with *Cerastes cerastes* venom (0.435 mg/kg BW) that represent 1/10 LD_{50}, dissolved in 100µl of 0.09% physiological saline. Group IV (*Cerastes cerastes* venom + *Alkanna orientalis* extract) group: rats injected (i.p.) with 1/10 LD_{50} *Cerastes cerastes* venom (0.435mg/kg/BW) dissolved in 100µl of 0.09% physiological saline then, immediately injected (i.p.) with *Alkanna orientalis* extract (250 mg/kg/BW) dissolved in 100 µl distilled water. Group V (a prophylaxis group): rats were given *Alkanna orientalis* extract orally (250 mg/kg/BW) then after 2 hours were injected (i.p.) with 1/10 LD_{50} *Cerastes cerastes* venom (0.435 mg/kg BW) dissolved in 100µl of 0.09% physiological saline. Group VI (*Cerastes cerastes* venom + antivenom): rats were injected (i.p.) with 1/10LD_{50} of *Cerastes cerastes* venom (0.435 mg / kg/BW) dissolved in 100 µl of 0.09 % physiological saline, then were immediately injected (i.p.) with 300 µl of antivenom immunoglobulin.

Blood sampling

Rats of each group were sacrificed after 2 hours of *Cerastes cerastes* venom injection and blood samples were collected in three sterile tubes, two containing anticoagulant (sodium citrate and EDETA) and one without anticoagulant.. The serum separated from clotted blood and used for the determination of biochemical parameters, while, citrate and EDETA tubes were used for determination of plasma prothrombin/partial thromboplastin concentration and blood component respectively.

Evaluation of some biochemical and hematological parameters

Biochemical parameters carried out by spectrophotometric (BT-260) analysis. Estimation of Aspartate and alanin transaminase (ALT and AST) was performed according to Reitman and Frankel (25). Alkaline phosphatase (ALP) (26) and total protein and albumin were detected (27**)**. Total and direct bilirubin, serum cholinesterase and plasma prothrombin concentration (PT) & plasma partial thromboplastin concentration (PTT) were measured as previously measured (28-30). Hemoglobin concentration (Hb) as well as, determination of red blood cells (RBCs), white blood cells (WBCs) and platelets count were performed (31, 32).

Evaluation of internal hemorrhage in rat's abdominal cavity

Rats in each group were sacrificed after 2 hours of *Cerastes cerastes* venom injection then, the rats' abdominal cavities were examined for internal hemorrhage.

Statistical analysis

The obtained data were analyzed by the statistical analysis software (SAS–2013 program), for obtaining Mean values ±standard error. Subsequent multiple comparisons between the different groups were analyzed by Duncan's multiple comparison tests (33), values at (P<0.05) were considered significant (34).

Results

Liver function parameters and cholinesterase

Table (1) depicting the effect of *Cerastes cerastes* venom and the ameliorative role of *Alkanna orientalis* extract and antivenom on some liver function parameters and cholinesterase in the different groups. Rats that injected with *Cerastes cerastes* venom (group III), showed a significant increase (P<0.001) in serum level of ALT, AST, ALP, total and direct bilirubin, and a significant decrease (P<0.001) in serum albumin, total protein and serum cholinesterase as compared with control group (group I). While, *Alkanna orientalis* extract (group II) showed a non-significant effect in all previous parameters after 2 h from injection of it, as compared with control group.

Whereas, when *Alkanna orientalis* was injected (i.p.) immediately after *Cerastes cerastes* venom or given orally 2 hours before *Cerastes cerastes* venom as a prophylaxis dose, (groups IV and V, respectively), it was able to ameliorate the hepatotoxic effect of the venom. As it showed a significant decrease (P<0.001) in ALT, AST, ALP, total & direct bilirubin and a significant increase (P<0.001) in serum albumin, total protein and cholinesterase as compared with venom treated group. On the same pattern, antivenom immunoglobulin showed a significant ameliorative effect against the venom when given (i.p.) immediately after *Cerastes cerastes* crude venom injection.

Table 1: Effect of *Cerastes cerastes* venom, *Alkanna orientalis*, antivenom and their combination on some liver function parameters in male albino rats

Parameter	Groups						*P* value
	Group I control	Group II AO	Group III Cc	Group IV Cc + AO	Group V AO + Cc	Group VI Cc + Anti	
ALT (U/l)	12.87±0.55^{e}	12.87±0.55^{e}	40.5±0.55^{a}	24.62±0.55^{cd}	23.57±0.55^{d}	31.62±0.55^{b}	< 0.001
AST (U/l)	15.12±0.77^{e}	14.87±0.77^{e}	44.5±0.77^{a}	29.37±0.77^{c}	24.65±0.77^{d}	34.87±0.77^{b}	< 0.001
ALP (U/l)	90.24±3.6^{e}	88.57±3.6^{e}	176.82±3.6^{a}	104.8±3.6^{c}	96.34±3.6^{d}	112.36±3.6^{b}	< 0.001
Total protein (g/dl)	6.26±0.05^{a}	6.21±0.05^{a}	4.88±0.05^{d}	5.85±0.05^{b}	5.9±0.05^{b}	5.51±0.05^{c}	< 0.001
Albumin (g/dl)	3.35±0.04^{a}	3.43±0.04^{a}	2.75±0.04^{c}	3.32±0.04^{ab}	3.39±0.04^{a}	3.23±0.04^{b}	< 0.001
Total bilirubin (mg/dl)	0.88±0.04^{d}	0.87±0.04^{d}	1.81±0.04^{a}	1.31±0.04^{bc}	1.23±0.04^{c}	1.41±0.04^{b}	< 0.001
Direct bilirubin (mg/dl)	0.3±0.02 ^{de}	0.26±0.02^{e}	0.59±0.02^{a}	0.36±0.02^{dc}	0.43±0.02^{b}	0.44±0.02^{b}	< 0.001
Cholinesterase (U/l)	3901±144^{ab}	4004±144^{a}	2145±144^{d}	3545±144^{b}	3568±144^{b}	2592±144^{c}	< 0.001

Indicated values were mean ± SE. Means within the same row in each category carrying different letters were significant at $P \leq 0.05$. AO: *Alkkanna orientalis* extract; Cc: *Cerastes cerastes;* venom and Anti: antivenom immunoglobulin.

Hematological parameters

Concerning to the effect of *Cerastes cerastes* venom and the ameliorative role of *Alkanna orientalis* extract and antivenom immunoglobulin on hematological parameters (Table 2) revealed that, group III showed a significant decrease in Hb level, RBCs count, platelets count, prothrombin concentration and partial thromboplastin concentration in comparison with group I. Meanwhile, a significant increase (P<0.001) in WBCs count was observed. While, *Alkanna orientalis* extract (group II) showed a significant decrease in WBCs count and non significant effect in the other parameters after 2 hours from (i.p.) injection of it when compared with control group as shown in Table (2). As well as, in groups IV and V *Alkanna orientalis* was able to ameliorate the hematological toxic effect of the venom. It showed a significant increase in Hb level, RBCs count, platelets count, prothrombin concentration and partial thromboplastin concentrations. In addition, it showed a significant decrease in WBCs count when compared with the venom treated animals (group III). On the same pattern, antivenom immunoglobulin showed a significant ameliorative effect against the venom when given (i.p.) immediately after *Cerastes cerastes* crude venom injection. It showed a significant increase in Hb level, RBCs count, platelets count, prothrombin concentration and partial thromboplastin concentrations. In addition, it showed a significant decrease in WBCs count when compared with venom treated group as shown in Table (2).

Table 2: Effect of *Cerastes cerastes* venom, *Alkanna orientalis*, antivenom and their combination on some hematological parameters in male albino rats

Parameter	Groups						*P* value
	Group I control	Group II AO	Group III Cc	Group IV Cc + AO	Group V AO + Cc	Group VI Cc + Anti	
RBCs × 106/ mm^3	5.32±0.13^{a}	5.35±0.13^{a}	3.83±0.13^{c}	5.45±0.13^{a}	5.51±0.13^{a}	4.66±0.13^{b}	< 0.001
Hb (mg\dl)	14.03±0.16^{a}	13.98±0.16^{a}	9.92±0.16^{c}	14.07±0.16^{a}	14.03±0.16^{a}	13.03±0.16^{b}	< 0.001
Platelets × 103/ mm^3	368.12±10^{a}	390±10^{a}	50.12±10^{d}	321.12±10^{b}	372.1±10^{a}	247.62±10^{c}	< 0.001
WBCS × 103/ mm^3	10.5±0.19^{c}	9.55±0.19^{d}	14.57±0.19^{a}	7.48±0.19^{f}	7.26±0.19^{f}	8.47±0.19^{e}	< 0.001
PT (%)	99.62±1.24^{a}	99.87±1.24^{a}	27.62±1.24^{e}	87.5±1.24^{b}	88.12±1.24^{b}	47.0±1.24^{d}	< 0.001
PTT (Sec)	37.25±0.85^{d}	36.25±0.85^{d}	53.5±0.85^{a}	41.12±0.85^{c}	42.62±0.85^{c}	46.37±0.85^{b}	< 0.001

Indicated values were mean ± SE. Means within the same row in each category carrying different letters were significant at $P \leq 0.05$. AO: *Alkkanna orientalis* extract; Cc: *Cerastes cerastes* venom; Anti: antivenom immunoglobulin, PT: prothrombin concentration and PTT: partial thromboplastin time.

Internal hemorrhage

Group I and II showed normal abdominal cavity with normal internal organs without any hemorrhage as shown in (Figure 1a and b). While severe internal hemorrhage was observed in group III (Figure 1c). Mild internal hemorrhage was noticed in groups IV and V (Figure 1d and e) and the abdominal cavity of group VI showed less internal hemorrhage than group III (Figure 1f). The obtained results showed that *Alkanna orientalis* extract when given as antidote for *Cerastes cerastes* venom hepatic, cholinergic, hematological toxicity and the internal hemorrhage was more effective than the synthesized antibody (antivenom immunoglobulin) and these results clearly appeared by comparing the findings of groups IV and V with group VI.

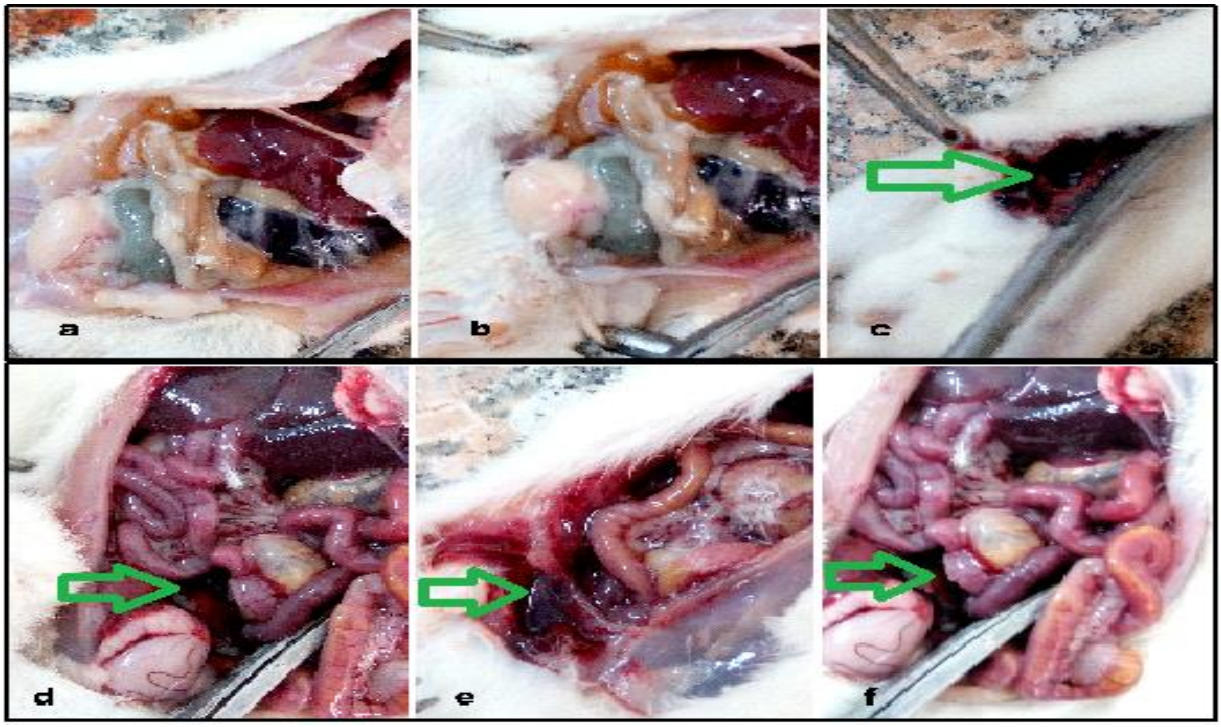

Figure 1: Illustrate the abdominal cavity of different groups in the study. (a) Showing the abdominal cavity of group I (control group) without hemorrhage, (b) showing the abdominal cavity of group II (*Alkanna orientalis* extract) without hemorrhage, (c) showing abdominal cavity of group III (*Cerastes cerastes* venom treated group) with sever internal hemorrhage as shown by the green arrow, (d) showing abdominal cavity of group IV (*Cerastes cerastes* venom + *Alkanna orientalis* extract) with less internal hemorrhage than group III as shown by the green arrow, (e) showing abdominal cavity of Group V (a prophylaxis group) with less internal hemorrhage than group III as shown by the green arrow and (f) showing abdominal cavity of group VI (*Cerastes cerastes* venom + antivenom) with less internal hemorrhage than group III as shown by the green arrow

Discussion

The intraperitonial injection of *Cerastes cerastes* crude venom caused a reduction in serum total proteins and albumin in male albino rats. These findings were in agreement with Salman (35), who reported a significant reduction in serum albumin and total proteins in Guinea pigs injected with *Cerastes cerastes* crude venom after different hours. The main factors that cause hypoproteinemia may be the disturbance in renal functions that result from the snake venom. As well as, the hemorrhage that commonly occurred with snakebites Also, the increase in vascular permeability due to the toxic effect of the crude venom can lead to the loss of protein in the tissues (36).

The current study showed a significant increase in serum total and direct bilirubin, ALT, AST and ALP enzymes after 2 hours of

Cerastes cerastes crude venom intraperitonial injection in comparison to control group. These results go hand in hand with previous findings (35-37). ALT and AST enzymes considered a major markers for hepatocellular damage as ALT enzyme is essentially present in hepatocytes, these enzymes acts as monitoring for liver disease, inflammation and necrosis (38).

Measurement of the enzymes activities is important in assessment of vital organs, as the crude viper's venoms reported to affect the activities of several serum enzymes. These enzymes activities increased significantly after the liver damage, skeletal muscles and myocardial damage (39). In the present study, increasing in the activity of ALT, AST and ALP indicate the damage of liver and other organs by the venom. These results were in agreement with previous researches on the venoms of other snake species such as *Cerastes cerastes gasperetti*, *Echis carinatus*, *Walterinessia aegyptia*,viper *B. arietans*, *Echis coloratusis*, *Naja haje* (40,41).

As well as, *Cerastes cerastes* venom according to the same program of treatment caused a significant decrease in serum cholinesterase. The decrease in serum cholinesterase level in rats following *Cerastes cerastes* venom injection is in agreement with Soares and Giglio (42). They reported that phospholipases A_2 constitute major components of *Cerastes cerastes* venom and it display a wide range of biological effects, including neurotoxic, myotoxic, cytotoxic. Also, Kini (43) reported that PLA_2 exhibit a presynaptic neurotoxicity. On the same pattern, a postsynaptic neurotoxin belonging to family *Viperidae* was isolated from the Egyptian sand viper *Cerastes cerastes* (44). These Postsynaptic neurotoxins bind specifically to the nicotinic acetylcholine receptor in the postsynaptic membrane of skeletal muscles, thus preventing the binding of the chemical neurotransmitter acetylcholine and thereby blocking the excitation of muscles. This block at the neuromuscular junction leads to flaccid paralysis and lead to respiratory failure (45).

Alkanna orientalis treatment provide a protective effect against *Cerastes cerastes* venom hepatotoxicity as, *Boraginaceae* family especially *(Alkanna orientalis* and *Echium humile)* rich with alpha linolenic acid (ALA), gamma linolenic acid (GLA), stearidonic acid (SDA), flavonoids in addition to rosmarinic acid (RA), all previous compounds have a powerful antioxidant properties and anti inflammatory effect. Also, flavonoids was reported as strongly inhibits the group II PLA_2 from Vipera and it weakly inhibits the group I PLA_2 from *Naja naja* (22). Therefore, they can restore the hepatic toxicity of *Cerastes cerastes* venom (16-19).

On the same pattern, the protective effect of *Alkanna orientalis* extract against the significant decrease in cholinesterase and the cholinergic effect of *cerastes cerastes* venom are in full agreement with Diab et al. (46). As they described the protective effect of *Echium humile* extract (family: *Boraginacea*) against malathion neurotoxicity and against the significant decrease in cholinesterase as a result of malathion cholinergic effect. In addition, the antagonist effect of *Alkanna orientalis* extract to cholinergic toxicity may be because of flavonoids inhibition to group II PLA2 from *Cerastes cerastes venom* that responsible for the major neurotoxicity of *Cerastes cerastes* venom (22).

The significant decrease in red blood cells and hemoglobin level that was reported in this study could be attributed to the intravascular hemolysis due to the venom indirect hemolytic activity, the micro thrombin formation with disruption of erythrocytes in the microvasculature (47) and a physiological stress that result from the envenomation (48). The observed decrease in platelets count that caused by the crude venom may be a result of some enzymes that have a major effect on platelets such as serine proteinases and phospholipases A2 enzymes (49). Another acceptable cause for the decreasing in platelets count may be because of the consumption of platelets due to hemorrhagic metalloproteinases effect on blood vessels damaging and rupturing them. Due to this rupture, platelets migrate to the lesion site and can bind to sub endothelial surfaces to form a haemostatic plug, which lead to the decreasing in platelet count (47,49).

In contrast, WBCs count increased significantly 2 hours after venom injection. This explained by the temporary immune response to crude venom that leads to liberation of white blood cells (50). Another acceptable hypothetical reason is that crude venom stimulates the liberation of pro-inflammatory cytokines by macrophages that responsible for the mobilization of both lymphocytes and monocytes (40). As well as, 2 hours after envenomation, the envenomed group showed a significant decrease in plasma prothrombin and partial thromboplastin concentration. This haemostatic disturbance may refer to the efficacy of C-type lectins, which considered a group of proteins that form about 2% of *Cerastes cerastes* venom (51). In addition, C-type lectins are responsible for the activation and inhibition of coagulation factors (52).

In addition, phospholipases A_2 display anticoagulant efficiency by suppressing the exterior passage of the haemostatic cascade. These groups of proteins can extend their activities by hydrolyze phospholipids or by overlapping with coagulation factor Xa and overlapping with its interaction with factor Va resulting in suppression of prothrombinase complex (48,53). These classes of protein comprise 19% of *Cerastes cerastes* venoms (51). The present study display that treatment with *Alkanna orientalis* was effective in restoring the depressed hematological parameters as well as leukocytosis due to the anti inflammatory, anti oxidative, antibacterial, and antiviral activities of *Alkanna orientalis* which rich in RA (19). RA has been reported to block both metalloproteinase and phospholipases A2 (20). Therefore, *Alkanna orientalis* can prevent most hazardous hematological effect of *Cerastes crrastes* crude venom.

The injection of rats with *cerastes cerastes* venom (i.p.) resulting in severe internal hemorrhage in the abdominal cavity because Cerastes *cerastes* crude venom contains 70% metalloproteinases, serine proteinases and 57.3% of hemorrhagic metalloproteinases P-III (51). These metalloproteinases exhibit proteolytic effects, leading to local hemorrhages because of the degeneration of vascular endothelium (54). As well as, serine proteases and phospholipases A_2 are able to form proteins that effect platelet aggregation, fibrinolysis and cascade of coagulation as well as, the complement system (55). On the other hand, *alkanna orientalis* extract when given orally 2 hours before *Cerastes cerastes* venom injection or (i.p.) immediately after *Cerastes cerastes* venom injection was able to minimize the hemorrhagic effect of the venom. As, it able to antagonize the hematological toxicity by preventing the thrombocytopenic effect of the venom as well as, preventing sever depression in prothrombin and partial thromboplastin concentration that considered the major cause of the internal hemorrhage .

On the same pattern, when *Cerastes cerastes* antivenom immunoglobulin was injected intraperitonial immediately after *Cerastes cerastes* venom injection, was able to minimize the hazardous venom hepatic and hematological effects. Antivenom reported as the specific antidote that prevents mortality and reduces hospital stay and morbidity, incase of snakebites (56). Antivenom is not compound that can cure or rollback the effects of snake venom, all they can do is to halt the effect of further activity of the snake antigens in the blood stream. Besides that, antivenom immunoglobulin therapy may cause a hypersensitive reaction in the body. This is called anaphylaxis that caused by the antibodies and other substances found in the blood-donor animals serum during antivenom synthesis (56). Hence, the creativity of this this work is the presentation of a new safe therapy for *Cerastes cerastes* venom with antidotal effect that clearly from previous results to be better than antivenom immunoglobulin therapy.

Conclusion

The natural medical herbs are showing a new area for development of better therapeutic and prophylaxis agents against expected envenomation by *Cerastes cerastes* venom toxicity. The alcoholic extract of *Alkanna orientalis* has many benefits, as it is cheap, easily available as it widely distributed in Saint Catherine (Egypt), stable at room temperature and able to antagonize the hazardous toxic effect of *Cerastes cerastes* venom. Therefore, we recom-

mend the usage of *Alkanna orientalis* extract as initial assist in *Cerastes Cerastes* bitted victims therapy to minimize mortality and morbidity. As well as, this extract can be used as a prophylaxis for researchers who work in the fields where *Cerastes cerastes* are abundant.

Conflict of interest

The authors have declared no conflict of interest

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Original Research Article

A REVIEW ON METABOLIC SYNDOME: BIOCHEMICAL INVESTIGATIONS

Samah S. Khalil[1,2]*, Amany I. Ahmed[1], Nada A. Mohammed[1], Azza A. El-Naggar[1], Haytham A. Ali[1,3], Hamad A. Al-Saadawy[1], Khalifa R.E. Ahmad[1]

[1]Biochemistry Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig, 44511, [2]Zagazig University Hospitals, Zagazig University, Zagazig, Egypt, [3]Biochemistry Department, Faculty of Science, University of Jeddah, Saudi Arabia

*Corresponding author, E-mail: samahsaid75@gmail.com

Abstract: In the last few years, the metabolic syndrome (MetS) has attracted increased attention. Metabolic syndrome defined as a group of interconnected biochemical, physiological, metabolic and clinical risk factors such as hypertension, obesity, glucose intolerance, dyslipidemia and inflammation that lead to many fatal diseases as atherosclerotic cardiovascular disease, stroke and type 2 diabetes mellitus. This review tends to go over the main points of the essential mechanisms involved in induction models of MetS by diet regimen based on high-fat high fructose/sucrose (HFHF) added to normal chow. Management of metabolic syndrome should undergo several axes such as increasing physical activity, modification of lifestyle and healthy food besides. Moreover, medications can be used to control the symptoms of different disease related to metabolic syndrome. Finally, we can conclude that the main and prevalent risk factors for the pathophysiology of Metabolic Syndrome are Insulin resistant, abdominal obesity. Also, physical inactivity and chronic inflammation which provide a possible explanation of the cause of the metabolic syndrome until now which designates the energetic relationship between a number of contributing factors.

Key words: metabolic syndrome; diabetes mellitus; insulin resistance; obesity

Introduction

Metabolic syndrome (MetS) is recognized as sequence of linked biochemical, physiological, clinical and metabolic factors that tend to directly raise the risk of atherosclerotic cardiovascular disease (CVD), type 2 diabetes mellitus and all of these factors could lead to mortality (1). It consists of a set of conditions that do not have a single reason but can be caused due to many contributing elements such as genetic or environmental factors (2). Insulin resistance (IR), hypertension, heredity type 2 diabetes mellitus and racial background are unavoidable hereditary elements that enormously elevated the hazard of creating MetS (3). Moreover, aging is another fundamental unalterable risk factor for MetS. On the other hand, environmental hazard factors for MetS are possibly controllable. These incorporate the sedentary life, physical latency, and bad dietary patterns. Metabolic syndrome could eventually induce other medical complications such as; extended hazard of type 2 diabetes mellitus, cardiovascular disease (CVD), non-alcoholic fatty liver

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disease, kidney and pancreatic dysfunction, liver, pancreas, bladder, and breast cancers (4).

Epidemiology of metabolic syndrome

The propagation of the MetS has been increasing all over the world together with obesity, influencing equally developing and developed countries (5), due to the presence of different definitions of MetS, it's difficult to estimate the frequency but it varies from 10-84% across racial groups, gender, countries, and age (6). Epidemiologic studies demonstrated that the metabolic syndrome appears widely in ethnic groups including Asian-Indians, African-Americans, Mexican-Americans, Caucasians, and Chinese with varying prevalences of both the metabolic syndrome and its components (6).

USA use data from The National Health and Nutrition Examination Survey (NHANES 1999–2000) and (2009–2010) to make the comparative analysis, using the 2009 harmonized definition of MetS (7), demonstrated that the last decades the prevalence of MetS reduced from 25.5 to 22.9% (8). This result was seen obviously in Caucasians may be due to the usage of the drugs for the treatment of dyslipidemia and hypertension. Although, higher rates of prevalence (almost 40%) elicit when using the NHANES 1999–2002 data and the definition of International Diabetes Foundation (IDF) of the syndrome (9), which need central obesity to be one of the three factors; As the prevalence of abdominal obesity increased from 45.4 to 56.1% since 1999 till 2010, the thresholds for waist circumference were strongly lowered, also hyperglycemia increased from 12.9% reach to 19.9%, while the other parameters, as blood pressure were decreases from 32.3 to 24.0% and hypertriglyceridemia reduced from 33.5 to 24.3% due to use of antihypelipidemic agents (8). On the other hand, the prevalence is increased for Asians and Mexican-Americans when using the lower thresholds of waist circumference (10). In an Australian population-based survey, according to the IDF definition, the prevalence of the MetS was 29.1% and 19.3% according to the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) definitions (11). Another study using various definitions of the metabolic syndrome In Brazil found that the mean prevalence was 30% (12). While in Europe, the prevalence ranges from 10 to 30% (13). Also, in the Norwegian the predominance of the MetS according to IDF was 29.6% when compared to the NCEP-ATP III was 25.9% (14). Although in France according to the NCEP-ATP III definition, the MetS was found in men 16% and in women 11% (15).

Recently in China statistical-analysis including about 220,000 people, reported that the incidence of MetS was 19.2% in men and 27.0% in women, while noticed that MetS increased with increasing age in urban areas. Another study notes that the most common MetS component is hypertension in men with percent 52.8% followed by abdominal obesity in women with percent 46.1% (16).

In India the prevalence was 18.3% according to the NCEP-ATP III definition and 25.8% according to IDF (17), and significantly increases in urban areas (18) especially in Indian immigrants in USA (19). While in Japan Rates were lower 8% in men and 10% in women (20). Harzallah et al. (21) reported that the prevalence of MetS in Arab countries increased in female more than male by using the IDF criteria, while the prevalence decreased when using World Health Organization (WHO) or NCEP-ATP-III criteria (21). In Tunisia the prevalence of the MetS about 27% in male and 39% in the female, while in Iran and Turkey was about 33.1%. Generally, by using any definition, the MetS risk factors are higher in women than in men due to the significant differences of obesity and HDL-C and hypertension (22).

The risk of IR also the risk of developing the MetS increases in the family with the history of CVD or type 2 DM (23). Also varying in metabolic syndrome phenotypes and different clusters of components across ethnic groups and countries (24). Another study noticed that the predominance of the MetS increased with age, disregarding of sexual category (6). These data harmonized

with another study done in several countries and ethnic groups (25). An important study in children showed that the prevalence of MetS increased with increasing of type 2 DM and severity of obesity degree (26). Another study in the USA done in youth reported that the occurrence of the MetS was 11.9% in the overweight, 3.3% in children and 29.2% in the obese (27). Also, in NHANES 2001–2006, the prevalence was higher in Hispanic and Caucasian boys when compared to black adolescents (28). The prevalence of MetS was higher in boys and older children (26).

Origination of metabolic syndrome

Over the years, numerous definitions for MetS have been developed. The first definition was provided by world health organization (WHO), which placed insulin resistance and/or impaired glucose tolerance as an important high risk factor and considered it to be a necessary condition for the diagnosis of MetS (29). Later on, other definitions were developed by different organizations (30-34). The newest defining criteria have been provided by the World Heart Federation, the International Atherosclerosis Society, and the International Association for the Study of Obesity (35) as presented in Table (1). Despite the fact that the grouping criteria contrast from each other, almost every one of them incorporates a combination of the following factors: abdominal obesity, blood pressure, diabetes and biochemical indicators (36).

MetS is a theory more than a diagnosis. The current definition of MetS includes many parameters such as hyperinsulinemia or IR, dyslipidemia, hypertension, and obesity, with a particular stress on central adiposity (40). Many other factors can influence MetS such as drugs like corticosteroids, antidepressants, antipsychotics, antihistamines which cause weight gain and obesity (41).

Obesity and MetS

The most causal component of MetS is obesity. Adipocytes generate different types of biologically active fragments (adipokines) including adiponectin, plasminogen activator inhibitor–1 (PAI-1), resistin, leptin, and TNF-α. Dysregulated generation of these adipokines participates in the pathogenesis of obesity associated with metabolic syndrome. In obesity, developing of IR and atherosclerosis is due to decrease in adiponectin in plasma (42). The correlation between obesity and type 2DM created the term ‘diabesity’ to confirm the strong relationship between these two conditions (43). Central obesity linked with insulin resistance, type 2 DM, metabolic syndrome and atherosclerotic cardiovascular disease (CVD). Central obesity is considered a low-grade inflammatory state, as it associated with increased secretion of interleukin-6(IL-6) and tumor necrosis factor-alpha (TNF-α) from adipose tissue that consequently elevated level of plasma C - reactive protein (CRP) which is an inflammatory marker associated with increased risk of myocardial infarction in these individuals. Moreover, obesity is associated with markedly decrease level of adiponectin, which has an important role in improving insulin signaling and protection against atherosclerosis (44) as showed in Figure (1).

Insulin resistance and MetS

Insulin resistance has a fundamental role in the progress of MetS. Multiple factors may lead to IR development. The most prominent of them are weight gain, sedentary lifestyle, and heredity factors. Moreover, other factors that could contribute to IR development are high-carbohydrate diets, ageing, drugs like steriods and androgens (45). Insulin resistance eventually will cause malfunction of β-cells in the pancreas and leads to hyperglycemia and T2DM (46). Patients who suffer from IR should not necessarily be obese, but may only suffer from poor distribution of fat, which is concentrated in the upper body especially the abdomen that strongly correlates IR with MetS (47).

Impaired glucose tolerance and IR are combined with increased CVD incidence. The mechanisms by which IR accelerate CVD risk include the production of glycation end products, elevated blood pressure, proinflammatory and prothrombotic states and dyslipidemia, which is caused by the increased flux of free fatty acids from adipose tissue to the

liver. Many organs could be affected metabolically by IR such as; adipose, hepatic and skeletal muscles tissues (48). IR may also have a direct effect on small and large blood vessels, which lead to hypertension, cellular proliferation and inflammatory responses in the wall of blood vessels (atherogenesis). Insulin resistance and MetS could also increase the risk for ischemic heart disease (49). Although obese people have increased the risk of insulin resistance, type 2DM and CVD, not every obese individual is insulin resistant or at high risk of diabetes and CVD (50).

In obese individual, impaired metabolism of non-esterified fatty acid (NEFA) could participate in developing the insulin-resistant state. The adipocytes in those obese individuals are characteristically in a hyperlipolytic states that are resistant to the insulin induced antilipolytic action. Moreover, the produced NEFA enter to the liver could affect liver metabolism, that increase liver production of hepatic glucose and IR .in summary impaired NEFA metabolism considered as insulin secretion stimulation the may cause of IR (51). Insulin is a hormone secreted by β-cells of pancreas responsible for in maintaining the glucose balance and also of some anabolic functions. It controls blood glucose level by regulating glucose uptake of muscle, liver, peripheral and fatty tissues. Moreover it has a role in fat metabolism by stimulating the synthesis of fat in the liver and fat cells (52).

Accumulation of free fatty acids (FFA) is the main reason for development of IR. The FFA is released in blood circulation in the lipolysis process of lipoprotein rich with TAG by action of lipoprotein lipase enzyme and through the action of the cyclic AMP-dependent hormone sensitive lipase enzyme and also by the action of lipoprotein lipase (53). Insulin hormone plays important role in stimulation of lipogenesis and prevention of lipolysis in adipose tissue and activation of lipoprotein lipase which play an important role in the transfer of triacylglycerol from blood lipoproteins into the tissues. IR cause inadequate secretion of insulin from pancreas so increase lipolysis process lead to accumulation of FFA. In muscles, FFA deactivates protein kinase C. In the rats fed a high-fat diet, IR cause deficiency in insulin-stimulated insulin receptor substrates in livers (54).

Type 2 diabetes and MetS

Diabetes mellitus is a metabolic disease that identified by hyperglycemia due to abnormalities either in insulin secretion, action or both (55). People who are not diabetic, but have metabolic syndrome are at higher risk to develop diabetes. The patients have a metabolic disorder syndrome are five times at risk of getting type2 DM since there is already glucose dysregulation (56). According to the American diabetes association diagnosing diabetes can fall under 4 major categories: Type 1, Type 2, Gestational diabetes mellitus (GDM) (57).

Type 2 diabetes mellitus (T2DM) is the commonest type of diabetes and is often referred as noninsulin-dependent diabetes" or "adult-onset diabetes" In T2DM there is a dysregulation of carbohydrate, lipid and protein metabolism as a result of impaired insulin secretion, insulin resistance or both. When a patient has T2DM, this puts him at a high risk for the suffering of CVD and MetS (58). Thus, the combination of these previous factors could lead to the development of MetS. Moreover, the incidence of MetS varies with the race and the age of the study population (59).

Table 1: Clinical diagnosis of metabolic syndrome by different organization

	ORGANIZATION				
Clinical measurements	**WHO (1998) (29)**	**EGIR (1999) (39)**	**NCEP/ATPIII (2001) (31)**	**AACE (2003) (38)**	**IDF (33, 37)**
Insulin resistance	IGT,IFG ,T2DMor lowered insulin sensitivity + any 2 factors of following	Plasma insulin > 75 thpercentile + any 2 of following	None Butany 3 of following 5 features	IGT or IFG + any of following	NONE
Body weight	Waist to hip ratio Men ≥ 90 cm Women ≥ 85 cm	WC in Men ≥ 94 cm Women ≥ 80 cm	WC in Men ≥ 102 cm Women ≥ 88 cm	BMI ≥ 25 kg/m^2	Increased WC (population specific)
Triacylglycerol	TAG ≥150 mg/dl	TAG ≥ 150 mg/dl	TAG ≥ 150 mg/dl	TAG ≥ 150 mg/dl	TAG ≥ 150 mg/dl
HDL-c	Men < 35 mg/dl Women < 39 mg/dl	Men < 39 mg/dl Women < 39 mg/dl	Men < 40 mg/dl Women < 50 mg/dl	Men < 40 mg/dl Women < 50 mg/dl	Men < 40 mg/dl Women < 50 mg/dl
Glucose	IGT, IFG or T2DM	IGT, IFG but not diabetes	>110 mg/dl includes diabetes	IGT, IFG but not diabetes	≥100 mg/dl include diabetes
Blood pressure	≥ 140/90 mm/Hg	≥ 140/85 mm/Hg	≥ 130/85 mm/Hg	≥ 130/85 mm/Hg	≥ 130/85 mm/Hg

IGT: impaired glucose intolerance, IFG: impaired fasting glucose, T2DM: type 2 diabetes mellitus, WC: waist circumference; BMI: body mass index, TAG: triacylglycerol. WHO: World Health Organization, EGIR: the European Group for Study of Insulin Resistance, AACE: American Association of Clinical Endocrinologists, IDF: International Diabetes Foundation, ATPIII: Adult Treatment Panel III.

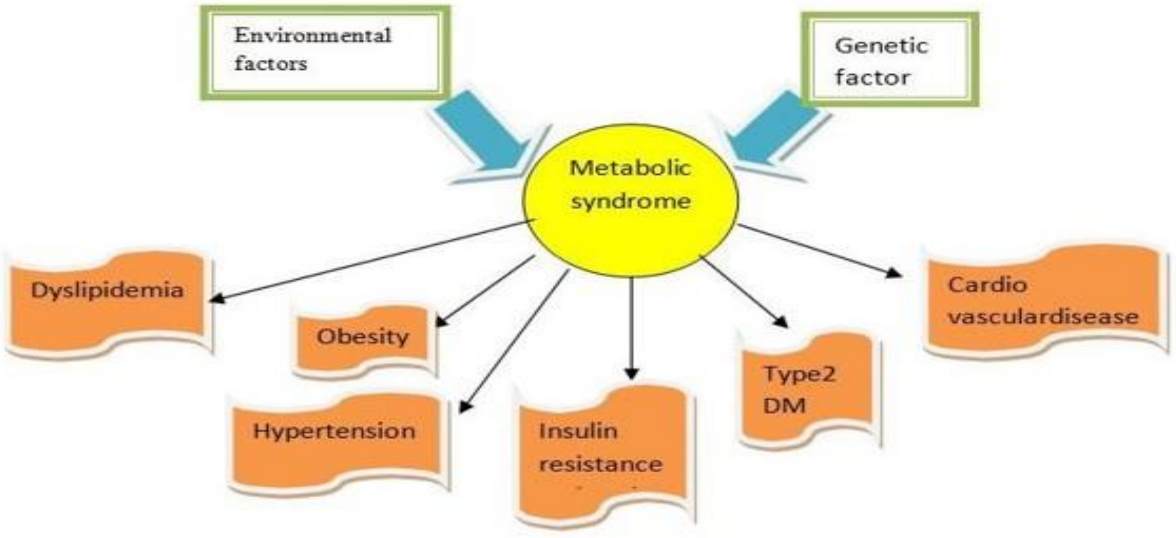

Figure 1: Etiology of MetS and risk factors affecting it

MetS and Oxidative Stress

Researches indicate that increased oxidative stress (OS) cause exaggeration in MetS symptoms such as T2DM, atherosclerosis, insulin resistance and inflammation (60). Oxidative stress generated due to the imbalance between reactive oxygen species (ROS) production and antioxidants efficiency, so OS occurred due to increase in ROS production or shortage in antioxidant system. ROS generated from the aerobic system through oxidation system ($O2^-$, H_2O_2, OH^- is a highly active product cause damage to the cellular component of the body, DNA denaturation and lipid peroxidation (61). ROS destroys many biological processes inside cellular system such as lipid peroxidation, carbohydrates oxidation, oxidation of nucleic acid base lead to deformation of DNA and mutation (62) (Figure 2).

Antioxidants divided into enzymatic catalase (CAT), superoxide dismutases (SOD), and glutathione peroxidase (GPX)) and non-enzymatic (vitamin C, vitamin E, β-carotene, reduced glutathione, and numerous phytochemicals). The body should keep its antioxidant level balanced either by dietary intake and/or de novo synthesis (63).

Oxidative stress plays a key role in the induction of many metabolic diseases such as diabetes, obesity, cardiovascular disease. In diabetes, it causes impairment release of insulin from β-cells of pancreas, inadequate uptake of glucose by cells. In obesity, it causes deformation of abdominal and visceral fats (64).

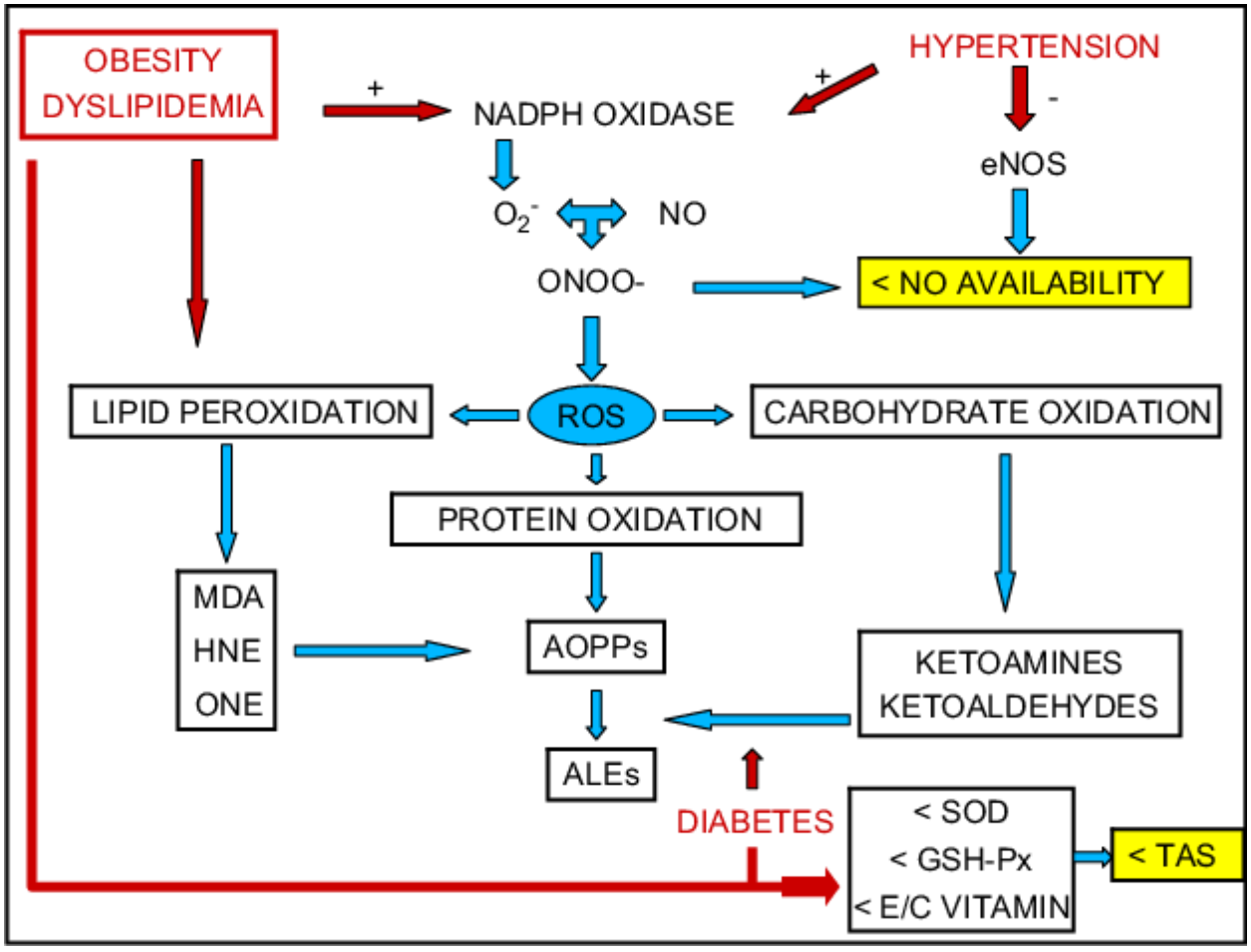

Figure 2: Role of oxidative stress in developing of MetS. MetS is affected by oxidative compounds lead to oxidation of lipids, carbohydrates and proteins (derived from Hopps et al. (64)

Abbreviations: O_2: superoxide anion; NO: nitric oxide; ONOO_: peroxinitrite; ROS: reactive oxygen species; eNOS: endothelial NO synthase; MDA: malonyldialdehyde; HNE: 4-hydroxynonenal; ONE: 4-oxy-2-nonenal; AOPPs: advanced oxidized plasma protein; ALEs: advanced lipoxidation end-products; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; and TAS: total antioxidant status.

Inflammation and MetS

According to previous studies, abdominal obesity results in systemic low-grade inflammation which causes IR and MetS. In patients who suffer from obesity or Type 2DM, investigate from plasma levels of coagulation, white blood cell counts, acute phase proteins, and some pro-inflammatory cytokines and chemokines are always elevated. Those previously mentioned inflammatory biomarkers often tend to decrease when the patient begin lifestyle changes in favor of reducing weight and weight loss. Some experimental data revealed that the site of inflammation in the obese and Type 2DM patient is mainly located in the liver, adipose tissue, muscle and pancreas (65). In animal models as well as humans suffering from obesity and DM there will be an infiltration of macrophages to these inflammation sites, these macrophages are important for the production of pro-inflammatory cytokines which are TNF-α, IL-6, and IL-1b (66). They promote insulin resistance through acting in the autocrine and paracrine way through interrupting with insulin signaling in peripheral tissue by stimulation of two pathways (the c-JUN N-terminal kinase (JNK) and nuclear factor-kappa B (NF-kB) pathway). In obese and Type 2DM patients, these pathways are activated and play major central act in building up tissue inflammation (67) as presented in Figure (3).

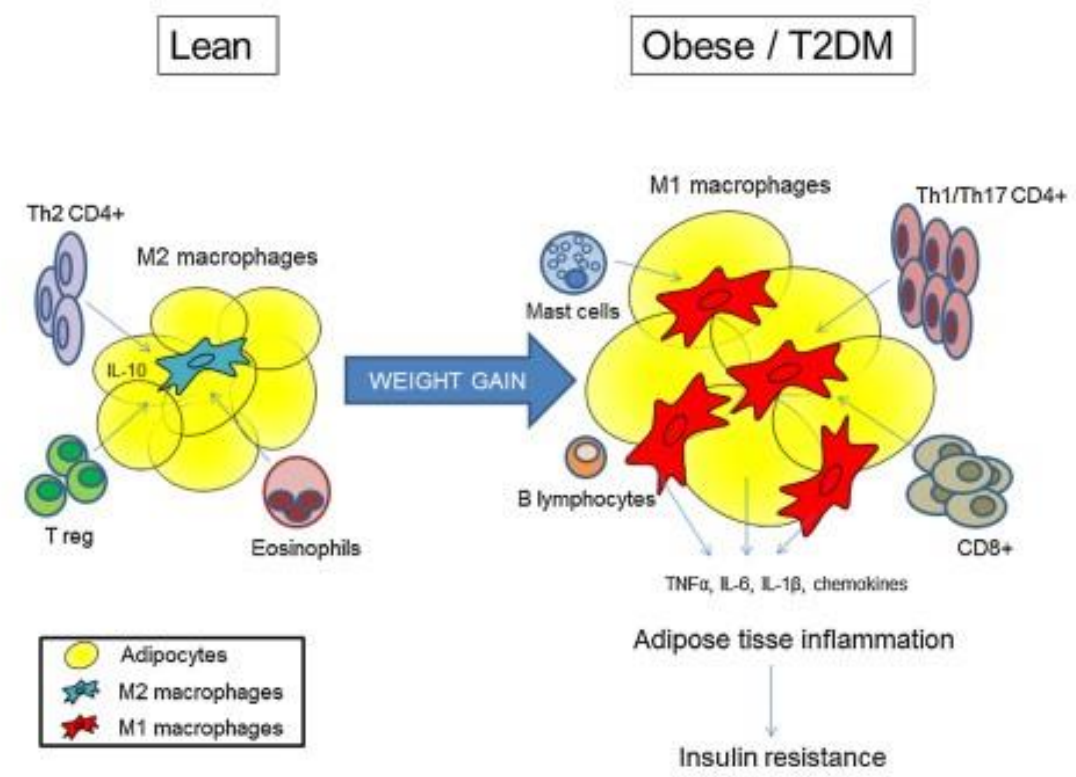

Figure 3: Role of inflammation of adipose tissue in developing of MetS and type 2 DM (derived from Esser et al. (65)

The main and prevalent risk factors for MetS are IR and abdominal obesity. Also, physical inactivity and chronic inflammation has a significant role in MetS (68). IR and hyperinsulinemia were closely correlated to the disturbed metabolic symptoms cluster (69). Visceral fat accumulation, which caused by physical inactivity and over nutrition, leading to an unusually high circulation of FFA, consequently appear of lipotoxic effects and IR and leading eventually to a state of hyperglycemia. Therefore, IR, ecological factors, inflammation and obesity provide a possible explanation of the cause of the metabolic syndrome until now which designates the energetic relationship between numbers of contributing factors (68).

Genetic mechanisms

Genes may affect in the propagation of the MetS in different ways each key of the syndrome parts high blood pressure, obesity, dysglycemia and dyslipidemia have a genetic basis, for which the main candidate genes can identify (70). This relation might facilitate the emergence of the syndrome. For an example, there is a difference in the expression of adiponectin gene (ADIPOQ), which associated with visceral obesity (71). Also, blood pressure has been correlated with differences in expression of angiotensinogen gene (AGT) (72), in addition to changes in apolipoproteins C-III genes (APOC3) and apolipoproteins E genes (APOE), which correlated with concentrations of plasma lipid (73,74). Thus, variants connected with the individual components of the MetS phenotype could interpretive correlation with the entire syndrome. Moreover, some candidate gene products may act within the main pathway which can affect different syndrome component, and possible single-gene associations have been identified. For an example glucocorticoid receptor gene (NR3C1), the gene associated with hypertension, obesity and insulin resistance (75). ADIPOQ associated with dyslipidemia, diabetes, and hypertension, β3 subunit of G protein (GNB3) gene, which associated with obesity and hypertension (76). Changes in expression of some genes which encode for

certain transcription factors as sterol regulatory element-binding protein 1 (SREBP1) and for-khead box protein C2 (FOXC2) are associated with concentrations of trigly-cerides in plasma and insulin sensitivity. These genes may be the maestro for association with the development of the whole metabolic syndrome (77,78).

The Metabolic Syndrome heritability

Study on family and twin showed a heritable interference in MetS factors clustering (79). For example, the study done in United States on 2,508 men twin pairs, the appearance of diabetes, obesity, and hypertension can be noticed in 31.6% of monozygotic pairs but noticed only 6.3% of dizygotic pairs (80). On the same line, a similar demonstration for heritable factors showed in a twin of female pairs. Also, another study on 432 people from 68 Japanese American families, significant genetic interference and effects were noted on all components of the MetS, especially dyslipidemia, about 50% of the changes were related to genetic influences (81). All above studies suggested that all causative genes underlying the MetS has motivated research to make more studies on the rare monogenic forms of the syndrome and also study the common trait by various approaches, including genetic linkage and association analysis (82).

Experimental Induction of MetS

There are different models of induction MetS in animals that allow scientists to study and investigate. One of these methods is genetic one by using specific species of rats; another method is by drugs that cause the destruction of β-cells of pancreas such as streptozotocin (STZ) or alloxan, but his type of induction is different from the human model. The most common and easiest way to induce MetS is by diet but it needs a long time of experimental, this method mimics to the human model of diet causing the metabolic disorder. As human dietary is more complex than animal diet, so the combination of the different types of foods (fat and carbohydrates) is added to reach the same model of human regimen (83).

In the field of MetS researches, rats and mice are the most well-known animal models. Different dietary systems have been used to initiate MetS in animals. They included the utilization of either a single kind of eating routine or a mix of weight control plans, for example a) high fructose b) high-sucrose c) high-fat d) high fructose/ high-fat e) high-sucrose/high-fat diets or f) high fat/high fructose/high sucrose diet. It was proved that using high-fat high-fructose/sucrose eating regimen (HFHF) in rodent chow is the fundamental method for induction of MetS mimic the human ones (84,85) (Table 2).

Accordingly, chow is a high fiber eating routine containing complex sugars with fats of vegetable sources. Chow is reasonable to make and is attractive to rodents. Fiber is regularly given by cellulose (86). The HFHF eating regimen used to induce MetS in animals usually contain high quantities of sugar (fructose/ sucrose), fat or both (87). This model had utilized generally little amounts of these added substances, planning to mimic nearly the cafeteria diet, these eating regimens were related with essentially higher caloric admission (88).

Table 2: Different dietary methods for induction of metabolic syndrome in animals

Methods	Effect on normal rats
1. High fructose 66% for 2weeks	This diet results in hyperinsulinemia , hypertriglyceridemia , increase in blood pressure and insulin resistance (89).
2. High sucrose 8% for 5weeks	This model results in mild hypertension and tachycardia (90).
3. High fat 60% lard for 120 days	Obesity, insulin resistance (91).
4. High sucrose34%+ high fat 21% for 2 or 4 weeks	High body fat percentage and impaired glucose tolerance along with hyperinsulinemia and insulin resistance (92).
5. High fructose 17.5% and fructose 25% in drinking water+ high fat 20% for 16 weeks	Metabolic syndrome and cardiovascular such as hypertension, hypertrophy, ventricular fibrosis and endothelial dysfunction (83).
6. High fat 30%+ high fructose 40% + high sucrose 10% (HFHF) diet for 12 weeks	The fundamental method for induction of MetS mimics the human ones (93).

Sugars can be separated into simple (e.g. monosaccharides and disaccharides) and complex (e.g. oligosaccharides and polysaccharides) forms. Carbohydrates are one of the basic supplements that act as short-term fuel in the body since they are the simpler process than fats. Carbohydrates digestion starts in the small digestive tract to form glucose particles, then it is absorbed by the circulatory system and transported to the liver through the entry vein. When carbohydrates exceed the daily limits of the body need, blood glucose concentration will stay high and insulin is secreted by the pancreas to enable cells to take up glucose. Then, glucose is metabolized in the following pathways (a) breakdown during the process glycolysis to supply the body with energy (b) changed over to glycogen in liver and muscles (c) transferred under insulin effect in the adipose tissue to develop fatty acids (lipogenesis) (94).

Extended utilization of carbohydrates causes elevated glucose levels in the blood. Therefore, the pancreas pumps more insulin into the bloodstream to decrease the blood glucose. So, the carbohydrates changes to fats that stored in adipose tissue. The sensitivity of insulin is also decreased. There is strong correlation between insulin resistance and high carbohydrate intake (95).

Fructose is a monosaccharide, which is available with glucose in general food items. The impact of mixing fructose and glucose together is more effective in inducing MetS than the impact of using fructose alone (96). Fructose is the favored sugar that usually added to the animal nourishment or drinking water and the most prominent model uses 10% fructose solution (97). After fructose absorption it's delivered via the portal vein to the liver, then it undergoes different metabolic pathways it may be oxidized, change into glucose, stored as glycogen, or enter the process of de novo lipogenesis (DNL). The major difference between fructose and glucose is the higher tendency of fructose to enter in the process of DNL, as the fructose is the key element in de novo lipogenesis (96). Massive fructose uptake by the liver is due to fructose consumption, fructose utilization brought about enormous fructose absorption by the liver. Fructose is changed to fructose-1-phosphate by the phosphofructokinase enzyme in the presence of ATP. It is trailed by the cleavage of fructose-1-phosphate into glyceraldehyde and dihydroxyacetone which may convert to triacylglycerol or glycogen. (97).

In glycolysis process phosphofructokinase (PFK) is the rate-limiting enzyme, enabling fructose convert to fructose-1, 6-bisphosphate, then changed to pyruvate through the procedure of glycolysis. At this status, fructose is associated with a few procedures: (a) some of the fructose is changed into lactate from pyruvate, (b) another part produces triose-phosphate which promptly changes into glucose or on the other hand glycogen through gluconeogenesis, (c) carbons determined from the fructose can be changed over into unsaturated fats, and (d) the hindrance of hepatic lipid oxidation by fructose favors very low density lipoproteins (vLDL)- triacylgly-

cerol synthesis and unsaturated fat re-esterification (98).

The negative effect of fructose on health is influenced by the amount of fructose consumed (99). An extensive inflow of fructose into the liver causes an increase of triacylglycerol and cholesterol production due to its lipogenic properties, in this way diminished insulin sensitivity and promoting insulin resistance and glucose intolerance (61, 100). Sucrose, or table sugar, is a disaccharide found in cane and beet sugar. It is converted into its constituent fructose and glucose by the catalyst sucrase enzyme (101).

Glucose uptake in glucose digestion is adversely controlled by phosphofructokinase, prompting the persistent passage of fructose into the glycolytic pathway. Overabundance fructose will be changed over into fat in the liver as fructose is a superior substrate for unsaturated fat combination contrasted with glucose. In this way, fructose is the primary dynamic fixing adding to the advancement of MetS in creatures after sucrose utilization (102). Administration of sucrose in drinking water in animal labs lead to stimulates insulin resistance, obesity, triacylglycerols, cholesterol, (LDL-c), and free unsaturated fats cause exaggeration of MetS (103).

High-fat diets have been widely used to initiate MetS in the clinical trial. It has been demonstrated that a high-fat eating regimen is potent in inducing hyperglycemia, insulin resistance, dyslipidemia and elevated plasma free fatty acids (104,105). Triacylglycerol is the main constituent of body fat; it is an ester derivative of three unsaturated fat chains and glycerol. Lipid digestion starts with the procedure of lipolysis. A lot of glycerol and fatty acids diffuse into the circulation system. Plasma free unsaturated fats are significant substrates for hepatic vLDL-c and triacylglycerol generation (106). Roughly 70 % of free fatty acids will be re-esterified (lipogenesis) to produce triacylglycerol. The rate of re-esterification is correlated with the rate of glycerol-3-phosphate generation through glycolysis process and the rate of unsaturated fat discharge from adipocytes (107).

A proper treatment and control of dyslipidemia, hypertension and an excellent glycaemic control are needed in case the patient suffers from diabetes while, already having a metabolic disorder. The clinical researchers recommended the drug therapy choice besides lifestyle modifications is essential to reach the ideal glycaemic goal as a prophylactic way against many long-term microvascular complications (i.e. retinopathy, nephropathy, and neuropathy) and macrovascular ones (i.e. heart attack and stroke) (58).

Management and controlling of MetS

The cornerstone in the treatment of MetS is depending on lifestyle modify, a development in physical fitness which leads to progress in body fat circulation and cardiovascular health, reduce body mass index (BMI) and treatment of diabetes mellitus type 2. ATP III launched some instructions for the healthy diet that must contain a lot of fibers from fruits and vegetables, also food that it has to contain omega-3 and omega-6such as olive oil, tuna, fish and canola oil. Individuals must avoid the cafeteria diet, junk food, and food contains saturated and hydrogenated fats. Sugars intake must be controlled in the MetS patients and preferred to replace with artificial sweaters sugar. Moreover, whole grains are healthier to use instead of rice and bakeries (108).

The first guidelines to control MetS directed to increase daily physical activities and exercises as walking, swimming, ride bikes, gymnastics, but the most important is to keep on these regular exercises and for a long time. Moreover, a proper diet control must be followed to reduce obesity, IR, type2DM; also we can keep blood pressure, TAG, LDL-c and cholesterol within normal ranges by following lifestyle regimen (108,109). The second guideline is to use drug medications for treatment of MetS disease such as metformin for treatment of IR and statins for treatment of high cholesterol as it inhibits reductase enzyme lead to improving CVD diseases. Also, fibrates used to relieve the symptoms of athe rogenic, dyslipidaemia and enhance CVD diseases (110,111).

Management of diabetes mellitus varies according to many factors such as type 2 DM, the age of patients and severity of the disease. These entire factors play role in putting the suitable strategy for managing the disease. Maintaining the glycemia and decreasing the long term hazards and risks of diabetes is done through pharmacological therapy. In case of T2DM several drug classes are used including: a) Insulin sensitizers (Biguanides – Thiazolidinediones); b) Insulin secretagogues (Sulfonylureas – Meglitinide derivatives); c) Alphaglucosidase inhibitors; d) Glucagonlike peptide–1 (GLP-1) agonists; e) Dipeptidyl peptidase IV (DPP-4) inhibitors; f) Selective sodium-glucose transporter-2 (SGLT-2) inhibitors; g) Insulin; h) Amylinomimetics (112). Although there are many pharmaceutical drugs in the markets, scientists tends to use medicinal plants to treat diabetes because of the less harmful side effects of plants medications and the natural formulation than manufactured ones (113).

Conclusion

Finally, we can conclude that the main and prevalent risk factors for the pathophysiology of MetS are IR and abdominal obesity. Also, physical inactivity and chronic inflammation have a significant role in MetS. For the understanding of the MetS, it starts from the noticing that IR and hyperinsulinemia were closely correlated to the disturbed metabolic symptoms cluster. Visceral fat accumulation, which caused by physical inactivity and overnutrition, leading to an unusually high circulation of FFA, consequently appear of lipotoxic effects and IR and leading eventually to a state of hyperglycemia. Therefore, IR, ecological factors, inflammation, and obesity provide a possible explanation of the cause of the metabolic syndrome until now which designates the energetic relationship between a number of contributing factors.

This review determines different issues related to the metabolic syndrome which needs additional research for an explanation. Firstly need to improve strategies to achieve and sustain long-term weight reduction and increased physical activity. Moreover, a lack of understanding of the genetic and metabolic contributions to the causation of the syndrome stands in the way of developing new therapeutic approaches. The need exists, therefore, for additional basic and clinical research designed to better understand pathophysiology from the standpoint of genetics, molecular biology, and cellular signaling.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

PREVALENCE, ANTIBIOGRAM AND MOLECULAR CHARACTERIZATION OF *Aeromonas hydrophila* ISOLATED FROM FROZEN FISH MARKETED IN EGYPT

Abd-Elsalam E. Hafez, Wageh S. Darwish*, Rasha M. Elbayomi, Mohamed A.M. Hussein, Sara M. El Nahal

Food Control Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt

*Corresponding author, E-mail: wagehdarwish@zu.edu.eg

Abstract: Fish is one of the most important foods because of its high nutritional value, high palatability and easy digestion. On the same time, it acts as a vehicle for many types of pathogenic microorganisms especially Aeromonas spp., which results in public health hazards. Therefore, the present study was conducted to evaluate the prevalence of Aeromonas spp. in frozen fish (mackerel, herrings and fish fillets) marketed in Zagazig city, Sharkia Governorate, Egypt. In addition, multiplex PCR was done to detect some virulence-associated genes in A. hydrophila isolates. Furthermore, antimicrobial susceptibility testing of A. hydrophila isolates to the commonly used antimicrobials in Egypt including cephalothin, ampicillin, chloramphenicol, sulphamethoxazol, oxytetracycline, cloxacillin, gentamicin, kanamycin, amikacin, ciprofloxacin, cefotaxime, erythromycin, streptomycin and neomycin was conducted using the disc diffusion method. The achieved results indicated contamination of frozen fish with different species of Aeromonas such as A. veronii, A. sobria, A. caviae and A. hydrophila. A. veronii was the predominant species isolated from the examined fish; its prevalence rates in mackerel, fish fillets and herrings were 62.5, 50 and 45%, respectively. A. sobria came second; it was isolated only from herrings (30%) and fish fillets (16.7%). The prevalence rates of A. hydrophila in mackerel, fillets and herrings were 12.5, 33.3 and 10%, respectively; while A. caviae was isolated only from mackerel (25%) and herrings (15%). The isolated A. hydrophila harbored some virulence attributes such as aerolysin (aerA) and haemolysin (ahh1). A. hydrophila isolates were resistant to different antimicrobial agents used in Egypt including cloxacillin, erythromycin and streptomycin (100% each); cefotaxime and sulphamethoxazol (80% each); and cephalothin, chloramphenicoland oxytetracycline (60% each); while it was sensitive to ampicillin (80%) and gentamicin (60%). Therefore, hygienic measures should be adopted to control the microbial contamination either in the aquatic environment or in fish markets.

Key words: fish; *A. hydrophila*; virulence genes; antibiotic sensitivity

Introduction

Fish is a healthy food, it plays an important role in human nutrition providing one third of the world's population by at least 20% of their

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protein intake (1). Fish protein is also a rich source of the omega-3 polyunsaturated fatty acids. In addition, fish contains some micronutrients as vitamin D, minerals (magnesium, calcium, iodine, zinc, and selenium) which provide a role in the deficiency eradication of human-related micronutrient diseases (2). Mesophilic *Aeromonas* spp., is an important fish pathogen that causes significant problems in aquaculture and leads to fish spoilage and food-borne disease (3). *Aeromonas* spp. is opportunistic microorganism, one of the normal microbial flora of many aquatic animals and widely distributed in the aquatic environments, including fresh, marine, and ground water. It is considered as a primary pathogen of fish and can be isolated from healthy or diseased ones (4). *Aeromonas* spp. is responsible for large economic losses because of high fish mortalities (5). Many *Aeromonas* spp. have been identified in fish diseases, including *A. hydrophila, A. allosaccharophila, A. salmonicida* and *A. veronii* biovar *sobria* (6). In human, motile *Aeromonas* spp. can cause food-borne gastroenteritis in addition to extra intestinal symptoms (septicemia, wound infections, meningitis, endocarditis and osteomyelitis) with a high mortality rate in immune-compromised persons (7).

The pathogenicity of *Aeromonas* spp. is attributed to the release of various virulent attributes, including enterotoxin, hemolysin, and adhesion-related factors which contribute to the adhesion of the bacteria into the host gastrointestinal tract and responsible for hemolysis and enterotoxin production; meanwhile, extracellular hemolysin and aerolysin potentially contribute to the occurrence of septicemia (8,9). Antimicrobial resistance in *Aeromonas* spp. is usually chromosomally mediated, but β-lactamases produced by aeromonads may occasionally be encoded by plasmids or integrons (6). However, the antibiogram of *Aeromonas* spp. isolated from fish in Egypt is less informed.

In developing countries, shortage and high price of animal proteins is the principle cause of the high demand on fish; so many trials have been developed by National Authorities during the last years in order to improve the fish quality**.**

There is little information about the prevalence of *Aeromonas* spp. in fish marketed in Egypt. Therefore, this study was conducted to detect the prevalence of *Aeromonas* spp. in different types of frozen fish retailed in fish markets in Zagazig city, Sharkia Governorate, Egypt. Due to the significant roles of *A. hydrophila* as food-borne pathogen, characterization of its virulence attributes and antibiotic resistance profile were further studied.

Material and methods

Collection of fish samples

A total of one hundred and fifty frozen fish samples of mackerel, herring and fish fillets (50 of each) were randomly collected from different fish markets at Zagazig city, Sharkia Governorate, Egypt. The collected samples were identified and packaged separately in a sterile plastic bag; then transported in cooled aseptic conditions without any delay to the Laboratory of Meat Hygiene, Faculty of Veterinary Medicine, Zagazig University, Egypt for bacterial isolation and identification.

Preparation of fish samples

After removal of the dorsal, pectoral and ventral fins by sterile scissors and forceps, the scales were removed from the body surface everywhere by a sterile scalpel. The surface was sterilized by a hot spatula and was removed. About 25 grams of the back muscles were aseptically homogenized with 225 ml of sterile 0.1% alkaline peptone water for 2.5 min and then were allowed to stand for 5 min (10).

Bacterial isolation

From the prepared homogenate, 1 ml was transferred into a sterile test tube containing 0.1% alkaline peptone water as an enrichment broth and incubated at 37°C for 18 h. After incubation, a loopful of the alkaline peptone water was streaked on the surface of Aeromonas agar medium (Himedia, Mumbai, India) and incubated at 37°C for 18-24 h (11). Typical colonies (Pale green with dark centers, their size is typically

between 0.5 and 1.5-mm) were sub-cultured on a non-selective medium (Nutrient agar, Himedia, Mumbai, India), and incubated for 24 h.

Primary characterization and identification of the isolates

Pure cultures of the isolates were morphologically, biochemically and physiologically identified (12). Morphological characters including shape, size, Gram staining, and motility of the isolated Aeromonas were confirmed (12,13). Biochemical identification was done using the following tests; oxidase, esculin hydrolysis, arginine hydrolysis, indole, methyl red, Voges–Proskauer test, citrate utilization, urease, hydrogen sulphide production, nitrate reduction, gelatin liquefaction, oxidation-fermentation, pigment formation, sugars fermentation and detection of ornithine decarboxylase, L- lysine decarboxylase, arginine decarboxylase and β- galactosidase (14). Biochemical reagents used were from Himedia, Mumbai, India. Physiological properties were investigated by observing the growth of each isolate at various temperatures (4, 24, 37 and 40°C) and different NaCl concentrations (0, 1, 2, 3 and 4%) (12,15).

Detection of virulence genes of A. hydrophila by multiplex PCR

The extraction of the bacterial DNA was carried using QIA amp kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Application of PCR for identification of *16SrRNA*, *aerolysin (aerA)* and haemolysin *(ahh1)* virulence genes of *A. hydrophila* was performed by using primers purchased from Pharmacia Biotech, Sweden. The 16SrRNA primers were sense 5′GGG AGT GCC TTC GGG AAT CAG A′3 and antisense 5′TCA CCG CAA CAT TCT GAT TTG′3 with a product size of 356 bp. while the primers used for detection of *aerA* were sense 5′ CAA GAA CAA GTT CAA GTG GCC A ′3 and antisense 5′ACG AAG GTG TGG TTC CAG T′3 with a product size of 309 bp. The primers used to detect *ahh1* were sense 5′GCC GAG CGC CCA GAA GGT GAG TT′3 and antisense 5′GAG CGG CTG GAT GCG GTT GT′3 with a product size of 130 bp (16).

The amplification reaction was performed on a thermal cycler (Master cycler, Eppendorf, Hamburg, Germany). The PCR reaction (10 µl) consisted of bacterial DNA (2µl), 10X EX Taq buffer (1µl), forward primer (1µl), reverse primer (1µl), 2.5 mM dntp (0.8 µl), EX Taq polymerase (TaKaRa, Japan) (0.05µl) and nuclease free water (4.15µl) (17). Amplification consisted of an initial denaturation at 95°C for 5 min, 50 cycles at 95°C for 30 sec., 59°C for 30 sec., 72°C for 30 sec., followed by final elongation at 72°C for 7 min. Amplified DNA fragments were analyzed by 2% of agarose gel electrophoresis (Applichem, Germany, GmbH). Finally, the gel was stained with ethidium bromide and captured as well as visualized on UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes (18).

Antibiotic resistance of the isolated A. hydrophila

Antimicrobial susceptibility of the isolated *A. hydrophila* strains was tested by Kirby-Bauer disc diffusion method (19) using the antibiotic discs *(*Oxoid Limited, Basingstoke, Hampshire, UK*)* with variable concentrations (µg) including cephalothin (CN, 30µg), ampicillin (AM, 10µg), chloramphenicol (C, 30µg), sulphamethoxazol (SXT, 25µg), oxytetracycline (T, 30µg), cloxacillin (Cl, 5µg), gentamicin (G, 10µg), kanamycin (K, 30µg), amikacin (AK, 30µg), ciprofloxacin (CIP, 5µg), cefotaxime (CF, 30µg), erythromycin (E, 15µg), streptomycin (S, 10 µg) and neomycin (N, 30µg). Interpretation of the results was carried out according to the guidelines stipulated by National Committee for Clinical Laboratory Standards (NCCLS) (20).

The tested isolates were evaluated as susceptible, intermediate and resistant and multiple antibiotic resistances (MAR) index for each strain was determined; MAR index = No. of resistance (isolates classified as intermediate were considered sensitive for MAR index) / Total No. of tested antibiotics (21).

Results

Prevalence of Aeromonas spp. in frozen fish

The prevalence of *Aeromonas* spp. in the examined mackerel, fish fillets and herrings was recorded in Table 1. Out of 150 fish samples analyzed, 68 (45.3%) were found to be contaminated with *Aeromonas spp*. High level of contamination by *Aeromonas* spp. was found in the herring fish (40/50; 80%), while the prevalence in mackerel and fish fillet was 32% (16/50) and 24% (12/50), respectively.

Identification of Aeromonas spp. isolated from the examined fish

Morphological and biochemical properties of the isolated species showed in Table (2) and revealed that the examined frozen fish samples were contaminated by *A. hydrophila*, *A. sobria*, *A. caviae* and *A. veronii*. Of the 68 identified isolates; *A. veronii* (34/68, 50%) was the predominant species isolated from the examined fish; its prevalence rates in mackerel, fish fillets and herrings were 62.5, 50 and 45%, respectively. *A. sobria* came second with 14 (20.6%) isolates; it was isolated only from herrings (30%) and fish fillets (16.7%). Ten isolates were identified as *A. hydrophila* and *A. caviae* (each). The prevalence rates of *A. hydrophila* in mackerel, fillets and herrings were 12.5, 33.3 and 10% respectively; while *A. caviae* was isolated only from mackerel (25%) and herrings (15%) (Table 1).

Table 1: Prevalence of *Aeromonas* spp in the examined frozen fish samples

Fish samples (No=50, each)	Positive samples*	*A. hydrophila***	*A. sobria***	*A. caviae***	*A. veronii***
Mackerel	16 (32)	2 (12.5)	0	4 (25)	10 (62.5)
Fillet	12 (24)	4 (33.3)	2 (16.7)	0	6 (50)
Herrings	40 (80)	4 (10)	12 (30)	6 (15)	18 (45)
Total	68 (45.3)	10 (14.7)	14 (20.6)	10 (14.7)	34 (50)

No: Number of examined fish samples. Data were represented by number (%)
*Percentages were calculated according to number of examined samples
**Percentages were calculated according to number of positive samples

Table 2: Morphological and biochemical characteristics of the isolated *Aeromonas* spp. from frozen fish

Test	*A. hydrophila*	*A. sobria*	*A. caviae*	*A. veronii*
Motility	+	+	+	+
Esculine hydrolysis	+	-	+	-
Oxidase	+	+	+	+
Arginine dihydrolase	+	+	+	+
Indole	+	+	+	+
Methyle red	+	-	-	V
Voges Proskuaer	+	-	-	-
Citrate utilization	V	+	+	+
Urease	-	-	-	-
H2S	+	+	-	V
Nitrate reduction	+	+	-	V
Gelatin liquefaction	+	+	+	+
Glucose fermentation (F)	+	+	+	+
Sucrose (F)	+	+	+	+
Rhamnose (F)	+	-	-	-
Mannose (F)	+	+	V	V
Arabinose (F)	+	-	+	V
Inositol (F)	-	-	V	-
Sorbitol (F)	-	-	-	-

F: Fermentation; (+): Positive response; (-): Negative response; V: variable in reaction

Molecular characterization of A. hydrophila using multiplex PCR

In the current study, a multiplex PCR was used as a reliable DNA based technique for detection of virulence associated genes in the isolated bacteria. It was found that 6 out of 10 (60%) of *A. hydrophila* isolated from the examined fish samples harbored *16S rRNA*, aerolysin *(aerA)* and haemolysin *(ahh1)* genes; while 2 (20%) harbored *16S rRNA* and aerolysin *(aerA)*; and 2 (20%) harbored *16S rRNA* and hemolysin *(ahh1)* genes (Table 3).

Antimicrobial susceptibility of A. hydrophila

Results achieved from the disc diffusion test in table 4 revealed that *A. hydrophila* isolates (n=10) were resistant to different antimicrobial agents used in Egypt including cloxacillin, erythromycin and streptomycin (100% each); cefotaxime and sulphamethoxazol (80% each); and cephalothin, chloramphenicoland oxytetracycline (60% each); while it was sensitive to ampicillin (80%) and gentamicin (60%).

The average MAR value was 0.614 for all *A. hydrophila* isolates (Table 3). Antimicrobial resistance varied among the obtained isolates. For instances, the two strains of *A. hydrophila* isolated from herrings, harbored *16S rRNA*, *aerA* and *ahh1* genes, differed in their antibiotic susceptibilities. The first strain was resistant to all tested antibiotics with MAR index 1; however, the other strain was resistant to cephalothin, chloramphenicol, sulphamethoxazol, oxytetracycline, cloxacillin, amikacin, cefotaxime, erythromycin, and streptomycin with MAR index value of 0.643 and sensitive to ampicillin, gentamicin, kanamycin, ciprofloxacin and neomycin.

While *A. hydrophila* isolated from fish fillets, harbored 16S *rRNA* and *aerA*, were resistant to cephalothin, chloramphenicol, sulphametho-xazol, oxytetracycline, cloxacillin, amikacin, cefotaxime, erythromycin, streptomycin, kanamycin, ciprofloxacin and neomycin with MAR index value of 0.857, and sensitive to ampicillin and gentamicin. The other strain that harbored *16S rRNA, aerA* and *ahh1* had a MAR index value of 0.357. It was resistant tostreptomycin, cloxacillin, erythromycin, sulphamethoxazol and cefotaxime; but sensitive to chloramphenicol, oxytetracycline, cephalothin, amikacin, kanamycin, ciprofloxacin, neomycin, gentamicin and ampicillin (Table 4). *A. hydrophila* isolated from mackerel fish that harbored *16S rRNA* and *ahh1* was resistant to only 3 of the tested antibiotics (streptomycin, cloxacillin and erythromycin) with MAR index value of 0.214; while sensitive to sulphamethoxazol, cefotaxime, chloramphenicol, oxytetracycline, cephalothin, amikacin, kanamycin, ciprofloxacin, neomycin, gentamicin and ampicillin (Tables 3 and 4).

Table 3: Virulence genes and antimicrobial resistance profile of selected 5 isolates of *A. hydrophila* of frozen fish

***A. hydrophila* source**	**Virulence genes**	**Antimicrobial resistance**	**MAR**
Herrings (No=1)	16S rRNA, *aer*A and *ahh*1	S, Cl, E, SXT, CF, C, T, CN, AK, K, CP, N, G,AM	1.000
Fillet (No=1)	16S rRNA and *aer*A	S, Cl, E, SXT, CF, C, T, CN, AK, K, CP, N	0.857
Herrings (No=1)	16S rRNA, *aer*A and *ahh*1	S, Cl, E, SXT, CF, C, T, CN, AK	0.643
Fillet (No=1)	16S rRNA, *aer*A and *ahh*1	S, Cl, E, SXT, CF	0.357
Mackerel (No=1)	16SrRNA and ahh1	S, Cl, E	0.214

n: Number of examined samples; MAR: Multiple antibiotic resistances. S: Streptomycin; Cl: Cloxacillin; E: Erythromycin; CF: Cefotaxime; C: Chloramphenicol: Oxytetracycline; AK: Amikacin; K: Kanamycin; CP: Ciprofloxacin; G: Gentamicin; AM: Ampicillin; SXT: Sulphamethoxazol; CN: Cephalothin; N: Neomycin; *aerA*: aerolysin gene and *ahhl*: haemolysin gene.

Table 4: Percentages of antimicrobial susceptibility of *A. hydrophila* (No=10)

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
S	0	0	0	0	10	100
Cl	0	0	0	0	10	100
E	0	0	0	0	10	100
SXT	0	0	2	20	8	80
CF	2	20	0	0	8	80
C	4	40	0	0	6	60
T	2	20	2	20	6	60
CN	4	40	0	0	6	60
AK	4	40	0	0	6	60
K	0	0	6	60	4	40
CP	2	20	4	40	4	40
N	4	40	2	20	4	40
G	6	60	2	20	2	20
AM	8	80	0	0	2	20

No: Number of examined samples. %: Percentage of positive samples. S: Streptomycin; Cl: Cloxacillin; E: Erythromycin; CF: Cefotaxime; C: Chloramphenicol: Oxytetracycline; AK: Amikacin; K: Kanamycin; CP: Ciprofloxacin; G: Gentamicin; AM: Ampicillin; SXT: Sulphamethoxazol; CN: Cephalothin; and N: Neomycin

Discussion

Fish flesh is an excellent substrate for growth of large number of bacteria with compositional attributes, which affect the bacterial growth and the related biochemical activities (22). Various sources are responsible for microbial contamination of fish such as water, soil and fish handlers. Bad handling of fish during fishing, transporting, freezing and shipping plays an important role in cross-contamination of fish from the surrounding environment and can act as a stress factor which results in bacterial migration from the gut to fish muscles. Some of these bacteria are associated with many diseases in humans, making the aquaculture products a potential risk factor for customer's health (23). *Aeromonas* spp. is considered as one of the emerging food-borne pathogens (24).

The present study examined the occurrence of Aeromonas spp. in three different types of frozen fish (mackerel, herrings and fish fillets) consumed in Egypt. High contamination (45.3%) of the examined fish by Aeromonas spp. was detected. Herrings were the most contaminated (80%), followed by mackerel (32%) and fish fillets (24%). This result indicated that the examined frozen fishes were subjected to contamination during storage, transportation and marketing. Leaving fish at room temperature in fish markets is a favorable condition for growth of Aeromonas spp. In addition, contaminated equipment and unhygienic standards of fish handlers during deboning process of fish fillets can increase the bacterial load of such products.

These findings were in agreement with Aeromonas-related studies conducted in other countries. For instances, in Malaysia, *Aeromonas* spp. was isolated from 69% of fish samples (25); while in Turkey, it was isolated from 82.8% of the examined seawater fish (26). Generally, the studies on prevalence of *Aeromonas* spp. in fish focused on 3 species, *A. hydrophila*, *A. sobria* and *A. caviae* (27). In the current study, identification of the isolated *Aeromonas* spp. from the examined fish samples revealed four different species; namely *A. hydrophila*, *A. sobria*, *A. caviae* and A. veronii. The most predominant species was A. veronii (50%) followed by *A. sobria* (20.6%) then *A. hydrophila* and *A. caviae* (14.7% each).

This result indicated the wide spreading of motile *Aeromonas* spp. in the aquatic environment; consequently fish can be easily contaminated by these microorganisms after exposure to stress as a result of rough handling during fishing and overcrowding in fish boxes. In addition, cross contamination after fish freezing and during transportation to fish

markets can increase the contamination by pathogenic motile Aeromonas spp.

In agreement with the current report, a study conducted in Serbia found that *A. hydrophila* and *A. sobria* were isolated from 66.7% and 33.3% of the examined fish, respectively (27), while in Malaysia, *A. caviae* was the predominant species isolated from fish samples (47.1%) followed by *A. hydrophila, A. sobria* and *A.veronii* (25). In India, *A. sobria* was the most common species isolated from apparently healthy fish (9,28). In China, *A. veronii* was the dominant *Aeromonas* spp. (29). Variation in the prevalence of *Aeromonas* spp. in mackerel, fish fillets and herrings could be attributed to the differences in the contamination levels during handling, transportation and storage.

Aeromonas spp. is recognized as an emerging pathogen that has the ability to cause various diseases in humans including food poisoning, gastroenteritis, septicemia, skin affections, soft-tissues and muscles infection (30). *A. hydrophila, A. sobria,* and *A. caviae* are the main causes of Aeromonas associated human diseases (31). Such microorganisms had been linked to an outbreak of Aeromonas infection due to ingestion of raw fermented fish (32). Identification of *A. hydrophila* using conventional methods remains difficult. Therefore, the multiplex PCR was used in the current study. In *A. hydrophila*, as with all pathogenic microorganisms, disease occurs because of complex molecular interactions between bacteria, host and environment (31). Virulence in *A. hydrophila* is a multifactorial due to the production of several virulence factors, such as cytotoxins, adhesins, hemolysins, proteases and lipases, as well as the ability to form biofilms by using a specific metabolic pathway and a mediate virulence factor expression (6). Enterotoxins, cytotoxins and hemolysins, are more frequently detected in isolates obtained from patients with gastrointestinal symptoms; there was a relationship between hemolysin production and human illness caused by motile *Aeromonas* spp. (33).

In the present study, selected five isolates of *A. hydrophila* were specific for 16S rRNA gene; while four isolates were producers to hemolytic toxins such as aerolysin (*aer*A) and haemolysin (*ahhl*). This result agreed with published reports in Canada, Turkey and India, where, ahh1 and aerolysin were detected (18). Hemolytic toxins were also detected in 82% of *A. hydrophila* (25); and hemolysin gene was detected in 78% of *A. hydrophila* isolates (34). Additionally, *ahh1* and *aer*A were expressed in 60.52% and 13.15%, respectively of *Aeromonas* spp. (35).

The 16S rRNA gene is an excellent and rapid way to assess the identity of *A. hydrophila*. It is complicating factor because the bacteria contain up to 15 copies of this ribosomal operon (36). It has been used for molecular identification of species by restriction fragment length polymorphism (37) or direct gene sequencing (38). Hemolysin is a group of multifunctional enzymes, which play important roles in the pathogenicity of *A. hydrophila*. Hemolysins include *aer*A, *ahh1*, *ahy*A, and *asa1*; *ahh1* is the most widely distributed extracellular heat-labile hemolysin, the synergistic combination of *aer*A and *ahh1*is the most cytotoxic genotype (18).

Other virulence factors encoded by *A. hydrophila* include adherence proteins, catalysts, nucleases, and toxins that may be expressed differently. Adherence proteins are responsible for mucosal adherence, biofilms formation, cell division and motility (39). These virulence-associated factors are very important in distinguishing between pathogenic and non-pathogenic strains. *A. hydrophila* enterotoxin, which is cytotoxic in nature, is the main cause of gastroenteritis, while aerolysin is the principle virulence-associated factor implicated in various intestinal disorders (34).

Antibiotics are widely used in fish farms for prevention and control of bacterial diseases. Using a wide variety of antimicrobial agents, aquaculture had been implicated in the development of resistant bacteria and a source of transmission of these resistant pathogens to other animals and humans (40). In addition, application of the same antibiotics in different fields including veterinary and human medicine facilitates the appearance of the microbial drug-resistance phenomenon.

In the present investigation, *A. hydrophila* showed variable degrees of resistance to the fourteen antimicrobial tested agents. The isolates were 100% resistant to streptomycin, cloxacillin and erythromycin; 80% of the isolates were resistant to sulphamethoxazol and cefotaxime; 60% were resistant to oxytetracycline, cephalothin and amikacin; 40% were resistant to kanamycin, ciprofloxacin and neomycin and 20% were resistant to gentamicin and ampicillin. In agreement with the current study similar resistance profiles were reported in Bangladesh, India and Egypt (34,41,42). However, higher resistance percentages were recorded in Turkey to gentamicin, ampicillin and cephalothin; while, low resistance rates were recorded to streptomycin (18.1%), erythromycin (54.1%), sulphamethoxazol and cefotaxime (63%) (25). In another report, resistance to sulphamethoxazol and cefotaxime was 67% (43). These differences in the antimicrobial resistance profiles may be related to the differences in fish species and the type of the used antimicrobials.

Conclusion

The results achieved in the present study revealed high contamination of mackerel, herrings and fish fillets by *Aeromonas* spp. The isolates of *A. hydrophila* harbored virulence-associated genes in addition to their resistance to different types of antibiotics. This might be due to the contamination of the aquatic environment by such pathogens as well as bad handling of fish. Such microorganisms may lead to several public health implications, especially, if such contaminated fish consumed. Therefore, hygienic measures should be adopted to control the microbial contamination either in the aquatic environment or in fish markets.

Conflict of interest

The authors declare that there is no conflict of interest

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Original Research Article

DETERMINATION OF MINOCYCLINE RESIDUES IN CHICKENS USING HPLC

Gamal El-Din A. Shams[1], Sohair A. Abd El-Lateef[1], Nahla S. Elshater[2], Samah R. Ebrahim[1]*

[1]Pharmacology Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig, 44511, [2] Reference Lab for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt

*Corresponding author,E-mail: samahroshdy11@gmail.com

Abstract: Residues of veterinary drugs in poultry meat have serious health effects on humans (e.g., increase antimicrobial resistance, carcinogenicity, mutagenicity and hypersensitivity) which make the control of veterinary drug residues an important parameter in ensuring consumer protection. This work was performed to quantitatively determine minocycline residues in different tissues of chickens (kidneys, liver, muscles and fat) and sera following multiple oral doses of the drug using High Performance Liquid Chromatography (HPLC). Moreover, the study aimed to estimate the withdrawal time of this drug in chicken tissues. Twenty five healthy chicks (Cobb 500) were used. Twenty one chickens were given minocycline directly into the stomach at a dosage of 7 mg/kg body weight once daily for five successive days. Samples were analyzed at 1st, 3rd, 7th, 14th, 21st and 28th day after last oral dose. The results indicated a widespread distribution of minocycline in the tissue samples, which remained within the detectable limit till the 3rd day (49.20-135.20 µg/kg) in all tested tissues. While in kidneys, minocycline remained till the 7th day (11.80µg/kg) following the last oral administration of the drug. Therefore, it is recommended to pay attention to the proper withdrawal periods before marketing to ensure the hygienic suitability of broilers edibles for safe human consumption.

Key words: minocycline; residues; chickens; HPLC

Introduction

In Egypt, broad spectrum tetracyclines are the most common used antibiotics in poultry husbandry. They are used for therapeutic and prophylactic purposes against both aerobic and anaerobic bacteria and some protozoa due to their relatively low cost, easily accessible and availability. They can be administered either in feed, in drinking water or by injection. Tetracyclines include a wide number of various types of compounds as oxytetracycline, chlortetracycline, doxycycline and minocycline (1). About 60% of an ingested dose of oxytetracycline is absorbed from the gastro-intestinal tract (GIT) and widely distributed in the body, particularly to liver, kidney, muscles, bones and teeth (2).

Minocycline is an effective semi-synthetic, second-generation tetracycline that was used for treatment of certain sexually transmitted diseases (3), comparable with oxytetracycline, it is more active against sensitive micro-organisms especially Staphylococci, *Mycopla-*

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sma pneumonia and Chlamydia (4). This may be due to its highly infiltration into the microbial cells (5) or its higher lipid solubility which leads to improved antimicrobial activity and antibiotic absorption and enhanced its distribution in the body (6).

The potential harmful human health effects presented by the presence of antimicrobial residues or their metabolites in poultry meat may be due to intensive uncontrolled use of antimicrobials in the poultry industry especially if the withdrawal times are neglected (7). To ensure human safety, World Health Organization/Food Agriculture Organization (WHO/FAO) (8) set a tolerance or Maximum Residue Limits (MRLs) for tetracycline and their metabolites at a level of 200µg/kg in muscle, 600µg/kg in liver and 1200µg/kg in kidney. Hazard of some tetracyclines` residues is decreased by heat treatment but complete removal of these antibiotic residues in broiler meat could not be achieved (9).

The use of High Performance Liquid Chromatography (HPLC) is an effective, accurate, rapid and sensitive separation technique for the analysis of minocycline residues (identification, confirmation and quantification) in the serum and tissue (10), varying its efficiency according to chromatographic condition and type of detector used (11). Different detectors have been described for analysis of minocycline residues in chicken tissues as ultraviolet (UV), diode- array detection (DAD), fluorescence (FLD) and mass spectrometry (12). Therefore, this study was planned in Egypt to determine minocycline residues and their withdrawal times from different chicken tissue parts (liver, kidneys, muscles and fat) and sera after administration of last oral drug dose (7mg/ kg BW) using HPLC technique.

Material and methods

Drug

Minocycline (Minocine) was supplied by Lederle Company for Medical Products, USA. It was prepared by dissolving the powder, bright yellow-orange amorphous solid, in normal saline.

Experimental design

The study was approved by the Committee of Animal Welfare and Research Ethics Faculty of Veterinary Medicine, Zagazig University, Egypt. A total of 25 apparently healthy live unsexed one-day old broiler chicks (Cobb 500) were acquired from Mansoura Poultry farm at Dakahlia Governorate. They were reared until 21 days old before the start of the experiment. They were fed on balanced commercial ration free from any medication, and the water was provided ad-libitum. They were kept under clean condition during the investigation period. The chicks were randomly allocated into two unequal groups; group 1 (negative control, n=4) was administered sterile normal saline and group 2 (n=21) was orally administered 7mg/kg BW minocycline directly into the mouth through a stomach tube once daily for 5 consecutive days. The dose was calculated according to Paget and Barnes (13). Five samples (liver, kidney, breast muscle, fat and serum) were collected from each slaughtered bird (n=3) at six withdrawal periods (1st, 3rd, 7th, 14th, 21st and 28th day) following the last oral medication dose.

Chemicals and reagents and apparatus used for HPLC analysis (14)

Chemical and reagents used throughout the study as Methanol HPLC, Acetonitrile HPLC trifluoroacetic acid and potassium hydroxide (Fisher Scientific Co, Fairlawn, NJ, UK); Triethylamine and phosphoric acid (Sigma-Aldrich Co, USA); Sodium hydroxide (Honeywell Co, Germany); Granular potassium dihydrogen phosphate monobasic (KH_2PO_4) (Sham Lab, Syria); Ammonium acetate (Fisher Scientific Co, Fairlawn, NJ, UK); Dithioerythritol, iodoacetamide, potassium chloride (Sigma Aldrich Co, UK); Calcium chloride (Merk Co, Darmstadt, Germany); Sodium borat e (Lobachemie PVT,Ltd., India); Deionized water (Milford, MA, USA) and McIlvaine buffer.

Agilent Series 1200 quaternary pump, Series 1200 auto sampler, Series 1200 UV Vis detector, Column (Agilent ZORBAX SB-C8 250mm × 4.6mm, 5µm), SPE vacuum manifold and solid-phase extraction (SPE) columns C18

(500 mg, 3 or 6 ml; Varian, Les Ulis, France) and nitrogen evaporator were used.

HPLC analysis

Preparation of the samples

Blood and tissue samples were collected from slaughter birds after 24 h from the last dose. Blood were gathered in sterile glass tubes and placed in a slant position for 20 min at 25°C to be coagulated then centrifuged at 3000 rpm for 10 minutes to obtain the serum which stored with chicken tissues (liver, kidney, breast muscle and fat) at -20 °C until used for HPLC investigation (14).

Extraction and determination of drug residues was performed as described previously (14)**.** Briefly, 5 g of homogenize sample were extracted with 20 ml of 0.1 mol/l Na_2EDTA-McIlvaine buffer solution (60.5g Na2EDTA. 2H2O (J.T. Baker, England) was added to 1625 mL McIlvaine buffer). The later was prepared by addition of 1000 ml of 0.1M citric acid (J.T. Baker, England) to 625 ml of 0.2 M disodium hydrogen phosphate (J.T. Baker, England), the reagents were mixed and PH adjusted to 4.0±0.05 with NaOH or HCl as needed. The extract was vortexed for 1 min followed by 10 min ultrasonic extraction in an ice bath, centrifugation was done at 3,000 rpm for 5 min in a cooling centrifuge (below 15 °C). The supernatant was filtered and the extract was saved in a clean tube. The extraction was repeated twice with 20 ml and 10 ml 0.1 mol/l Na_2EDTA-McIlvaine buffer solution, successively. The supernatant (50 ml in 0.1 mol/l Na_2EDTA-McIlvaine buffer solution) was collected, centrifuged at 4,000 rpm for 10 min (below 15 °C) and filtered with fast filter paper.

Solid phase extraction

Ten mL extract (equivalent to a 1g sample) was passed through the SampliQ OPT cartridge (Varian, Les Ulis, France) at a speed of 1 ml/min which was previously conditioned with 5ml of methanol HPLC (Fisher Scientific Co, Fairlawn, NJ, UK) then 5ml of a 10mmol/l trifluoroacetic acid (TFA) solution. (Fisher Scientific Co, Fairlawn, NJ, UK). After the sample effusion, the cartridge was washed with 3 ml of water (pH adjusted to 4.5 with TFA), the entire effluent was discarded and the cartridge was dried under negative pressure below 2.0 kPa for 3 minutes. Finally, minocycline was eluted with 10ml of 10mmol/l oxalic acid in methanol, collected and dried under nitrogen below 40°C. The resulting residue was dissolved and made to a constant volume of 0.5ml using the methanol/10 mmol/l TFA solution (1/19) then filtered through a 0.45-µm filter membrane (p/n 5185-5836) and analyzed (14).

Liquid chromatography operating conditions

Flow rate, 1.5 ml/min; column (Agilent ZORBAX SB-C8 250mm × 4.6mm, 5µm) temperature, 30°C; injection volume, 100 µL and detector (Series 1200 UV Vis detector) wavelength, 350nm were adjusted for HPLC analysis. Moreover, the mobile phase; Methanol-acetonitrile-10 mmol/l TFA solution, gradient elution and its gradient is shown in Table (1).

Quantification of residues

Quantification of the antibiotic residues in the samples was obtained and calculated from the area under curves extrapolated automatically by the HPLC 2D Chemstation Software (Hewlett-Packard, Les Ulis, France).

Table 1: Composition of gradient mobile phase

Time (minutes)	Methanol %	Acetonitrile %	10 mmol TFA %
0	1	4	95
7.5	6	24	70
13.5	7	28	65
15	1	4	95

TFA: trifluoroacetic acid

Standard curve

A reference standard, minocycline powder, was obtained from Sigma-Aldrich Co, USA. The stock solution (1mg/ml) was prepared in methanol, kept in the freezer at –20 °C for 6 months in the dark. Intermediate standard solution (100µg/ml) was prepared from stock solution in methanol. Working solutions at concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 µg/g were prepared using the stock solution diluted with a mixture of methanol/10 mmol/l trifluoroacetic acid solution (1/19). The working solutions were prepared daily.

Spiked samples of minocycline

Minocycline standard prepared at concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 µg/gm and homogenized with 5 g of control chicks muscles (blank samples) then treated according to the abovementioned extraction procedure. The calibration curve was calculated by linear regression equation method as y = 0.10391546 x + 3.4880808 where y symbol indicated area under peak and x symbol indicated concentrations of minocycline.

Statistical analysis

Data was analyzed by using computerized SPSS (Statistical Package for Social Sciences) program version 25 (IBM Corp., Armonk, NY). Statistical evaluations of the results were done by using methods as mixed model analysis of variance (ANOVA) followed by Tukey test. Significant difference (Tukey's HSD) test as post hoc test was used. The level of significance was taken as $P < 0.01$.

Results

High performane liquid chromattography analysis recorded that the corresponding peak responses (area under peak) of minocycline standard concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5µg/gm as illustrated in Table (2) and Figure (1). Linearity existed witin range of 0.025 and 5µg/gm with a correlation coefficient (r^2=0.99982). The percentage recovery of spiked samples ranged from 95-98%. The limit of detection for minocycline was 0.0025µg/ml, while, LOQ (limit of quantification) was 0.01µg/ml.

Minocycline distribution in chicken tissues (liver, kidney, breast muscles and fat) and serum was represented in Table (3). The typical chromatogram of minocycline is shown in Figure (2). There was a widespread distribution of the minocycline in the tested tissues and serum. The mean concentration of minocycline residues were 139.6±1.4, 157.4±2.5, 89.2±2.2, 100.6±2.6µg/kg and 294.6±3.7 µg/kg at the 1st day after the last dosage in liver, kidney, breast muscles, fat and serum, respectively. In the 3rd day of slaughter, the residues minimized in all organs to be 73.4±1.3, 135.2±1.8, 63±1.4, 49.2±1.7and 68.4±1.2 µg/kg for liver, kidney, breast muscles, fat and serum respectively. Moreover, in the 7th day post treatment the residues disappear in all organs except kidney (11.80±0.97 µg/kg), while minocycline residues were not detected in all organs after the 7th day post treatment.

From the obtained results it was found that the highest minocycline residual level was detected in kidney followed by liver while the lowest level was recorded in muscle and fat samples. There was a highly significant difference ($P<0.01$) in detection levels of minocycline residues in serum and tissues (kidney and that of liver and muscle) and along the investigation periods. Our detectable levels of minocycline residues in kidney, liver and muscle samples were lower than the established MRL of WHO/FAO.

Table 2: The concentrations of minocycline spiked tissues (µg/gm) and their corresponding peak response automatically using HPLC

RT	Level	Amount (µg/gm)	Area
7.331	1	0.025	7.640
	2	0.050	9.386
	3	0.100	14.850
	4	0.250	32.359
	5	0.500	55.571
	6	1.000	101.140
	7	2.500	268.160
	8	5.000	521.680

RT: Retention Time.

Table 3: The concentrations of minocycline in tissues of slaughtered chickens at various intervals following treatment with 7 mg/kg BW once daily for 5 consecutive days (n=3) automatically using HPLC

Tissue	concentration (µg/kg) after minocycline administration Mean ± SE					
	1st day	3rd day	7th day	14th day	21st day	28th day
Liver	139.60±1.40^{c}	73.40±1.30^{f}	ND	ND	ND	ND
Kidney	157.40±2.50^{b}	135.20±1.80^{c}	11.80±0.97^{i}	ND	ND	ND
Breast muscle	89.20±2.20^{e}	63.00±1.40^{g}	ND	ND	ND	ND
Fat	100.60±2.60^{d}	49.20±1.70^{h}	ND	ND	ND	ND
Serum	294.60±3.70^{a}	68.40±1.20^{fg}	ND	ND	ND	ND

Means with different superscripts were statistically significant according to Tukey test.
ND: Not Detected

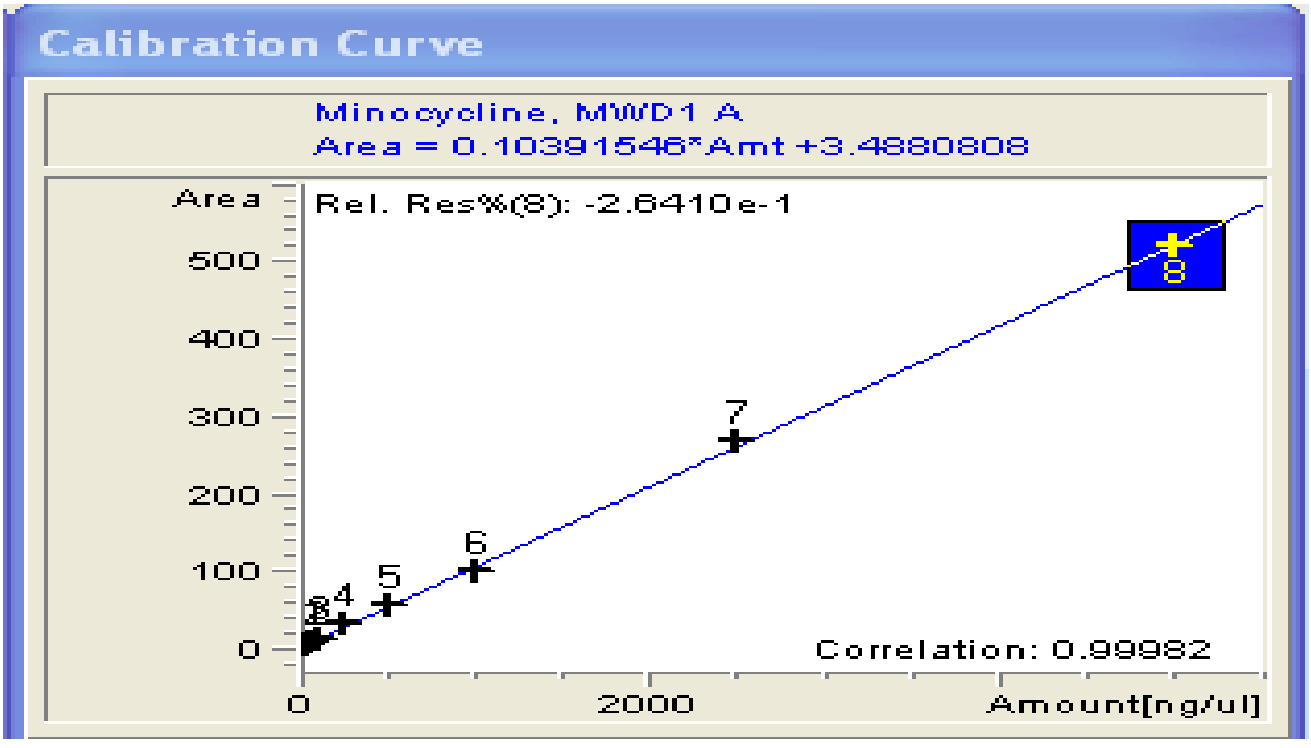

Figure 1: HPLC chromatograms standard curve of minocycline

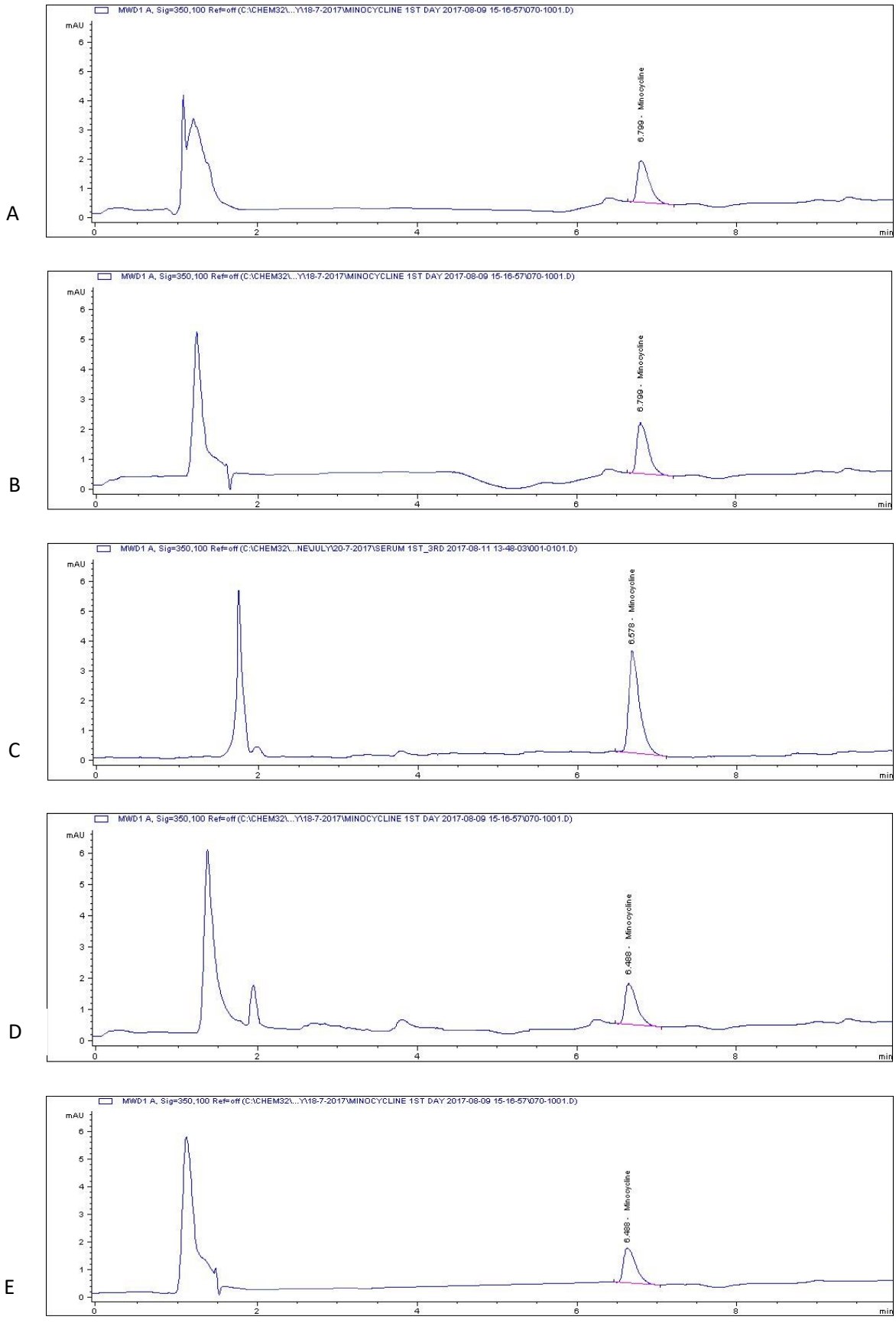

Figure 2: HPLC chromatograms of minocycline concentration in chicken samples (at 1st day following last oral dose (7 mg/kg BW). A: Liver; B: Kidney; C: Breast muscle; D: Fat and E: Serum

Discussion

Minocycline is a semi-synthetic, second generation tetracycline closely related to doxycycline, which could be used as an anti-microbial and growth-promoter in feed for livestock and poultry. This may be due to the positive control effect on intestinal microbial population, which is potentially fatal (15); its low poisonous quality or impact ahead against Gram-positive bacteria, Gram-negative bacteria and Mycoplasma (16). Minocycline is similar to doxycycline; rapidly and highly absorbed from GIT; has longer half-life (15-22 h); widely distributed through the body with the highest level in the kidney and liver; relatively high liposolubility (5 to 10 fold increase if compared to older tetracycline) which make it an effective antibacterial agent so its price in the Egyptian market is high compared with the tetracycline group (17).

It was clear from this study that the highest minocycline residual level was detected in kidney followed by liver while the lowest level was recorded in fat and muscle samples. Similar findings of tetracycline residues were reported in previous studies (18-20) in which higher tissue levels of doxycycline and tetracycline were reported in liver and kidney than muscle samples. The higher antibiotic residues in liver and kidney could be attributed to the role of liver and kidney in drug metabolism (detoxification, filtration and blood purification); unpaid attention to the withdrawal period because long-term administration of these antibiotic in feed, while the lower antibiotic residues in muscle due to most of the antibiotic are eliminated from the body via the kidney and bile (17).

In our study, we found that liver, kidney and muscle samples were lower than the MRL of WHO/FAO for tetracycline group. A several studies have been reported that levels of the tetracycline residues in muscle, liver and kidney (19-20) were exceeded above the recommended MRL of WHO/FAO in Egypt, also it was reported in many countries such as Mexico (21), Belgium (22), Saudi Arabia(23), Iran(18) and Pakistan(24).

From the obtained results it was found that minocycline remained in all tissue and serum till 3rd day post treatment but not detected in all organs or serum after this time except in kidney as the drug residues were detected at 7th day post drug treatment. The effective withdrawal period for doxycycline is 7 days to ensure that no harmful residues remain in food products after slaughter. The antibiotic concentrations in the edible tissues were detected to be below the MRLs (25) and did not enter the human food chain as reported previously (26). The possible human hazards related to antibiotic residues have been reported including; allergic/toxic reactions, chronic toxic effects (microbiological, carcinogenicity, reproductive and teratogenic effects) which may occur with prolonged exposure to low levels of antibiotic residues and development of antibiotic-resistant bacteria in treated animal (27)**.** So**,** the microbiological ADI of 3µg/kg of minocycline/kg BW×60kg (standard body weight of human) =180 µg/person was established as the overall ADI for minocycline, which can be ingested by human over a life time without appreciable risk (28).

Conclusion

The results confirmed the presence of minocycline residues in chicken samples. Higher level of minocycline residues usually observed in kidney and liver rather than in muscle due to their role in metabolism and excretion of antibiotics. This may pose potential hazard to public health. Thus, it is recommended that rules should be taken to ensure observing proper withdrawal periods before marketing and drug control in veterinary use. In addition, a monitoring policy should be implemented to ensure the conformity of poultry meat sold in Egypt with international standards.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Original Research Article

STEM CELLS IN VETERINARY MEDICINE – FROM BIOLOGY TO CLINIC

Gregor Majdic[1,2]

[1]Institute for Preclinical Sciences, Veterinary Faculty, University of Ljubljana, Ljubljana,
[2]Institute of Physiology, Medical Faculty, University of Maribor, Maribor, Slovenia

E-mail: majdic.gregor@vf.uni-lj.si

Abstract: Mesenchymal stem cells are adult stem cells found in different adult tissues such as adipose tissue, bone marrow, and also in umbilical blood. Mesenchymal stem cells are capable of differentiation into bone, cartilage and adipose tissue, and several reports also suggest that mesenchymal stem cells might be capable of transdifferentiation into muscle and neural cells. In addition to differentiation potential, mesenchymal stem cells might have other beneficial properties for pathological processes and studies in recent years suggest that mesenchymal stem cells have immunomodulatory, antiinflamatory and trophic actions, contributing to the healing processes in injured/ diseased tissues. Osteorthritis is a chronic, progressive disorder with debilitating effects in both animals and humans. It is particularly common in some dog breeds, but also fairly common in humans. Currently, there is no cure for such conditions, but studies in recent years in both human and veterinary medicine suggest that mesenchymal stem cells might have beneficial effect on chronic osteoarthritis. In our laboratory, we have developed a novel method of treating osteoarthritis using autologous adipose tissue derived mesenchymal stem cells in dogs and horses. Stem cells are collected from patients' adipose tissue, prepared in the laboratory and injected directly into affected joint(s). To prove the efficacy if this method, we have performed blind placebo study in dogs with bilateral osteoarthritis in knees, by treating one knee with stem cells and other knee with placebo (buffer used for cell delivery). Results of clinical examination revealed beneficial effect of stem cell treatment in osteoarthritic knees and x-ray imaging, and although with some limitations, results do suggest that degenerative processes in the knees treated with stem cells were limited or even reversed by the application of stem cells. Stem cells hold a great promise for the future of regenerative medicine, both veterinary and human, however, many questions about their use, potential and efficacy remain open and these will have to be studied and answered in the future years.

Key words: dog; horse; stem cells; veterinary medicine

Stem cells are special cells that have unique properties to divide indefinitely and are able to trans-differentiate into different tissues. Stem cells started to attract a lot of attention due to their possibility for use in regenerative medicine, both in animals and humans. Stem cells are usually divided into three groups:

- Embryonic stem cells, derived from early embryos that are capable of differentiating into majority of tissues

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- Adult stem cells that re present as resident cells in many adult tissues such as muscle, adipose, brain and many others and have limited potential for trans-differentiation
- Induced pluripotent stem cells (IPS cells), which are differentiated adult cells that are transformed into stem cells by genetic manipulations

Stem cells attract a lot of attention with the ideas to use these cells in regenerative medicine. In that respect, each of the three types of stem cells (embryonic, adult and IPS cells) have their own advantages and disadvantages, and there is still no clear answer which stem cells are the most suited for clinical use (1-3).

Embryonic stem cells are the cells with the higher differentiation potential. They could develop into any tissue or organ in the body and would, at a first glance, seem the best cells for use in regenerative medicine, as one cell could be used to treat many different disorders. However, embryonic stem cells have major disadvantages. One major disadvantage is collection of these cells. Cells can be only harvested from very young embryos (blasotcysts) and in human medicine, this present a large ethical dilemma whether working with such cells is ethical or not. Clearly, the ethical dilemma is not so important in the veterinary medicine although embryonic stem cells still have several disadvantages. Even if collection of embryonic stem cells in animals is not ethically questionable as with human stem cells, collection and culturing of these cells is challenging.

Furthermore, several studies have demonstrated that embryonic stem cells could form tumors *in vivo*, raising the question of safety of embryonic stem cells. Another important question in using embryonic stem cells is the potential immune response to such treatment. It would be certainly unpractical and too expensive (definitely in veterinary medicine) to prepare embryonic stem cells for individual patients (although there are ideas that in humans, cloned human embryos would be produced from patients needing stem cell treatment, and these cloned embryos could be used as a source of embryonic stem cells). Therefore, at least in veterinary medicine, embryonic stem cells from donors would have to be used and this does raise a question of rejection of foreign cells by the patient's bodies. Although some studies suggest that embryonic stem cells are immunoprivileged and do not elicit immune re-sponse in the recipient's organism, this is far from being clear and thus, safety concerns about immune reaction to transplanted stem cells remains.

IPS cells are derived from adult differentiated cells by genetic modifications. Four genes usually have to be activated to dedifferentiate adult cells into IPS cells and these are Oct4, Sox2, c-Myc and Klf4. Although this method of producing stem cells seems very promising, easy and would overcome the problem of immune rejection of cells as cells from patients could be transformed into IPS cells, there is an important concern about the safety of these cells. Namely, several of the genes need to be activated are actually oncogenes, and IPS cells do form teratomas when injected into mice. Therefore, there are a lot of ongoing studies trying to replace activation of aforementioned 4 genes with other methods such as temporal activation, transfection of proteins instead of activating genes, use of micro RNAs and others.

The third type of stem cells is adult stem cells. Adult stem cells are present in many organs/tissues and are thought to present a reservoir of cells that can be used in the event of tissue damage. For example, myoblasts, undifferentiated muscle cells, could be activated and develop into adult muscle cells after the damage to the muscles, and similarly, neural stem cells, found mostly in the hippocampus, along the ventricles and in subventricular zone, could be activated to produce new brain cells after damage in the central nervous tissue. Adult stem cells are very interesting for clinical medicine, both human and veterinary, as they are easy to obtain, they can be obtained from the patients and thus used for autologous treatments without any danger for tissue rejection. Studies in recent years have shown that adult stem cells do not have the potential to develop into tumors such as embryonic stem cells and IPS cells. However, on the other hand, adult stem cells have much more limited trans-differentiation

potential than embryonic stem cells. It is generally thought that adult stem cells could develop into the tissues of the same embryonic layer from which they arose initially, According to this theory; ectodermal cells would develop only into other ectodermal tissues, mesodermal cells to mesodermal tissues and endodermal cells into endodermal tissues. This is not completely confirmed yet, and several studies do suggest that cells might be able to cross this embryonic layer barrier.

Two types of adult stem cells that are mostly used in development of regenerative medicine in both human and veterinary medicine today are cells from bone marrow (hematopoietic cells) and adipose tissue derived stem cells. Both are classified as mesenchymal stem cells or MSC. In particular, adipose derived MSC are very promising in regenerative medicine as they are easy to obtain in large number from adipose tissue. Although characterization of these cells is somewhat difficult, as currently we do not know any really specific biological markers for these cells, there are currently three separate lines of experiments confirming that cells obtained from the adipose tissue are really MSC. These are multiple divisions of cells in cell culture, trans-differentiation into bone, cartilage or adipose tissue and expression of certain cell surface markers such as CD 29, CD 44, CD105, CD 73 and Oct 4, although expression of these markers is not completely specific only for MSC (4, 5). Adult MSC shows a lot of potential to be used for regenerative medicine, either for producing damaged tissues *in vitro* and then using this tissues for transplantation into patients, or using cells as a treatment. Many studies in recent years are suggesting that stem cells have the capability to promote regenerative healing processes in damaged/diseased tissues. Although in many cases, such studies are still in their early phases, there is quite a lot of evidence already present that cells could indeed promote the endogenous healing processes. Studies in laboratory mice and rats, veterinary patients and also in human patients have shown beneficial effects of adult stem cells, derived from different sources in orthopedics, cardiac problems (in particularly in humans with cardiac insufficiency, neurological trauma and even conditions like asthma. It is not yet clear what are the beneficial mechanisms through which adult stem cells induce healing, although accumulating evidence suggest that stem cells do not differentiate into damaged tissue but rather stimulate regenerative process, most likely by secreting certain, yet unknown, immunomodulatory factors and growth factors that modulate immune response /inflammation and promote endogenous healing processes.

Stem cells are attracting a lot of attention in the field of regenerative medicine. Although still in the early phases of development, stem cell treatments do show great promise for the future in both veterinary and human medicine. One of the areas where stem cells are already used is orthopedics, in both human and veterinary patients. One of the most common indications for stem cell treatments is osteoarthritis, a common disease in dogs that affects dogs of all ages. The incidence of osteoarthritis in general population of dogs is about 20 - 30 %, while in aging (older than 8 years) dogs, it can reach up to 80 %. Currently, osteoarthritis cannot be cured and dog owners are usually offered pain management. With proper pain management the quality of life of dogs is improved, although the disease cannot be cured and regular pain management therapy is both financial and life-style burden for dogs and their owners, and could have significant side effects (6). Several studies have suggested beneficial effect of adult mesenchymal stem cells in the treatment of osteoarthritis in different species from laboratory animals to dogs and horses (7-11), although there is still need for more clinical trials to confirm the benefit of MSC treatments in dog osteoarthritis.

We have performed a clinical trial in dogs with bilateral knee osteoarthritis (12). Ten privately owned dogs were included in the study with the consent of the owners. Dogs have received 2 – 3 millions of MSC into one knee and placebo (Phosphate buffer saline) in the other knee. Dogs were closely followed for one year. After one year, clinical improvement in limping was noted in 9 out of 10 dogs in stem cell treated knees. Interestingly, x-ray imaging did not reveal improvement in size of

osteophytes one year after treatment. However, while the disease progressed in all 10 joints receiving placebo, in 7 out of 10 joints treated with stem cells the radiographic score remained the same suggesting that stem cells did stall the progress of the osteoarthritis. This difference was statistically significant ($p<0,001$).

Our results, together with other similar clinical trials do show beneficial effect of stem cell treatment in dog osteoarthritis. However, it is not yet known, how these beneficial effects are achieved. Several studies, mostly in rodents, have trackedMSC after injection into diseased joints but most studies could not find evidence of cells engrafting into the cartilage or bone tissue (13-17), some engrafting cells are detected only in synovial membranes and menisci in rabbits and goats (13, 16). However, it has been shown that in rabbits, joints treated with MSC had reduced expression of TNF-alpha, an inflammatory cytokine, and reduced expression of matrix metalloproteinase 1(13). Similarly, studies in mice (18), goats (16) and horses (19) have shown anti-inflammatory effects of MSC in osteoarthritic joints. Therefore, it is currently thought that MSC affect in osteoarthritis might be more due to the anti-inflammatory effects, rather then regenerative capabilities. This is supported by our study as significant clinical improvement was observed in dogs on average 3 months after the treatment, but this was not accompanied by the improvement in the structure of the joints at the same time.

Beneficial effect of MSC treatment was prolonged and was evident by clinical examination at least a year after the treatment, and even more interestingly, the improvement of the cartilage appeared in some dogs 18 months after the treatment. We were not able to trace the cells in the dogs, as all dogs were clinical patients and not laboratory dogs, and therefore we could not use labeled cells for treatment. Therefore, we do not know if there was an engraftment of MSC in our study, although prolonged effect of MSC does suggest that stem cells must have engrafted into some tissues. However, this does not mean that stem cells had to engraft into the cartilage and differentiate into cartilage tissue, perhaps, as suggested by previous studies, MSC engraft only in soft tissues and secrete anti-inflammatory and trophic factors that contribute to the healing of the joint.

In addition to osteoarthritis, stem cells, in particularly mesenchymal stem cells are used in many other clinical trials/experimental treatments. Some of most promising areas are wound healing, neural trauma, cardiac and skeletal muscle disease/injuries, treatments of asthma and others. Although none of these treatments have been proven in large clinical trials, many smaller studies in both animals and humans have shown beneficial effects in various conditions. Stem cells have been shown to promote healing after myocardial infarction and tear of different muscles, in particular the semitendinosus muscle in dogs. MSC are also being studied in nerve damage, both in the spinal cord and in peripheral nerves, with very promising results using autologous porcine skin-derived mesenchymal stem cells(20). Furthermore, clinical trials are currently performed with corneal ulcers (21) and several studies, although none yet in veterinary patients, suggest an important beneficial effects in asthma (22).

Stem cells use in clinical veterinary medicine is very novel field and therefore, it is not surprising that evidence about beneficial effects are still weak. However, more and more studies, published in peer-reviewed journals do show important health benefits of stem cells in different conditions. At the moment, stem cells are most often used in treatments of osteoarthritis and tendon pathology in both dogs and horses, but large number of preclinical studies and clinical trials currently underway suggest, that the plethora of diseases treated with stem cells will most likely expand majorly in the near future.

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Original Research Article

RETROSPECTIVE REVIEW OF TRAMADOL ABUSE

Amany I. Ahmed[1]*, Khalifa El-Dawy[1], Medhat M. Fawzy[1], Haytham A. Abdallah[1,2], Heba N. Abd elsaid[1], Wessam O. Elmesslamy[1]

[1]Biochemistry Department, Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt,
[2]Biochemistry department, Faculty of Science, University of Jeddah, Saudi Arabia

*Corresponding author, E-mail: drmonmon_vet@yahoo.com

Abstract: In the Egyptian community, tramadol abuse is considered an increasingly alarming phenomenon. The popularity and massive use of tramadol especially among Egyptian youth contribute to alleged usages for treatment of premature ejaculation also for the extension of orgasm and also increase sexual pleasure as it available in many online drug media and stores. However, this abuser life on a blade of a knife and is susceptible to another sexual dysfunction, memory and learning disorder and different metabolic disorders. This literature consults the phenomenon of tramadol abuse and its relation to sexual function, memory learning disorder and other metabolic disorders. It could be concluded that the increase in the prevalence of tramadol HCl dependency over other substances in the Egyptian community, calls for paying more attention from family, health and educational institutes. Tramadol may apply a very useful intervention for treating premature ejaculation, but its abuse bad effect may supply a possible demonstration for the unexplained delayed fertility as well as behavioral and the associated psychological changes. Also it could involve malfunction of the cerebral cortex which includes deficits in memory and the reduction in cognitive function which appeared in chronic abusers of tramadol.

Key words: tramadol; abuse; apoptosis; oxidative stress; cognitive

Introduction

Tramadol is an unscheduled atypical artificial opioid analgesic that prompts μ-opioid as well as monoamine receptor systems. This has been proved for marketing as safe analgesic in Germany since 1977 also in Sweden and the United States since 1995. Tramadol has a fundamental expansive scope of purposes in acute pain in case of postoperative trauma and also in chronic pain, as pain killer in case of cancer; however, it may also have rewarding/enhancing effects (1). It believed to lead to the possibility of restricted abuse in contrast with another μ-opioid receptor agonists; however, lab information has suggested that it collaborate some of these other μ-opioid receptor agonists pharmacodynamic effects (2). Acute dosages of tramadol display a profile of outcomes comparable to those of opioid receptor agonists and may additionally impede abuse in populations (1). The rate of incidence for the tramadol abuse has been shown to be 69/1,000 individuals per year, and its annual rate of dependence is about 6.9/1,000 individuals (3). In the latest International Narcotics Control Board survey, of the 77 countries that responded on the issue of

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tramadol abuse, it appears to be problematic for 32 countries (4).

The relevance between tramadol and sexual function is dialectical. However, there is a proof that males with premature ejaculation (PE) can profit by the off-label use of tramadol (5). These patients are prone to other problems such as sexual dysfunctions, including desire disorder or erectile dysfunction (6), diminished sexual confidence and overall satisfaction on sexual relationship (7), secondary hypogonadism (8), drug tolerance (6) and dependency (9) as well as risky sexual behaviours.

There are some definitions for the hazardous use of analgesics are proposed according to two recent consensus statements (10), the associated commentaries of Butler (11) and Sullivan (12).

A. Misuse: Opioid use in opposition to the guide or specific pattern of utilization, in spite of the absence or presence of unfavourable outcomes or harm.

B. Abuse: tramadol or opioid used for other purposes nonmedical, such as altering one's state of consciousness or euphoria.

C. Addiction: A continues using of drug in spite of harm, and craving or in ability to stop using the drug despite of its harmful effect.

Epidemiology of tramadol HCl abuse

According to the data from Intercontinental Marketing Services (IMS) Kilochem, global tramadol consumption rate of tramadol from 2006 to 2012 increased from 290 tons to 424 tons (a 42% increase) (13). The International Narcotics Control Board (INCB) included data at 2013 annual record on international increasing and development in the nonmedical use and abuse, illicit manufacture and illicit home and global spread of tramadol. This record (14) confirmed that thirty three countries, about 42% of those responding, reported abuse and nonmedical use of tramadol, typically supplying narrative data. Abuse of tramadol (two thirds of which is oral-dosage-form abuse) has shown to be growing in twelve of the nations (38% reporting such abuse) and was stable in another thirteen nations (about 42%).

Also, five nations announced suffering from widespread risk of tramadol abuse, while unlawful deal was listed in a restricted number of countries. Tramadol abuse was previously reported to be a dangerous issue in Togo, Mauritius, Iran, Libya, Egypt, Lebanon, Jordan, Gaza and Saudi Arabia (4). Epidemiological reviews and surveillance research have reported that it's consuming, abuse liability, dependency, diversion and overdoses have increased, which lead a number of nations to set tramadol under national control (1). These nations consist of both developing and developed countries, such as Mauritius, Egypt, Bahrain, Venezuela, Sweden, Iran, Saudi Arabia, Ukraine, Nigeria, Brazil, China, Japan, Australia, Lithuania, Jordan, the United States and the United Kingdom. Otherwise when compared with morphine, tramadol is said to have low dependence and abuse potentials worldwide (13), and these low potentials have been proven both in preclinical studies (15) and through scientific observation.

It has been proved that higher (200 and 400 mg), but not lower doses (50 and 100mg), also chronic oral tramadol intake, even in the therapeutic concentrations (100 to 300 ng/ml) for a dose of tramadol which always prescribed in treatment of mild to moderate pain, may lead to physical dependence, boost its effects and dose-dependent opioid like withdrawal symptoms (16). Therefore, tramadol was listed as a controlled substance in many countries in the world. Recently in Egypt, although tramadol is not manufactured in the country, it has become a major health problem, despite the Egyptian Ministry of Health having listed it as a controlled substance in October 2002. Also a national survey of Substance Use Disorders in Egypt proved that tramadol is one of the most frequently abused drugs (17).

In Egypt, 20% to 40% of adults (18) and 83% of adolescents (19) with substance use disorders were found use tramadol. Recently, according to data from the Anti-Addiction Fund's hotline, tramadol considered as the number one of tabulated drug abused about 40.7% of Egyptian drug users. This elevated level may be due to that people use tramadol to increase their performance and work power. So,

tramadol moved from schedule 3 to 1 by the Egyptian Ministry of Health as it highly addictive substances Tramadol was considered a very popular street drug in Cairo according to the information from the United Nations Office for Drugs and Crime (UNODC) that found 5 billion pills available for use in 2014 (20). On the other hand, International Narcotics and Law Enforcement Affairs reported that although Egypt was not a producer for this drug, there was highly significant consumption of the tramadol in the country, and this medicine has gone from being used as a pain reliever to being used as a recreational drug bought easily on the street and online store (21).

The main cause which make tramadol as popular drug and used massively, especially between the young and middle-aged people as it used in treatment of premature ejaculation and for orgasm extension and to increase sexual pleasure (22). Another study reported the prevalence of tramadol dependency among substance abusers, and also assesses the severity of addiction. About 43.94% of the patients used poly substances, while individual who used one substance were as follows: tramadol 30.30%, heroin 11.52%, sedatives and hypnotics 4.24%, alcohol 3.64%, cannabinoids 3.03%, nalbuphine 1.82%, and cocaine 1.52% (23).

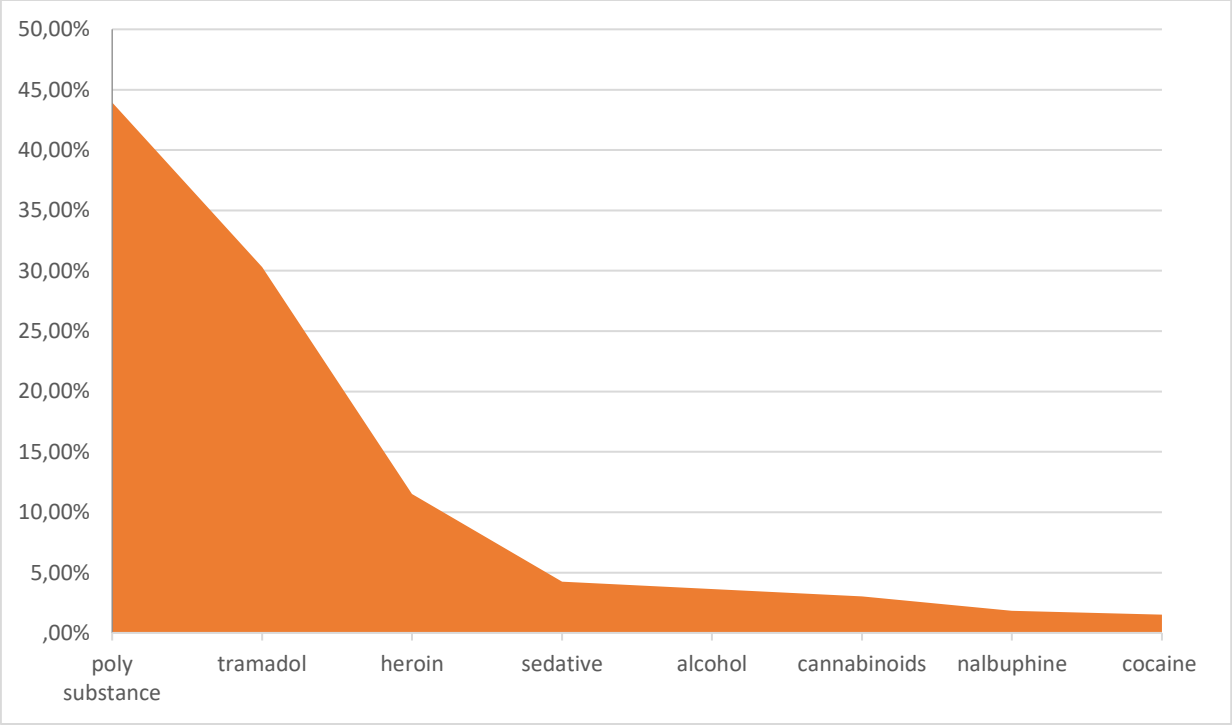

Figure 1: The prevalence of tramadol dependency among substance abusers, and assesses the severity of addiction in period of 12 months

Mohamed et al. (18) when analysed the percent of each drug among polysubstance, reported that the prevalence of cannabis within the polysubstance group was 83.4%, heroin 46.9%, sedatives and hypnotics 48.97%, tramadol 43.45%, and the prevalence of alcohol was 19.31% (23). Also, Hatata (24) reported that 61.9% of their participants used opiates, 18.5% used cannabis, 15.8% used sedatives, and 3.9% used alcohol. Moreover, Mohamed et al. (18) showed that opioids were the main substance in 30% of their patient, especially tramadol. With regard to the gender, 80% were males and 20% were females. These findings make us wonder: the low number of female detected in the sample because of the low percentage of drug intake in between females or due to the difficulty and lack of availability of treatment options for women, or because of shame and stigma a woman, would encounter if she joins a substance dependence treatment program?

The prevalence of male over female reported in the WHO global survey (25), found that the estimated attributable burden due to illicit use of drugs is 0.8% among men and 2% among women. Also, Mohamed et al. (23) reported that 53% of the patients were single, 36% were married, 6% widowed and 5% divorced. Also 21.1% of the participants were married, 64.9% single, and 14% separated.

Abolmagd et al. (26) declared that 60% of the opioid users were from the middle socioeconomic level, while, 27% were from the high socioeconomic level, and 12.5% were from the high socioeconomic level. More than one-third of the substance abusers' fathers and almost half of their relatives were substance abusers. This indicates the effect of exposure to drug-related stimuli and the distorted models of fathers and relatives (27). The age of drug abuse onset was reported in the studies by Hafeiz (28), who found that, the age of onset was in the range of 21–32 years in 83% of the patients, which can proved the fact that most substance users are also within this age range.

Pharmaceutical form of tramadol

Hydrochloride salt is the form of marketing tramadol. Primarily route of administration is orally, although there are other available forms including intramuscular, subcutaneous, intranasal, intravenous and sublingual forms as well as rectal suppositories. Also we may found tramadol available in compound with acetaminophen and in extended-release or immediate-release formulations (29). After oral administration it is absorbed rapidly and completely, but its absolute bioavailability is only about 66–77% because of first-pass metabolism and bioavailability increases to almost 100% after an intramuscular administration or after multiple doses (30). The relative bio-availabilities in rats of the buccal and nasal-administration forms in comparison with the oral administration are about 183.4% and 504.8% respectively (31).

Pharmacokinetic of tramadol

Tramadol reach to peak levels after rectal administration in 3 hours, oral administration in 1-2 hours and intramuscular intake in 45 minutes. It has about 5–6 hours a terminal half-life (32). So tramadol need frequencies daily dosage about four to six times due to short half-life (33). While, it is extensively metabolized by demethylation, oxidation and conjugation in the liver (34) about 26 metabolites can be detected (fourteen phase I metabolites and twelve conjugates) (35) The O-desmethylation of tramadol metabolized to its main active metabolite, and then cytochrome P450 CYP2D6 catalyse O-desmethyltramadol (M1)(34) furthermore, CYP2B6 and CYP3A4 catalyse the N-desmethylation to N-desmethyltramadol (M2) (35) found only M1 and also N,O-didesmethyl tramadol (M5) are pharmacologically active Among all its metabolites (36). 90% of tramadol is primarily eliminated by the renal system and the remain part excreted with the stools (37). Tramadol excreted as the following percentages in 72-hour urine: 29% unchanged, 20% N,O-didesmethyl-tramadol and its conjugates, 20% O-desmethyl- tramadol and its conjugates,17% N-desmethyl-tramadol, and the remainder percentage being other metabolites (38).

Mode of action of tramadol

Pharmaceutical formulation of it formed from racemic mixture of (+) tramadol and (-) tramadol, the tramadol antinociceptive effects included both nonopioid ingredients, which is serotonergic and noradrenergic components, also opioid ingredients which mainly act on the central nervous system (CNS) (39). Also other actions include influences on other several G protein coupled receptors (GPCRs), ion channel and transporters (40). On the other hand, clinical and preclinical studies demonstrated that the parent drug [(±)-tramadol, the two isomers] consider the only weak μ-opioid receptor which encoded by gene OPRM1 agonist (4,000-fold less than that of morphine), while the primarily responsible for μ-opioid activity of tramadol and is significantly more powerful than tramadol binding is metabolite M1 (400-fold higher affinity for the m-opioid receptor than that of tramadol, one-tenth that of morphine for the m-opioid receptor) (41). In case of analgesic effect (approximately one-third as potent as morphine

when each is administered orally) (42), while metabolites of tramadol M2, M3 and M4 have low affinity for the μ-opioid receptor of human (43). Otherwise another researches on acute administration reported minimal opioid-like effects. While chronic administration leading to opioid physical dependence and withdrawal on discontinuation (44). The dose of tramadol intake affect on the level of physical dependence. So large doses of tramadol can make a signal of abuse potential, also followed by high level of some prototypic measures like "drug liking" (1). CNS adaptations which result from accumulation of M1 likely are typical of other μ-opioid receptor agonists (45). The affinity of μ-opioid receptor only is not sufficient to interpret the analgesic action of tramadol. In addition to the action of tramadol on μ-opioid receptors, it act on noradrenergic and serotonergic systems may explain the involving of tramadol in the analgesic effect. Reuptake inhibition of serotonin by tramadol, with (+) tramadol about 4 times more effective than (-) tramadol, while M1 is about 10 times less potent than (-) tramadol (46). Likewise, in rat hypothalamic synaptosomes chronic and acute dose of tramadol holds the potential to block noradrenaline reuptake with (-) tramadol being approximately ten times more effective than (+) tramadol (47). There are other activities of tramadol including experimental evidence of inhibition of 5-hydroxytryptamine (5-HT) type 2C receptors (48), 5-nicotinic acetylcholine receptor (49), GABAA receptor (50), M1/M3 muscarinic receptor (51), N-methyl-D-aspartate (NMDA) receptor (50). Tramadol blocks catecholamine production at least partly by inhibiting functions of nicotinic acetylcholine receptors at clinically relevant concentrations in a manner independent of opioid receptors. One of the antinociceptive mechanisms exerted by tramadol is the inhibitory effects of tramadol on nicotinic acetylcholine receptor (52), and inhibition of transient receptor potential ankyrin 1 channel (TRPA1) (53). In the mouse formalin model there is a proof of involvement of cyclic guanosine monophosphate pathway, nitric oxide (NO) and ATP-sensitive K channels in antinociceptive function of tramadol (54). Tramadol may make activation to the transient receptor potential vanilloid 1 channel (TRPV1) (55). Until now the significance of these later findings on the tramadol abuse is unknown; however, most of these receptors are involved in the regulation of sexual function.

Side effect of tramadol abuse

Oxidative stress can defined as in ability of antioxidant enzyme to neutralize reactive oxygen species (ROS) production. High level of reactive intermediates lead to damage in cell component which lead to production of secondary toxic compounds e.g., ketones and reactive aldehydes (56). Bajic et al. (57) concluded that chronic opioid administration may lead to oxidative stress which differs in relation to the age.

Previous *in vitro* studies, showed death of cell when exposed to opioid receptor agonists by apoptotic mechanisms (58). Also another studies in rats demonstrated that chronic opioid administration lead to serious changes in the proteins which involved in the apoptotic signalling pathway leading to induction of apoptosis (59). Otherwise at cellular level tramadol has toxic effects by increasing lipid peroxidation which may be used as a marker of damaged cell (60). In addition, Zhang et al. (61) showed that treatment with tramadol lead to increase of malonaldiahyde (MDA) concentration, which suggests an increased level of lipid peroxidation. A reduction in the reduced glutathione level in the hepatocytes isolated from rat when incubated with different opioids concentration, a low level of reduced glutathione, glutathione peroxidase, superoxide dismutase and catalase activities were detected.

Long term administration of tramadol have histological abnormalities on both testicular tissues and cerebral cortex with oxidative stress in these organs due to generation of lipid peroxidation and inhibition of gene expression of antioxidant enzymes leading to oxidative stress (62). Also, there is increase in apoptosis in the two organs which confirmed by increase in expression of Bax gene. Light and electron microscopic examination of both organs showed the toxic effects of tramadol through

two possible synerergestic way, oxidative stress as well as apoptosis.

Nna et al. (63) conclude that chronic exposure to high doses of tramadol has negative effects on reproductive hormones in male rats, with poor reversibility following withdrawal. Apart from testicular oxidative stress previously reported by other researchers, also shown that up–regulation of prolactin a hormone known to correlate negatively with serum concentrations of testosterone, FSH and LH (hormones that support spermatogenesis) in males may play a major role in the etiology of reproductive deficit when chronically exposed to PDE5 inhibitors and opioids.

Lemarie and Grimm (64) Noticed the oxidative stress generation by administration of tramadol in the brain. Also, Mohamed et al (65). They explained effect of high dose of tramadol in mitochondrial electron transport chain (ETC) as it inhibited complexes I, III and IV. generation of reactive oxygen species due to Inhibition of complex III as an outcome of the intrinsic characteristics of electron transfer process to this complex from reduced ubiquinone. So, due to its high levels of oxygen consumption brain is mostly susceptible to oxidative stress (66). In mice chronic administration of tramadol leading to oxidative damage in tissue of brain, which followed by low level in brain non-enzymatic antioxidant, intracellular reduced glutathione level also glutathione peroxidase activity (67).

Furthermore, it has been reported that oxidative stress lead to lowering of enzyme activity and loss of function (68). Another study (69) demonstrated that the chronic effect of tramadol administration on insulin signaling pathway and neuronal glucose metabolism in the cerebral cortex which also leads to oxidative stress. Oxidative stress happened in different organ by administration of tramadol through induction of inflammatory reaction which reduced after withdrawal period (70). In rabbits after long term of chronic tramadol administration this inflammatory reaction confirmed previously to be a main factor for generation of oxidative stress by make changes in fatty acid composition of cell membrane leading to a decrease of its fluidity, which reduce formation of pseudopodia and detect pathogens and foreign particles (71).

Recently, Ahmed and Kurkar (72) proved that administration of tramadol increased the testicular levels of lipid peroxidation and nitric oxide also significantly decreased the anti-oxidant enzymes activities in comparison with the control group as well as at level of immunohistochemical. They showed that tramadol in testicular tissues increased the endothelial nitric oxide synthase expression. Finally they concluded that the testicular function of adult male rats affected by tramadol treatment through generation of nitric oxide and oxidative stress stimulation by this drug.

Liu et al. (73) announced that the several effects due to long term use of tramadol and other opioid on neuronal structure (cytoskeleton) whish considered as the signs for neuronal damage in the lymphocytes and mouse spleen, heart and lung. It may also stimulate the mRNA expression of pro-apoptotic receptors, through opioid receptor activation. Also large amount of apoptotic neurons in the hippocampus of these rats have been detected, and the expressions of the apoptosis related proteins bcl-2, Fas and caspase-3 presented with alteration. In comparison with control normal group, they noticed that marked increase in the expressions of Fas and caspase-3, while expression of Bcl-2 reduced significantly. They suggested that long term use of opioid drug lead to increase the apoptotic neurons through apoptosis related signaling pathway. This result was also confirmed previously by Atici et al. (74). They showed that chronic administration of tramadol and/or morphine with increase in the doses caused degeneration in red neuron and rat brain apoptosis, leading to cerebral dysfunction.

Recently, Sharifipour et al. (58) showed that chronic admiration of tramadol in rats is a combined with a significant increase in the expression level of the pro-apoptotic Fas receptor, and upregulation in pro-apoptotic protein like caspase-3, which combined with decrease in the expression level of the anti-apoptotic oncoprotein Bcl-2.

Another study proved that under chronic tramadol administration the apoptotic index

showed highly significant increase in rat's testis tissue than that in the control group and decreased in rats under withdrawal (62). These results harmonized with another study using other opioids similar in action to tramadol (75). They confirmed that opioids have effects on secondary sex organs and spermatogenesis through a histological and pathological picture of apoptosis in rats receiving these drugs.

Another study showed neurobehavioral, neuropathological and neurochemical brain changes after co administration of nicotine and tramadol to male albino rats. Nicotine magnify oxidative stress produced by chronic administration of tramadol as proved by increase in nitric oxide and thiobarbituric acid reactive substances followed to the high amount of nitric oxide synthases. Also reduction of non-protein sulfhydryls was detected (76).Aggravation of oxidative damage by tramadol followed by increase level of nuclear factor kappa B (NfkB) and also setup in the proinflammatory cytokine as tumor necrosis factor α (TNFα) and appear of apoptosis obviously by the increased caspase-3 immunoreactivity as well as increased tyrosine hydroxylase in midbrain a comhgvrlbined by high of butyryl cholinestrase and acetyl cholinesterase level. These results provide evidence that such combination aggravated neurotoxic effects and elicited negative effects regarding learning and memory.

Lees (77) stated that (Na+ & K+-ATPase) enzyme supports the cells ionic homeostasis also support keeping of neuronal resting membrane potentials and production of neural impulses. Subsequently, impair the activity of this enzyme will lead to cellular depolarization followed by brain dysfunction. As well as, on the same lines, the significant reduction in the activities of brain Na+ & K+-ATPase enzyme was reported (78)administration of either morphine or tramadol at chronic dose level, leading to cell injury and death of neurons, followed by brain disorders, which is in the same line with previous studies (79). Another illustration is that the tramadol may labor their inhibitory actions by its direct effect on the brain enzyme protein itself. According to previous findings (78), the considerable change in activity of Na+, K+-ATPase enzyme, which is consider an integral membrane enzyme that enhance the process of Na+ and K+ ions transportation against concentration gradients, whish lead to changes in Na+ and K+ concentrations as Na+ and K+ ions have a very important role in body functions which include nerve signals and impulse transmission, various chemical reactions and fluid balance. Impair the function of Na+ & K+-ATPase enzyme in brain considered the main cause of various types of neurological disorders, which is in the same line with another study done by Horvat et al. (80), who proved that abnormal excessive release of certain neurotransmitters amount caused by depolarization of cell membrane.

Another study done by Elwy and Tabl (78) demonstrated the potent effects of tramadol abuse on the activities of Acetylcholine esterase enzyme The actions of the drug were contributed to reduce in cognitive function and the memory deficits in chronic users. As a consequence, this bad effect of tramadol considered as a signs for drug-induced neurotoxicity.

Effect of chronic administration of tramadol in rat hippocampus may disturb learning and memory. It has also bad neurotoxicity effects on inducing dark neurons DNs formation and apoptosis (81). In addition, this result harmonized with another study which reported that tramadol deteriorates memory when administered in acute or chronic dose. Also, tramadol when was administrated in a single dose reported more damaging effect on the memory when compared with multiple doses. This interpreted by the inhibitory effectiveness of tramadol on different type of neurotransmitters and numerous receptors as N-methyl D-aspartate, muscarinic, AMPA. It affect on some second messenger like cGMP and cAMP or it has stimulatory effect on the gama amino butyric acid ,opioid, serotonin or dopamine receptors in the brain (82).

Abuse as well as acute overdose of tramadol has led to reported cases of hepatotoxicity and even death in humans (83). Many studies in animals have proved hepatotoxicity with alteration on levels of liver function biomarkers (84) and histological damage was also reported

(85). Although, oxidative stress may be involved in tramadol induced hepatotoxicity due to decrease in the antioxidant enzymes and lipid peroxidation observed in treated animals (86).

On the other hand, another study showed biochemical and histopathological changes in kidney and liver of rats due to chronic usage of tramadol. Aspartate, Serum aspartate amino transferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), lactate dehydrogenase (LDH), and creatinin levels were significantly increased when compared with the control group. Also Serum LDH level was significantly increased. The mean malondialdehyde (MDA) level was significantly decreased. Examination with Light microscope showed severe centrolobular congestion as well as focal necrosis in the liver of rat administrated chronic dose of tramadol. The main histopathological changes observed in group treated by tramadol were vacuolization in tubular cells. They also illustrated the hazard of exacerbated lipid peroxidation, renal and hepatic destruction due to long term use of tramadol (87).

While, activities of Super Oxide Dismutase (SOD), glutathione (GSH) and catalase (CAT) were significantly reduce. Whilst an increased in the level of MDA in both liver and kidney tissue of chronic administrated tramadol rats was observed. Long term tramadol treatment induced histopathological changes in both tissues. These alterations were manifested by severe hydropic degeneration, with congested central veins of the liver and degenerated renal tubules and atrophied glomerulus. While at level of mRNA expression of proapoptotic marker as expression of Bax gene showed a highly significant increase and the antiapoptotic Bcl-2 expression decreased significantly. Therefore, tramadol have harmful effect at the cellular level and can induce apoptotic changes in these tissues (88)

On the other hand, Boshra (89) evaluated the effect of tramadol administration on the adult female rats bone. In chronic administration; tramadol had the lowest osteoporotic effect in comparison with other opioid. Hence, use of tramadol is safe in case of treating patients who suffering from chronic pain specially associated with osteoporosis.

Opioid drugs have a profound effect on sleep. Opioid receptors present in the ventrolateral preoptic nucleus, which is the same nucleus that is involved in regulation of sleep and considered as a sleep promoting area. It has been suggested that opioid peptides are involved in the induction of the sleep. They also cause excessive daytime sleepiness and fatigue (90). In the case of use of tramadol and opioid analgesics, despite the particular amelioration in sleep reported by patients on low doses of opioids, long-term administration of opioids affect sleep physiology. Opioids tend to disrupt the quality and quantity of sleep (91). Opioids can cause irregular and slow respiration leading to hypercapnia which followed by hypoxia (92). Chronic use of tramadol has also been accompanied with both obstructive and central sleep apnea through relaxation of the throat muscles in 30% to 90% of abusers (93). Tramadol is suspected to affect sleep. In addition to its effect on opiate receptors, it inhibits the reuptake of serotonin and norepinephrine, which in turn enhance its stimulating properties, that may further affect sleep quality (94).

In 2007, illustration in Ireland showed that the different critical complications like severe liver failure also heart failure, which may led to the death of patients, have been demonstrated. Only two cases presented that the only reason for his death was announced net tramadol. In autopsy examination of the cadaver post-mortem noticed changes as alveolar haemorrhage (bleeding in the air sacs), acute necrosis in renal tubular and ischemia in liver (95).

Tjäderborn (96) studied unintentional poisoning and fatal tramadol among Swedish forensic autopasy cases between 1995-2005. Seventeen patients (11 men and 6 women) have been recognized. For these cases, the range of age was from 18 to 78 years with average age 44 years and the median tramadol concentration was 2 μg/g.

In another study, ten cases (59%) of numerous drug poisoning was considered as the cause of death. while, in seven cases, tramadol

was the only drug which present in toxic concentrations. Also a History of different substance abuse in 14 cases (82%) was reported and recent history of tramadol abuse detected in eight patients (47% women). This demonstration proved that tramadol fatal poisoning may have occurred unintentionally and people with a history of substance abuse may be life on knife edges and high risk, so when history of tramadol use in these patients, they need caution (97). Another study in mice, revealed that tramadol acute intoxication when compared with control group, showed patholo-gical changes in the livers and inflammatory cells infiltration into alveolar, haemorrhage and pulmonary oedema congestion in 95% of the mice. But in the control group which received normal saline they noticed normal histological liver tissue (98).

According to fatal and risky symptoms appear after chronic administration of tramadol such as prolonged hospitalization for poisoning, cardiac arrest and loss of consciousness. Type of subsequent complica-tions, required attention to tramadol use poisoning. As in the case of death for poisoning was seen within tramadol user, mortality was most prevalent in young men. More attention and care for the elderly, in terms of risk of aspiration, which indicates a lack of defence mechanisms of prevention of these complications. Therefore prevention of intractable use of tramadol and care after poisoning with it is clear. After the death, the most important diagnostic aid will be biological samples with drug poisoning, first stomach contents and then urine samples (99).

Conclusion

The increase in the prevalence of tramadol HCl dependency over the other substances in the Egyptian community calls for pay more attention from family, health and educational institutes. Tramadol may apply a very useful interference in treatment of premature ejaculation, but its abuse risky effect may supply a possible demonstration for the unexplained delayed fertility as well as behavioral and the associated psychological changes. Also it could involve malfunction of cerebral cortex which includes memory disturbance and decrease in cognitive function. Moreover, the appearance of various metabolic disorders in different body tissues in chronic users was detected.

Conflict of interest

The authors declare no conflict of interest.

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