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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)

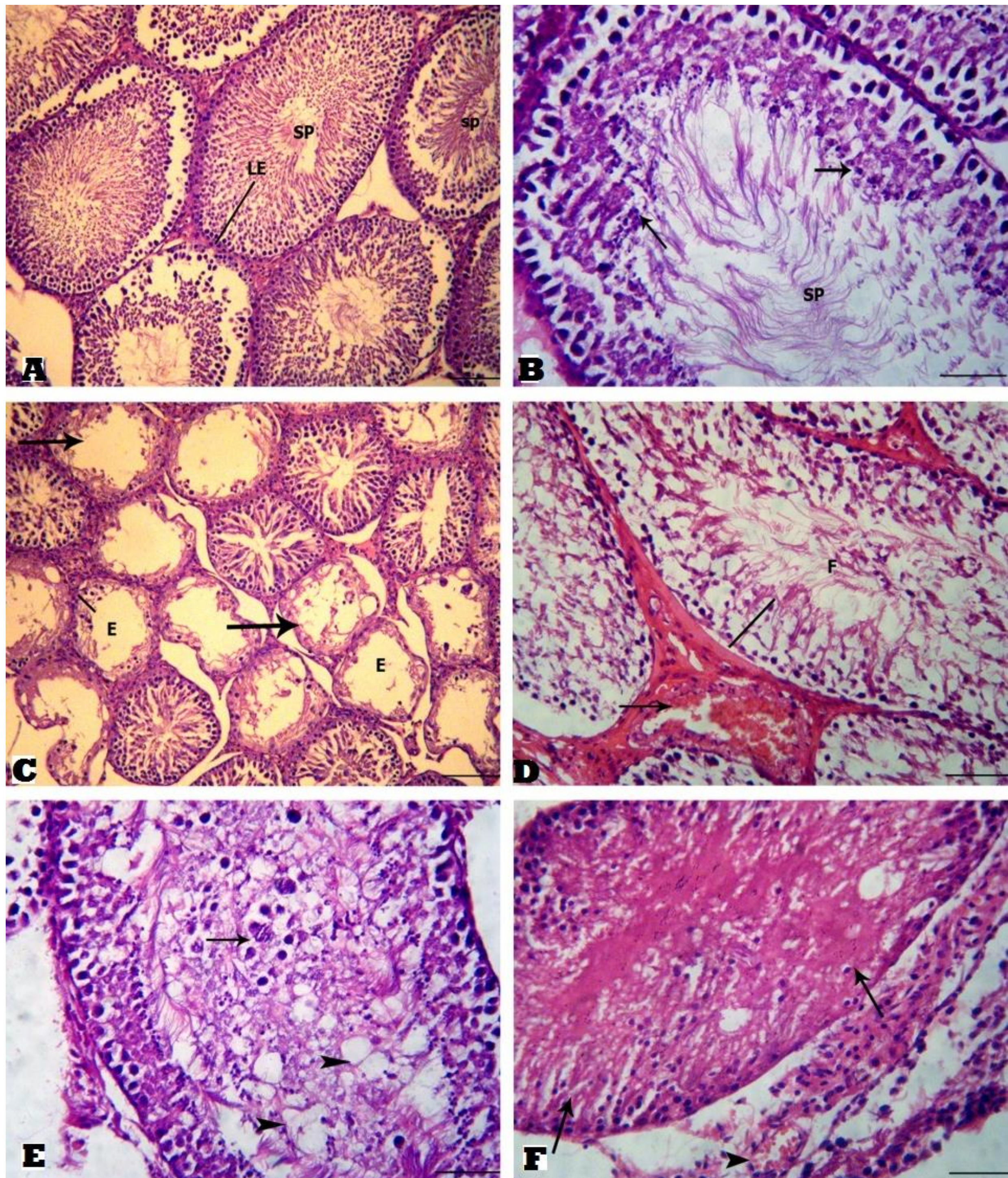

Figure 1: Histopathological effects of carmoisine on rat testes as studied with H &E staining. It showed normal lining epithelium and spermatogenesis in testes of control group (A and B). Carmoisine-treated rat testes were with focal mild testicular degeneration of vacuolated spermatocytes (C and D) 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)

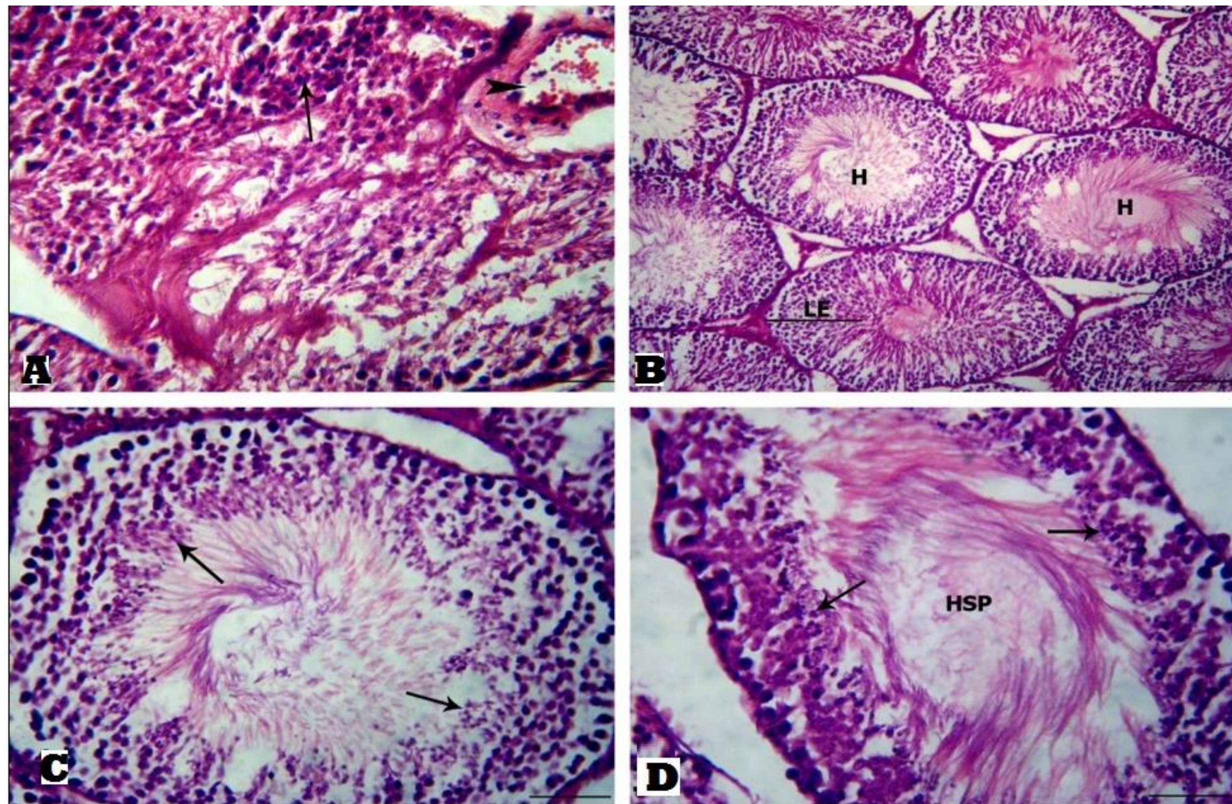

Figure 2: Histopathological effects of curcumin on rat testes as studied with H&E staining. Curcumin treated rat testes showed normal testicular tissue, improvement of spermatogenesis and presence of elongated spermatids and huge numbers of spermatozoa in all the lumina of the seminiferous tubules. (H&Ex 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).

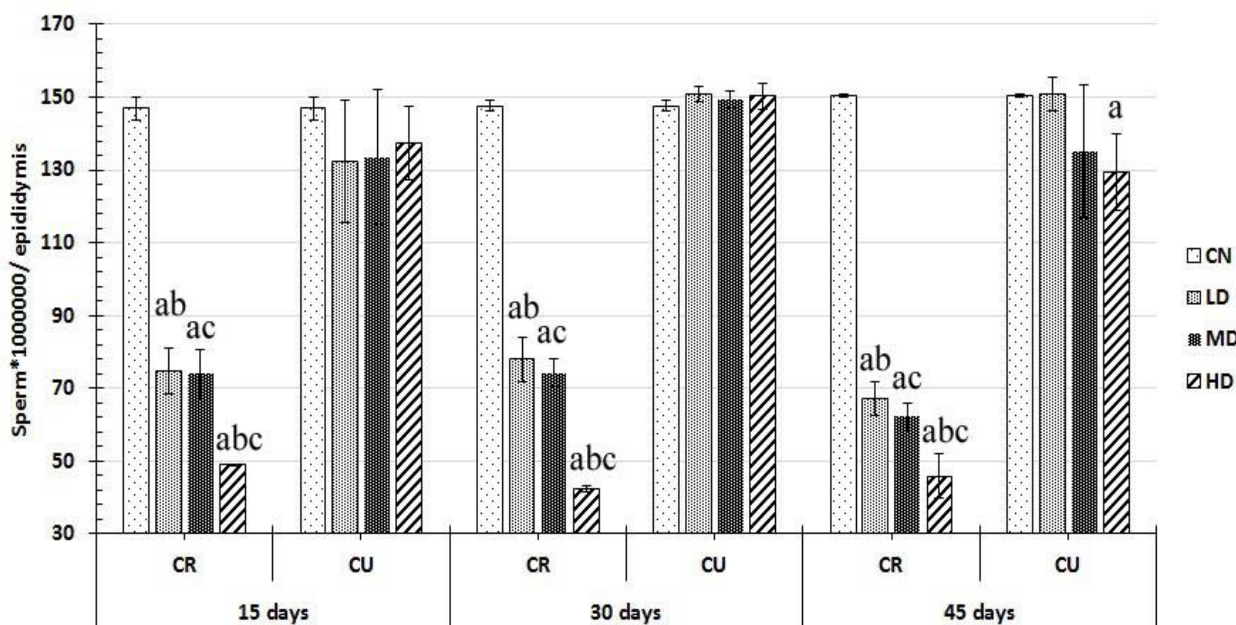

Figure 3: Histogram represents epididymis sperm counts in control and experimental rats. The letter (a) denotes significant (P <0.05) difference as compared to the control; however column with the same letter are in significant relation. Carmoisine (CR), curcumin (CU), control (CN), low dose (LD), medium 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 epidydimal 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).

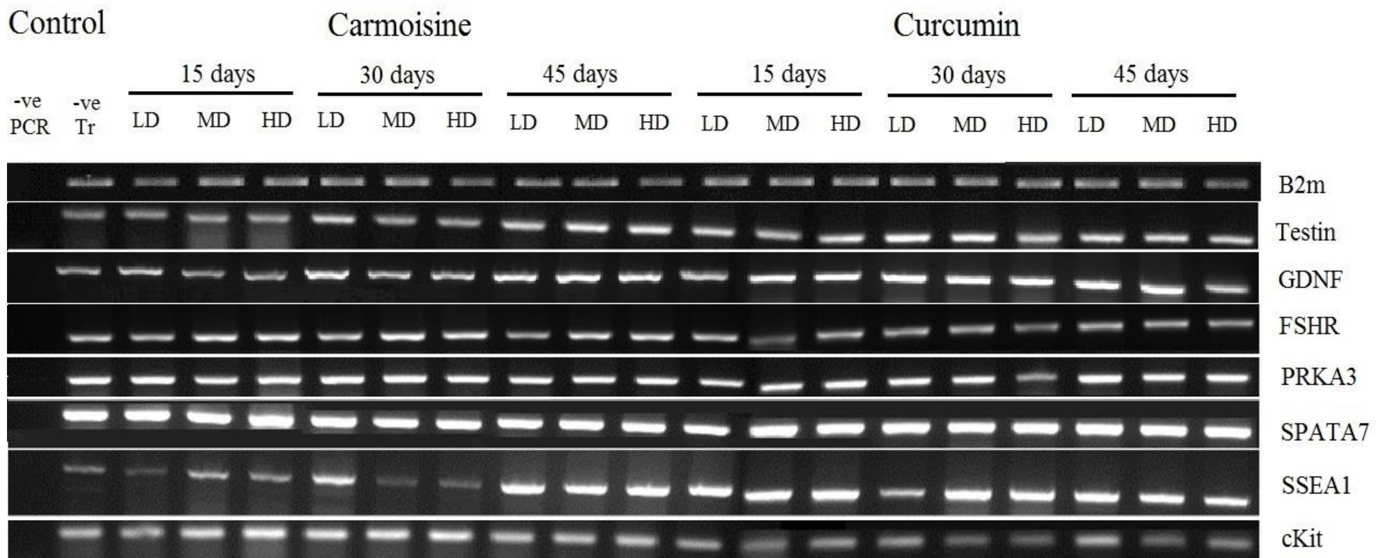

Figure 4: A panel represents mRNA expression patterns of control and rats given the food colorants carmoisine and curcumin. Glial cell derived neurotrophic factor (GDNF); tyrosine kinase receptor (c-KIT); follicle stimulating hormone receptor (FSHR); A kinase anchor protein 3 (PRKA3); spermatogenesis 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

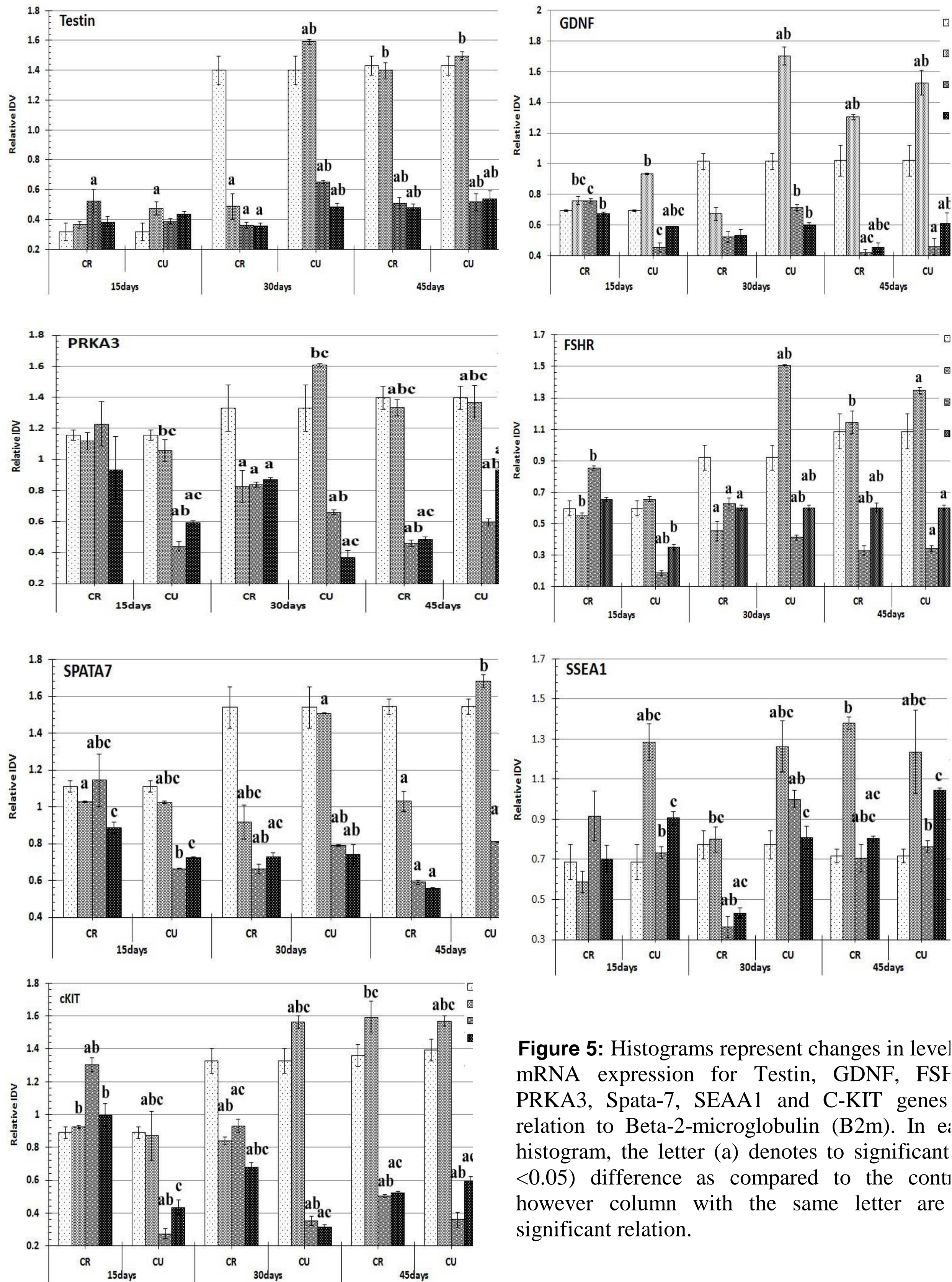

Figure 5: Histograms represent changes in level mRNA expression for Testin, GDNF, FSH PRKA3, Spata-7, SEAA1 and C-KIT genes relation to Beta-2-microglobulin (B2m). In ea histogram, the letter (a) denotes to significant <0.05) difference as compared to the contr however column with the same letter are 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.

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