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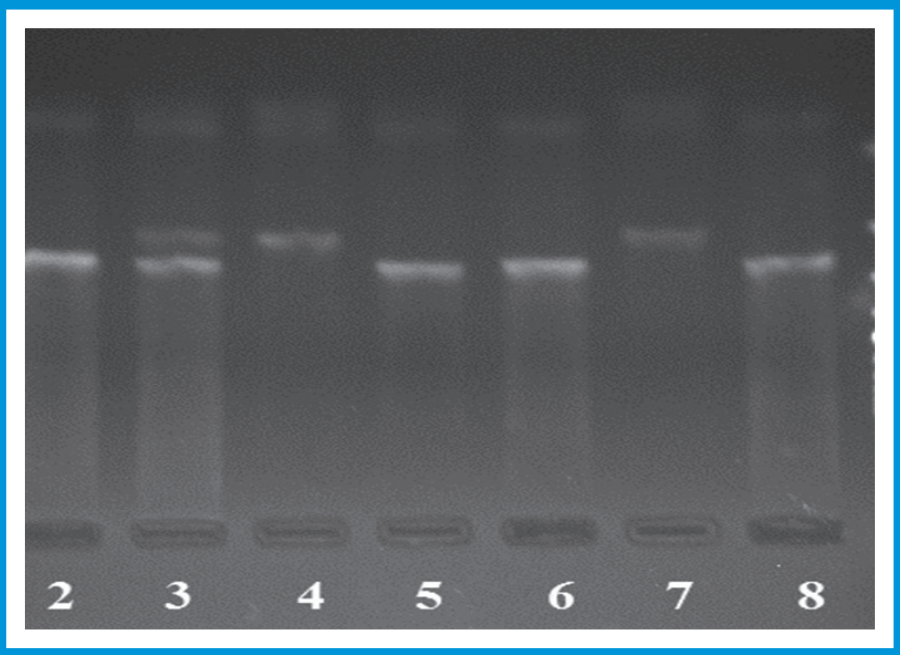

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Original Research Article

ASSESSMENT OF CIRCULATING TOTAL AND FREE IODOTHYRONINES' PATTERNS IN ADULT OVINE AND CAPRINE SPECIMENS: INFLUENCES OF ENDEMIC GOITRE AREA

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Abstract: North-eastern Sicily is an area with iodine deficiency disorders occurring in both humans and animals. The aim of this study was to test the influences of an endemic goitre area on iodothyronine ranges and their pattern in adult ovine and caprine specimens stabled in different locations of Sicily, taking into account the different sexes. A total of 48 Comisana sheep and 51 Maltese goats was studied. The sheep included 10 females and 6 males stabled in a non-endemic goitre area (farm A: control group), and 16 females and 16 males stabled in an endemic goitre area (farm B: observational group). The goats included 6 females and 13 males stabled in a non-endemic goitre area (farm A: control group), and 16 females and 16 males stabled in an endemic goitre area (Farm B: observational group). The results showed lower T3 and higher fT4 ($P<0.0001$) levels in female and male sheep, and higher T4 levels in males ($P<0.0001$) stabled in farm B than in farm A. In comparison to farm A, goats stabled in farm B showed higher fT3 ($P<0.0001$) levels; males stabled in farm B showed lower T4 levels ($P<0.0001$), and females showed higher fT4 levels ($P<0.0001$). Significant effects of sex and of endemic goitre area on the total and free iodothyronines of sheep and goats were observed. The obtained data showed that an endemic goitre area for humans is not necessarily the same for ovine and caprine species, which seem to be able to adopt an adaptive strategy without presenting any clinical signs of thyroid disorders.

Key words: thyroid hormones; sheep; goat; endemic goitre area

Introduction

Thyroid hormones play a pivotal physiological role in metabolic turnover and homeostasis (1) and in animal thermogenesis (2). These hormones exert pleiotropic effects in many different organs, including central nervous system development, and changes in cognitive function in animals and humans (3). Thyroid diseases are well described in companion animals, but less knowledge is available for livestock, in which nutritional iodine deficiencies represent a serious problem, especially in endemic goitre areas. In ewes, severe iodine deficiency results in neonatal lamb death and goitre, alopecia, and poor skeletal development; at the radiographs and necropsies the epiphyses of most of the long bones were not mineralised and the remainder of the bone was poorly mineralised (4). Placental restriction of foetal growth and small size of lambs at their birth may have increased the activation of T_4 to T_3 and the sensitivity of soft tissues to thyroid hormone, which may have contributed to catch-up postnatal growth (5).

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Maternal goats' hypothyroidism, displayed from mid-gestation, resulted in decreased brain and cerebellum weights of affected goitrous foetuses; T_4 and fT_4 levels in affected goat foetuses were dependent on the maternal phenotype, as was the degree of enlargement of the goitre (6). Nevertheless, in lactating goats, long-term dietary iodine supplementation significantly increased the fT_3 and fT_3/fT_4 ratios (7). The quantity of T_4 and T_3 available to new-born lambs in milk suggested that thyroid hormones ingested with the colostrum may have a physiological role during the early postnatal life of suckling goats (8); in addition to that, T_3 seemed to act as metabolic modulators for the establishment of puberty in goats (9). There is an assumption that the hairless gene is often responsible for congenital hypertrichosis in mammalian species, and the protein codified by this gene is a transcriptional corepressor for thyroid hormone receptors (10). Therefore, thyroid hormones affect the expression of the neuronal gene RC3 mRNA preferentially in the striatum in prenatal and adult caprine brains, suggesting a region-specific sensitivity for thyroid deficiency (6), and the mRNA expression levels of skin monodeiodinase II and III in Cashmere goats (11). The effects of iodine deficiency, especially in endemic goitre areas, have been reported both in humans and in different animal species (12-14). North-eastern Sicily is an area with iodine deficiency disorders occurring both in humans and in different domestic animals. Partial beneficial effects of the so called "silent iodine prophylaxis", an additional percentage of iodine salt given in water and food, on iodine deficiency disorders have been described in humans (12). In contrast, even in endemic areas, the iodine deficiency disorder in both adult and young farm animals is sporadically treated, and routine iodine supplementation of pregnant animals is not recommended, because it is economically unsustainable. The aim of this study was to compare the total and free iodothyronine levels of sheep and goat specimens stabled in non-endemic and endemic goitre areas, by taking into account the different sexes.

Material and methods

Animals, diets and, experimental design

The study was carried out on a total of 48 Comisana sheep and 51 Maltese goats, ranging in age from 2 to 3 years and weighing 38.3±0.57 kg, and 34.9±0.56 kg, respectively, which were stabled in two different areas of Sicily (Figure 1). The sheep included 10 females and 6 males stabled in a non-endemic goitre area (farm A), and 16 females and 16 males stabled in an endemic goitre area (farm B). The goats included 6 females and 13 males stabled in non-endemic goitre area (farm A), and 16 females and 16 males stabled in an endemic goitre area (farm B). The post-partum ewes and goats had delivered from 60-90 days previously. The rams and billy goats were destined for meat production. Farm A was situated near the city of Messina (at 100 meters above sea level; 38°13'19"92 N; 15°14'20"76 E); farm B was situated on the slopes of Nebrodi mountains (at 660 meters above sea level; 38°5'9" 96 N; 14°48'28"08 E). The last farm is in an area of severe endemic goitre with the presence of abnormalities in human thyroid function in the population. The stable management of farms A and B was superimposable: both sheep and goats were kept together with their respective flocks of more than 100 ovine and caprine herds on local pastures for most of the time, and fed twice a day on a similar diet of commercial concentrate (barn, corn and soya) (15.5% protein, 2.5% fat, 6.8% cellulose and 6.7% ash) and cereal straw; water was available *ad libitum*. No iodine supplementation was introduced into the diet. No plant known to be goitrogenic or a member of the Brassica family was present on pastureland.

Figure 1: Map of Sicily

Non endemic goitre area = farm A
Endemic goitre area = farm B

Measurements

The study was carried out in late spring and early summer; the mean environmental temperature was 22 °C (18 °C to 26 °C), and the mean relative humidity was 44.60% (38.20% to 51.5%). These were monitored using a Hygrothermograph ST-50 (Sekonic Corporation, Tokyo, Japan). Blood samples from the jugular vein (5 ml) were collected, twice a month for three months (April–June), using evacuated tubes (Venoject, Terumo®, Leuven, Belgium) at 09:00–10:00 h in order to minimise the effect of circadian rhythm on hormone measurements. All samples were taken in quiet conditions by the same veterinary team.

Laboratory analysis

Blood samples were kept at 4 °C until centrifugation at 1500 × g for 15 min, and the serum was harvested and stored in polystyrene tubes at -20 °C until total and free iodothyronines were determined. Hormone assays were analysed in duplicate using a commercially available immunoenzymatic kit and carried out per the manufacturer's instructions (SEAC-RADIM, Pomezia, Rome).

Limits of detection were 0.24 nmol/L for T_3, 5.79 nmol/L for T_4, 0.15 pmol/L for fT_3 and 1.3 pmol/L for fT_4. Intra- and inter-assay coefficients of variation (CV) were 7.3% and 11.4% for T_3, 2.3% and 5.7% for T_4, 4.2% and 11.9 % for fT_3, 6.6 % and 9.6% for fT_4, respectively, based on measurements, in three different samples. The commercial kits were validated for total and free iodothyronines by establishing that dilutions of ovine serum resulted in curves identical to those obtained with the human standards supplied with the assay kits.

Statistical analysis

All the results are expressed as mean ± SD (Figures 2 and 3). Two-way analysis of variance for repeated measures (2-way RM ANOVA) was applied to test for the effects of different sexes and locations of flocks (farms A and B) and sampling times, as well as the interaction between them, on hormonal concentrations; when the F statistic was significant, the differences between individual mean over time were then assessed using a *post hoc* multiple comparison test (Bonferroni). The level of significance was set at $P<0.05$. All calculations were performed using the PRISM package (GraphPad Software Inc., San Diego, CA, USA). The ratios for T_3/T_4 and fT_3/fT_4, and the percentages of fT_4/T_4 and fT_3/T_3 were also calculated.

Results

Ovine T_3 levels ranged from 1.56 to 4.76 nmol/L, T_4 levels ranged from 102.51 to 120.78 nmol/L, fT_3 levels ranged from 5.15 to 8.63 pmol/L, fT_4 levels ranged from 17.95 to 49.18 pmol/L. Compared to the non-endemic area (farm A), female and male sheep stabled in the endemic area (farm B) showed lower T_3 ($P<0.0001$) and higher fT_4 ($P<0.0001$) levels, and only in males were higher T_4 levels found ($P<0.0001$) (Figure 2). Compared to the females of farm B, the males showed higher T_4 ($P<0.01$) and fT_4 ($P<0.0001$) levels. Males of both farms showed higher fT_3 ($P<0.001$) levels than females did (Figure 2). Two-way RM ANOVA showed a significant effect of ovine sex on the fT_3 ($F=30.18$; $P<0.0001$), and of the endemic goitre area on the T_3 ($F=30.18$; $P<0.0001$) and fT_4 ($F=20.37$; $P<0.0001$) changes.

Caprine T_3 levels ranged from 2.20 to 3.60 nmol/L, T_4 levels ranged from 101.50 to 110.87 nmol/L, fT_3 levels ranged from 3.85 to 12.14 pmol/L, and fT_4 levels ranged from 12.77 to 33.39 pmol/L. Compared to females, males showed higher T_3 (farm A: $P<0.0001$; farm B: $P<0.001$) and fT_4 (farm B: $P<0.0001$) levels (Figures 2 and 3). Compared to farm A, female and male goats stabled in farm B showed higher fT_3 ($P<0.0001$) levels. Males stabled in farm B showed lower T_4 levels ($P<0.0001$) and females higher fT_4 levels ($P<0.0001$) (Figure 3). Compared to females of farm B, males showed higher fT_3 ($P<0.0001$) and fT_4 ($P<0.0001$) levels. Two-way RM ANOVA showed a significant effect of caprine sex on the T_3 ($F=29.40$; $P<0.0001$) and fT_4 ($F=86.91$; $P<0.0001$) levels, and of endemic goitre area on the fT_3 ($F=172.20$; $P<0.0001$) and fT_4 ($F=18.64$; $P<0.0001$) levels.

The T_4/T_3 and the fT_4/fT_3 ratios were lower in both female and male sheep stabled in farm A (non-endemic goitre area) than those stabled in farm B (endemic goitre area) (Table 1). The percentages of fT_4 to T_4 and fT_3 to T_3 were lower in sheep and goats of both sexes stabled in farm A than in farm B (Table 1).

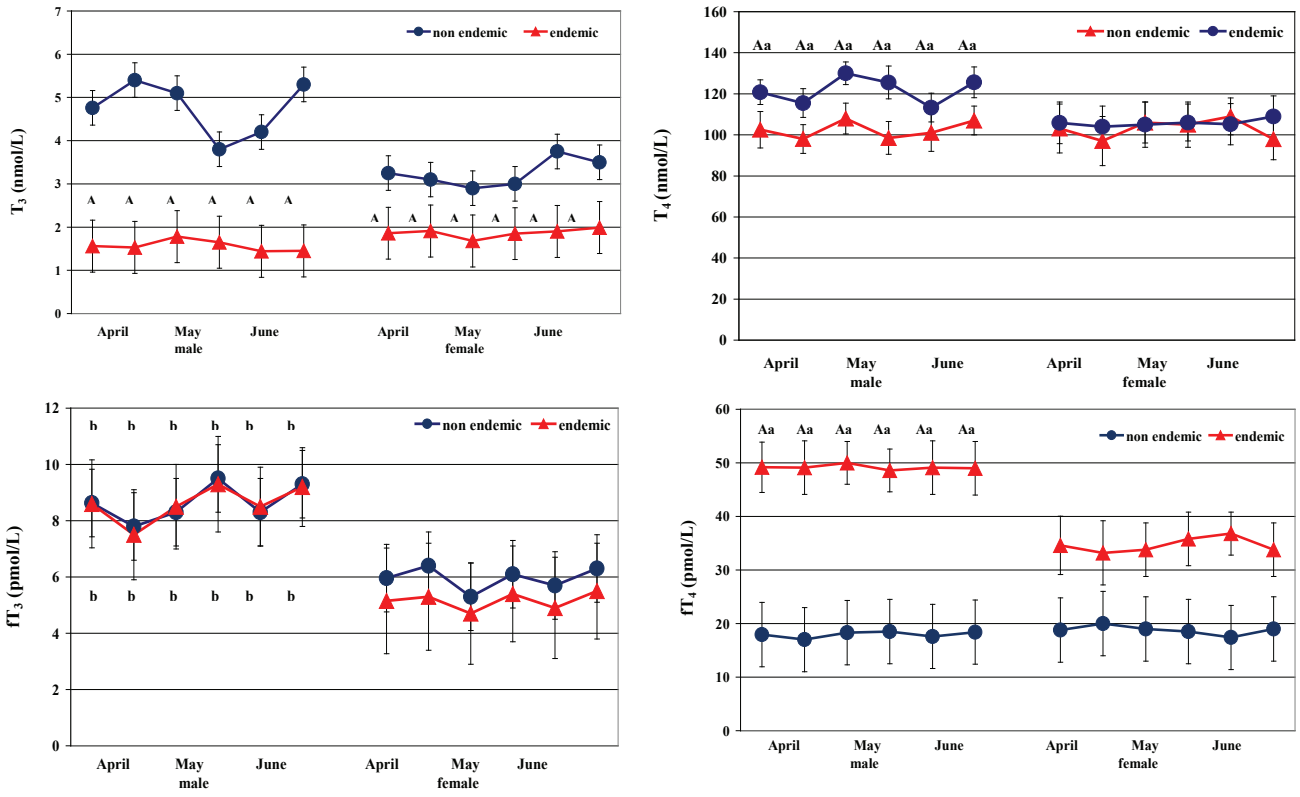

Figure 2: Total and free iodothyronine concentrations (M ± SD) in female and male sheep stabled in non-endemic (farm A) and endemic (farm B) goitre area

Different superscripts show significant differences versus control group (non-endemic): A=P<0.001; and versus female: a=P<0.01; b=P<0.001

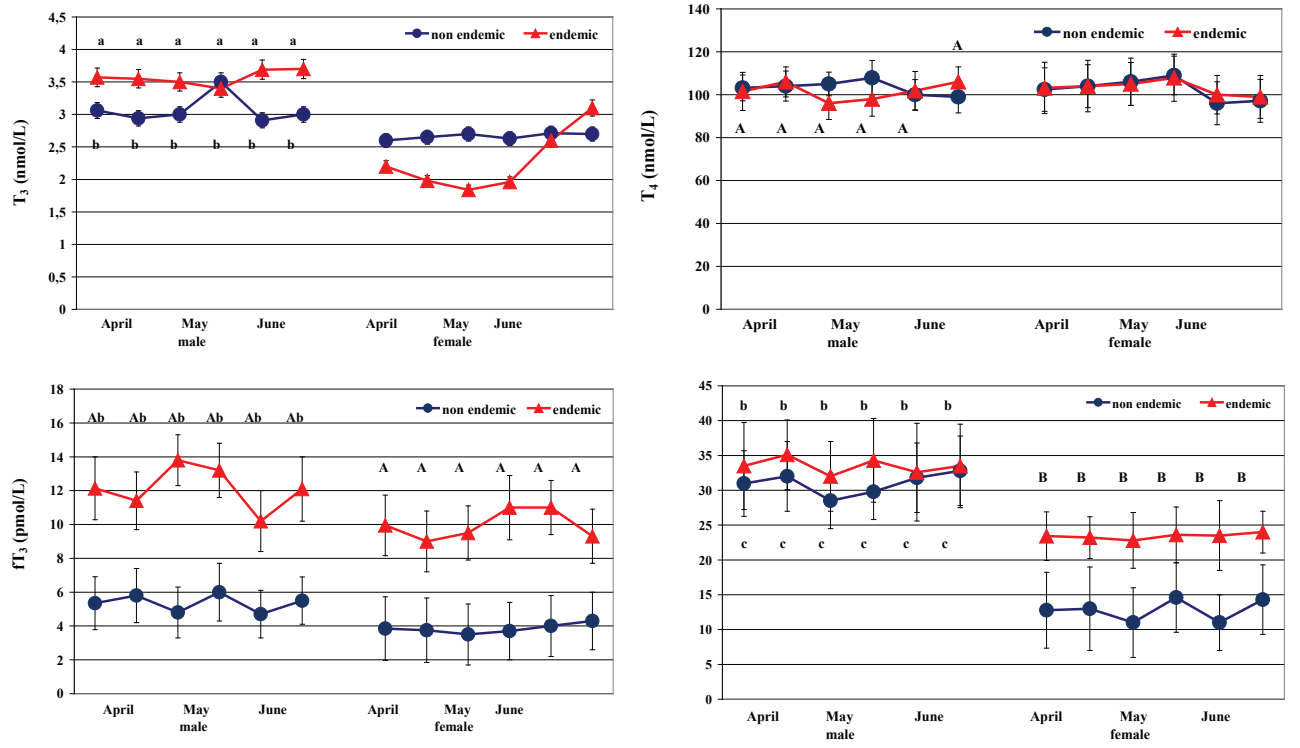

Figure 3: Total and free iodothyronine concentrations (M ± SD) in female and male goats stabled in non-endemic (farm A) and endemic (farm B) goitre area

Different superscripts show significant differences *versus* control group (non-endemic): A=P<0.001 and *versus* female: a=P<0.01; b=P<0.001; c=P<0.0001

Table 1: Ratios and relative percentages in female and male sheep and goats stabled in non-endemic (farm A) and endemic (farm B) goitre area

	Farm A: non-endemic		Farm B: endemic goitre area	
	female	male	female	male
Ratio of T_4/ T_3	31.7:1	21.4:1	56.4:1	77.4:1
Ratio of fT_4/ fT_3	3.15:1	2.08:1	6.72:1	5.72:1
Percentage free T_4 to total	0.018 ± 0.010	0.017 ± 0.005	0.032 ± 0.003	0.040 ± 0.004
Percentage free T_3 to total	0.183 ± 0.02	0.181 ± 0.01	0.027 ± 0.01	0.055 ± 0.01
Goats				
Ratio of T_4/ T_3	39.4:1	30.8:1	46.9:1	28.4:1
Ratio of fT_4/ fT_3	3.32:1	5.79:1	2.35:1	2.75:1
Percentage free T_4 to total	0.012 ± 0.001	0.027 ± 0.003	0.022 ± 0.007	0.032 ± 0.005
Percentage free T_3 to total	0.149 ± 0.01	0.148 ± 0.02	0.452 ± 0.02	0.340 ± 0.01

Discussion

Thyroid hormone levels were within normal ranges in adult sheep and goats. The comparison of our results with previously published data reporting the circulating thyroid hormones of adult ovine (T_3: 2.04-5.85 nmol/L; T_4: 49.68-146.46 nmol/L; fT_3: 3.63-4.35 pmol/L; fT_4: 23.93-25.49 pmol/L) and caprine (T_3: 2.21-3.56 nmol/L; T_4: 65.64-142.85 nmol/L; fT_3: 9.39-11.31 pmol/L; fT_4: 35.24-47.38 pmol/L) species (15, 16) did not reveal any significant discrepancies. Some slight differences may also occur because of the different techniques, age, nutritional factors and/or geographic environmental variations (17–19). The higher fT_4 levels recorded in female and male sheep and in male goats stabled in the endemic goitre area (where no clinical symptoms of thyroid disorders were present), in comparison to the physiological ranges reported in literature (15, 16), suggest that hypothyroidism found in humans from the same endemic goitre area does not necessarily correlate with lower T_4 levels and with evident clinical signs in lambs, kids and/or their mothers. Indeed, like sheep and goats stabled in the endemic goitre area, with the highest T_4 levels only in males, sheep and goats stabled in a non-endemic area also showed high T_4 levels. It is well known that the fT_4 fraction represents the biologically active hormone for tissues. Thus, the ratio of fT_4 represents a primary factor to determine the fractional turnover of thyroid hormones. Moreover, the binding fraction represents the hormonal reserve that balances the sudden increase and decrease of hormonal release to tissues. Although T_3 is mainly an intracellular hormone and its serum measurement is a less representative value of the total hormonal complex than serum T_4 measurements, inherent physiological effects are attributed almost exclusively to T_3 (20). Furthermore, peripheral T_3 concentrations are influenced mainly by extrathyroidal 5'-deiodinase activity, which represents an important control point for the regulation of metabolic status (21). In contrast, T_4 has been known as the predominant product of the thyroid gland, ant it has an intrinsic thyromimetic activity, as protection against hypothyroidism, which could be more pronounced in animals stabled in an endemic goitre area. Therefore, the lack of effects of an endemic area, and/or iodine deficiency, on thyroid hormones' metabolism could show that enzyme activity is homeostatically regulated, and iodine is probably incorporated, so as to ensure the maintenance of total and free hormone homeostasis, in accordance with the species.

Furthermore, sheep stabled in the endemic goitre area had higher T_4/T_3 and fT_4/fT_3 ratios, and higher percentages of fT_4 and fT_3, compared to T_4 and T_3. Another point is that goats stabled in the endemic goitre area also had high percentages of fT_4 and fT_3, compared to T_4 and T_3. These results confirm that changes in free iodothyronines generally follow those for total iodothyronines (22) and suggest the presence of a synergism between total and free amounts. For this reason, it is

possible to presume that, under circumstances of endemic iodine deficiency, a shift in T_4/T_3 balance will occur in favour of T_4, considered to be a reserve hormone.

Unexpectedly, the influences of an endemic area on the thyroid function of sheep and goats were different, with the significant involvement of T_3 and fT_4 in both ovine females and males, and of fT_3 changes in both caprine sexes. The obtained data suggest that sheep stabled in an endemic goitre area are probably capable of synthesizing more adequate T_4 and/or reducing its conversion to T_3, or to increase its metabolic clearance in peripheral tissues, as shown by the lowest T_3 levels observed in animals stabled in the endemic area; this hypothesis is partially superimposable on the goats stabled in an endemic goitre area, with T_3 levels unchanged.

These findings could probably be supported by suitable amounts of iodine in the diet for thyroid function for the species, or by an efficient iodine recycling system via gastrointestinal tract, which conserves iodine and can protect the animals against low dietary iodine, as has been reported in cows (23); however, animals stabled in endemic goitre area were kept on pasture most of the time, with plants being the primary source of iodine.

The obtained data confirm that the physiological ranges of thyroid hormones in ovine and caprine species are wide, because of the many intrinsic and extrinsic variables that can influence physiological thyroid hormone concentrations. In addition, in these small ruminants, a relative lack of information has been found regarding the use of thyroid hormone measurements to evaluate thyroid disorders and dysfunctions (24); therefore, the diagnosis of thyroid diseases in adult ovine and caprine populations, as well as in other species, has usually been difficult to perform, and the synthesis and mechanism of action of the thyroid hormones in ruminant physiology have been extrapolated from the extensive canine and feline knowledge (18).

Our data confirm the existence of significant differences between ovine and caprine sexes in thyroid hormone concentrations, although 2-way RM ANOVA showed a significant effect of sex on the fT_3 and fT_4 changes in sheep, and on T_3 and fT_4 changes in goats. The role of thyroid hormones in controlling seasonal reproduction in several mammalian species, including small ruminants, is well known (20). In ewes' rendered hypothyroids, the end of the reproductive season occurred later than in the controls (25). The permissive role of thyroid hormones seemed to be represented by the increase of the responsiveness to the oestradiol negative feed-back, but they are also involved in steroid-independent seasonal cycles in luteinising hormone pulse frequency (26). In male sheep, thyroidectomy abolished seasonal cycles of gonadotropin secretion and testicular size (27), and in male goats' testes T_3 level stimulates androgen release (28). Moreover, these observations do not provide additional evidence for the different involvement of thyroid hormones in accordance with female and male total and free iodothyronine concentrations**.** Moreover, our data are not in accordance with the results obtained in sheep, in which no statistically significant differences in thyroid hormone levels were found, due to sex (29), neither with lower T_3 levels reported in juvenile male goats than female (30). Higher T_4 levels observed in male than female sheep and goats are not in accordance with data reported by Celi et al. (30), which showed that T_4 levels of goats were not affected by sex. Thus, differences in T_4 levels could be explained on the basis of oestrogen-reduced catabolism of thyroxine-binding globulin, and androgen-inhibited TSH secretion by the pituitary (31).

Moreover, in sheep and goats stabled in the same areas of endemic human goitre, with severe hypothyroid cases of goitre, cretinism and deafness, no signs of any abnormal clinical symptoms associated with hypothyroidism were observed. Indeed, sheep and goats stabled in the endemic goitre area showed paradoxically higher T_4 concentrations, than the physiological and control values. It would, therefore, appear that ovine and caprine species, as also observed in equines (32), cope with an endemic environment through a significant thyroidal response in order to synthesize a representative reserve of T_4. However, it is not possible to exclude the existence of an inhibition of the enzyme 5'- deiodinase, which is responsible for the conversion of T_4 to T_3, which could represent an adaptive mechanism to decrease the metabolic rate during iodine deficiency, as reported by Duckett (33) during illness and stress**.** The results would suggest a physiological adaptive response of the ovine and caprine species to a low iodine environment. In addition to that, animals of small farms A and B, which did not receive iodine supplementation,

are fully dependent on the natural local iodine source. Therefore, the results obtained in sheep and goats, fed roughage of local harvests, can be regarded as another valuable information on the iodine status in the Sicilian area.

Using the values found in humans (34) as the basis for comparison, the obtained data showed that the sheep exhibit higher total circulating T_4 at 102.79 nmol/L (non-endemic goitre area) and 113.31 nmol/L (endemic goitre area), and the goats exhibit lower total circulating T_4 at 106.64 nmol/L (non-endemic goitre area) and 102.33 nmol/L (endemic goitre area), than the values found in euthyroid humans (95.23 nmol/L) and goitre humans (82.36 nmol/L).

The possibility that hypothyroidism cases are present in adult sheep and goats is thus excluded; this is supported not only by the lack of clinical signs, but also by the rarity of the cases reported, being limited exclusively to lambs and lambings and as a possible consequence of foetal or precocious death.

In conclusion, endemic areas for human goitre are not necessarily so for small adult farm animals that show an adaptive strategy without presenting any clinical signs of thyroid disorders. Evidence to prove this hypothesis should be obtained by assessing iodine status not only in urine, but also in the milk of sheep and goats, especially in animals living and stabled in endemic goitre areas, as suggested in humans (35) and reported by the World Health Organization (36). However, the goitre areas of Sicily remain a serious environmental and health problem for humans and represent a potential economic threat for animal production.

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DOLOČANJE VEZANIH IN PROSTIH ŠČITNIČNIH HORMONOV V KRVI PRI ODRASLIH OVCAH IN KOZAH: VPLIV ENDEMIČNIH OBMOČIJ Z GOLŠAVOSTJO

E. Fazio, P. Medica, C. Cravana, A. Ferlazzo

Povzetek: Severovzhodna Sicilija je območje, kjer se redno pojavljajo motnje, povezane s pomanjkanjem joda pri ljudeh in živalih. Cilj raziskave je bil raziskati pojavnost endemične golšavosti pri odraslih ovcah in kozah z merjenjem ravni jodotironinov na različnih lokacijah na Siciliji. Upoštevane so bile tudi razlike med spoloma. Skupno je bilo v raziskavo vključenih 48 ovc pasme comisana in 51 malteških koz. Pri ovcah je bilo vključenih 10 samic in 6 samcev z območij brez golšavosti (kmetija A: kontrolna skupina) ter po 16 samic in samcev iz endemičnih območij z golšavostjo (kmetija B: opazovana skupina). Pri kozah je bilo vključenih 6 samic in 13 samcev iz območij brez golšavosti (kmetija A: kontrolna skupina) in po 16 samic in samcev iz endemičnih območij z golšavostjo (kmetija B: opazovana skupina). Rezultati so pokazali nižje T_3 in višje T_4 vrednosti ($p < 0,0001$) pri samcih in samicah ovac in višje ravni T_4 pri samcih ($p < 0,0001$), ki so bili nastanjeni na kmetiji B v primerjavi s kmetijo A. V primerjavi s kmetijo A so pri kozah, ki so bile nastanjene na kmetiji B, ugotovili višje ravni prostega T_3 ($p < 0,0001$), pri samcih na kmetiji B nižje ravni T_4 ($p < 0,0001$), pri samicah pa višjo raven prostega T_4 ($p < 0,0001$). Ugotovljene so bile statistično značilne razlike med skupinami tako glede na spol kot glede na endemičnost področja na ravni vezanih in prostih jodotironinov pri ovcah in kozah. Pridobljeni podatki so pokazali, da območja z endemično golšavostjo pri ljudeh nimajo nujno enakega učinka pri ovcah in kozah, za katere se zdi, da so sposobne ustvariti prilagoditveno strategijo, zaradi katere se ne pokažejo klinični znaki bolezni ščitnice.

Ključne besede: ščitnični hormoni; ovce; koze; endemična območja z golšavostjo

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Original Research Article

EFFECTS OF CERTAIN DRUGS ON DIHYDROPYRIMIDINE DEHYDROGENASE ENZYME PURIFIED FROM BOVINE LIVER

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Abstract: The inhibitory effects of certain drugs on dihydropyrimidine dehydrogenase from the bovine liver have been investigated. Dihydropyrimidine dehydrogenase [5, 6-dihydrouracil: NADP+ oxidoreductase, EC 1.3.1.2; DPD] enzyme was purified from the bovine liver. The purification was performed by preparation of homogenate, ammonium sulphate precipitation, and affinity chromatography. Moreover, some important modifications were made in the purification procedure. Purification of bovine liver DPD enzyme was obtained with a yield of 12.5%. SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme, and the electrophoretic pattern is discussed in this article. In addition, the effects of certain drugs on bovine liver dihydropyrimidine dehydrogenase enzyme activity were investigated. Oxytetracycline, ciprofloxacin, ceftazidime, cefoperazone, amikacin, ornidazole, metronidazole, cefuroxime, cefepime, ampicillin, and amoxicillin, were used as drugs. All the drugs indicated the inhibitory effects on the enzyme. IC_{50} values of the drugs were determined by plotting activity % vs [I]. IC_{50} values of oxytetracycline, ciprofloxacin, ceftazidime, cefoperazone, amikacin, ornidazole, metronidazole, cefuroxime, cefepime, ampicillin, and amoxicillin 0.030, 0.046, 0.140, 0.610, 1.820, 2.500, 3.600, 4.330, 4.370, 4.920, and 6.300, mM; the Ki constants were 0.050±0.01, 0.090± 0.06, 0.130±0.045, 0.185±0.057, 2.010±0.55, 2.096±1.06, 2.115±1.00, 2.700±0.56, 3.730±1.48, 5.240±1.04, and 9.570±2.84, mM for bovine liver DPD, respectively. Ki constants for dihydropyrimidine dehydrogenase were determined by Lineweaver-Burk graphs. All drugs showed non-competitive inhibition patterns.

Key words: dihydroprimydin dehydrogenase; bovine liver; drug; inhibition

Introduction

Dihydropyrimidine dehydrogenase [5, 6-dihydrouracil: $NADP^+$ oxidoreductase, EC 1.3.1.2; DPD] the initial rate-limiting enzyme in pyrimidine catabolism, catalyses the conversion of pyrimidine and NADPH, to dihydropyrimidine and $NADP^+$. The enzyme DPD has an important function in the synthesis of the neurotransmitter β-alanine. It is the first enzyme in a series of enzymatic reactions converting Uracil to β-alanine, 5-florouracil to α-fluoro-β-alanine and thymine to β-amino isobutyrate (1, 2). DPD is a cytosolic enzyme that is present in various mammalian tissues, especially liver, kidney, pancreas, and lung (3). It has been purified from different species, including from human, rat, porcine and bovine livers. The molecular size of homodimeric DPD is roughly 220 kDa. It is known that each monomer of DPD contains a flavin adenine dinucleotide (FAD), a flavin mononucleotide (FMN), and four FeS groups (4, 5, 6). Recently the importance of the DPD enzyme has been more and more appre-

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ciated. Especially in cancer therapy, it has been found that the function of DPD is related to the efficacy of chemotherapy. Drugs used in chemotherapy such as 5-fluorouracil (5FU), a widely used anticancer drug (7), inhibit thymidylate synthetase which catalyses the synthesis of thymine. Inhibition of thymidylate synthetase prevents DNA synthesis in cancer cells. However, accumulation of 5FU causes toxic effects in the organism. In liver 5FU is normally catabolised and thus removed from the system. In this process DPD is essential as it first converts 5FU to 5-fluoro 5, 6-dihidrouracil and then into α-fluoro-β-alanine in a two-step mechanism. Genetic deficiency or inhibition of DPD might cause a lethal effect in cancer patients. Therefore, it is crucially important to know the inhibitors and activators of this enzyme (8).

The aim of this study was to purify bovine liver dihydropyrimidine dehydrogenase and the determination of inhibition or activation effects of some drugs (oxytetracycline, ciprofloxacin, ceftazidime, cefoperazone, amikacin, ornidazole, metronidazole, cefuroxime, cefepime, ampicillin, and amoxicillin) on the purified DPD.

No reports could be found in the literature on the *in vitro* and *in vivo* effects of the above drugs on bovine liver DPD.

Materials and methods

Materials

Uracil, dithiothreitol (DTT), NADPH, Tris and the other chemicals were purchased from Sigma, and the drugs were purchased from Hoechst Marian Roussel (Turkey).

Activity determination

DPD activity was measured spectrophotometrically at 25 °C as described by Podschun et al. (9). Briefly, the enzyme sample was added to a 1mL (final volume) incubation mixture containing 280 mM K_3PO_4 (pH 7.4), 8 mM DTT, 20 mM $MgCI_2$, 600 μM NADPH and 1.5 mM uracil and the decrease in absorption at 340 nm measured due to the oxidation of NADPH at 25 °C. One enzyme unit represents the oxidation of 1μmol of NADPH min^{-1} at 25 °C, pH 7.4.

Preparation of the homogenate

Bovine liver (60 g) was obtained from a slaughterhouse in Erzurum, Turkey. A liver sample was cut with a knife. Excess blood, foreign tissues and membranes were removed from the samples. The livers were washed three times with buffer A (35 mM K_3PO_4, 2.5 mM $MgCl_2$, 0.25 M sucrose, 10 mM ethylenediaminetetraacetic acid (EDTA), 2 mM DTT, 1 mM aminoetilizotiyoürebrobür, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4) and were homogenized by liquid nitrogen, transferred to the same buffer, and centrifuged at 4 °C, 13,500 g for 120 min. Supernatant was used in further studies (9).

Ammonium sulphate precipitation

The homogenate was subjected to orderly precipitation with ammonium sulphate (10–20%, 20–30%, 30–40%, 40–50%, 50–60%, 60–70%, 70-80% and 80–90%). Ammonium sulphate was slowly added to the haemolysate for complete dissolution. This mixture was centrifuged at 4 °C, 10,000 g for 15 min and the precipitate was dissolved in buffer A containing 1 mM DTT. For each respective precipitation, the enzyme activity was determined both in the supernatant and in the precipitate. The enzyme was observed to precipitate at 40–60% saturation. It was then dialysed at 4 °C in buffer A containing 0.5 mM DTT for 2 h with two changes of buffer (9).

Purification of bovine liver DPD

The column was equilibrated with buffer A. The ammonium sulphate fraction (40–60%) of the homogenate obtained above was loaded onto a 2', 5'-ADP Sepharose 4B affinity column, and the flow rate was adjusted to 20 mL/h. The column was then sequentially washed with buffer A. Washing continued until an absorbance of 0.05 at 280 nm was obtained. Some important modifications had been made during the purification process. The protein fractions within the solution were precipitated with ammonium sulphate and loaded onto an affinity column. However, high DPD enzyme activity was observed in the washing fractions. We suspected that Glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) and

glutathione reductase (GR) enzymes have higher affinities than DPD to the used column, DPD enzyme was unable to bind to the column; thus, it was present at very high levels within washing fractions. To solve this problem, firstly G6PD, 6PGD and GR enzymes were removed from the column by using their own eluting solutions. Then, these elutes, which were free of G6PD, 6PGD and GR enzymes, were reloaded onto the same column, which had been washed and regenerated. The washing solution was collected and subsequently analysed. No DPD activity was detected indicating that DPD enzyme was bound to the column. Then, elution of the DPD enzyme was carried out with buffer A containing 0.5 mM DTT, 0.2 mM NADPH and 1 M KCl. Enzyme activity was measured in all fractions, and the activity-containing fractions were pooled, then dialysed in buffer A containing 1 mM DTT for 2 h with two changes of buffer. All procedures were performed at 4 °C (9).

Protein determination

The protein content in all samples was quantified spectrophotometrically at 595nm according to Bradford's (10) method using bovine serum albumin as standard.

SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

Enzyme purity was examined using Laemmli's (11) procedure with 3% and 8% acrylamide concentrations for running and stacking gel, respectively. *E. coli* β-galactosidase (116,000 Da), bovine albumin (66,000 Da), chicken ovalbumin (45,000 Da), bovine carbonic anhydrase (29,000 Da), and stokrom c (12,400 Da), were used as standards (Sigma) (See Fig. 1).

In vitro effect of drugs

In order to determine the effects of some drugs on bovine liver DPD, concentrations of oxytetracycline (0.022–0.154 mM), ciprofloxacin (0.018–0.12 mM), ceftazidime (0.078–0.47 mM), cefoperazone (1.49–7.45 mM), amikacin, (1.28–3.40 mM), ornidazole, (0.038–0.303 mM), metronidazole, (0.87–2.04 mM), cefuroxime, (2.94-–14.70 mM), cefepime, (1.74–8.70 mM), ampicillin, (9.12–27.36 mM), and amoxicillin, (2.05–20.50 mM), were added to the reaction mixture and the enzyme activity was measured. An experiment in the absence of drug was used as a control (100% activity). The IC_{50} values were obtained from activity (%) vs. drug concentration plots (Fig. 2). In order to determine the K_i values, the substrate (Uracil) concentrations were 0.0075, 0.015, 0.075, 0.15, 0.225mM for drugs. Inhibitor (drug) solutions were added to the reaction mixture at 3 different fixed concentrations. Lineweaver-Burk graphs were drawn using 1/V vs. 1/[S] values and the K_i values were calculated from these graphs (see Fig. 3).

Results

Purification of the enzyme led to a specific activity of 1.66 EU/mg proteins, a yield of 12.5% and 1660-fold purification (Table 1). SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme, and the electrophoretic pattern is shown in Fig. 1. IC_{50} values of oxytetracycline, ciprofloxacin, ceftazidime, cefoperazone, amikacin, ornidazole, metronidazole, cefuroxime, cefepime, ampicillin, and amoxicillin 0.030, 0.046, 0.140, 0.610, 1.820, 2.500, 3.600, 4.330, 4.370, 4.920, and 6.300, mM, respectively, and the K_i constants were 0.050±0.01, 0.090±0.06, 0.130±0.045, 0.185±0.057, 2.010±0.55, 2.096±1.06, 2.115±1.00, 2.700±0.56, 3.730±1.48, 5.240±1.04, and 9.570±2.84, mM for bovine liver DPD, respectively. Whole drugs showed non-competitive inhibition. Representative graphs are shown for oxytetracycline (Fig. 2 and 3).

Discussion

Dihydropyrimidine dehydrogenase [5, 6-dihydrouracil: $NADP^+$ oxidoreductase, EC 1.3.1.2; DPD] the initial rate-limiting enzyme in pyrimidine catabolism, catalyses the conversion of pyrimidine and NADPH, to dihydropyrimidine and $NADP^+$. The enzyme DPD has an important function in the synthesis of the neurotransmitter β-alanine. It is the first enzyme in a series of enzymatic reactions converting Uracil to β-alanine, 5-florouracil to α-fluoro-β-alanine and thymine to β-amino isobutyrate (1, 2). In the liver, 5FU is normally catabolised and thus removed from the system. In this process, DPD is essential as it first converts 5FU to 5-fluoro 5, 6-dihidrouracil. Genetic deficiency or inhibition of DPD might cause

Table 1: Purification scheme of DPD from bovine liver

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity	Specific activity (EU/mg)	Yield (%)	Purification factor
Homogenate	0.020	35	19.26	672	0.70	0.001	100	1
Ammonium sulphate precipitation (40-60%)	0.055	11	4.90	53.90	0.605	0.011	86.4	11
2',5'-ADP Sepharose 4B affinity hromatography	0.011	8	0.0066	0.048	0.088	1.66	12.5	1,660

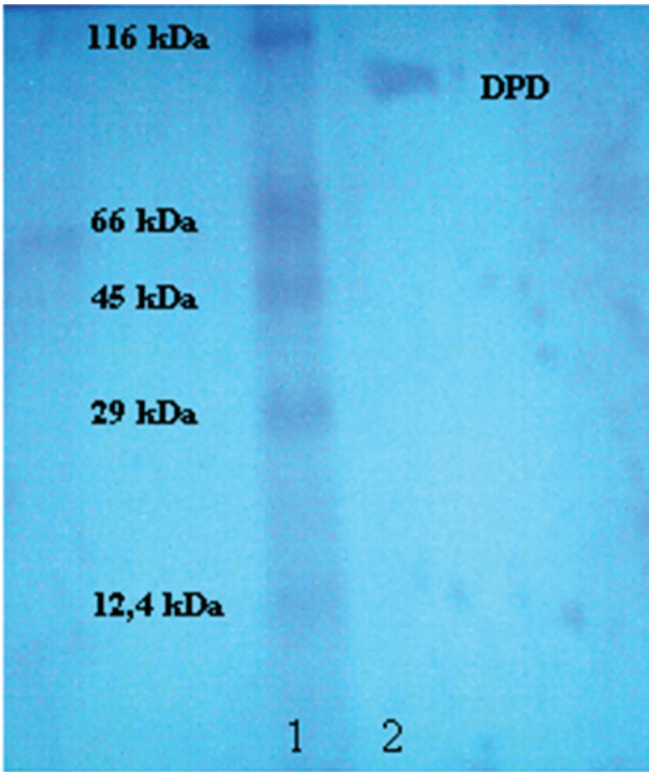

Figure 1: SDS-PAGE bands (Lane 1: Standards: E. coli β-galactosidase (116,000 Da), bovine albumin (66,000 Da), chicken ovalbumin (45,000 Da), bovine carbonic anhydrase (29,000), and stokrom C (12,400 Da) Lane 2: Bovine liver DPD

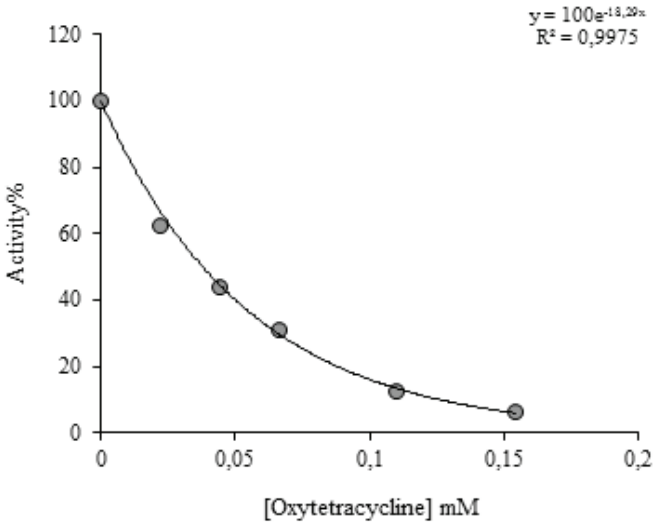

Figure 2: Activity % *vs* [Oxytetracycline] regression analysis graphs for bovine liver DPD in the presence of 5 different oxytetracycline concentrations

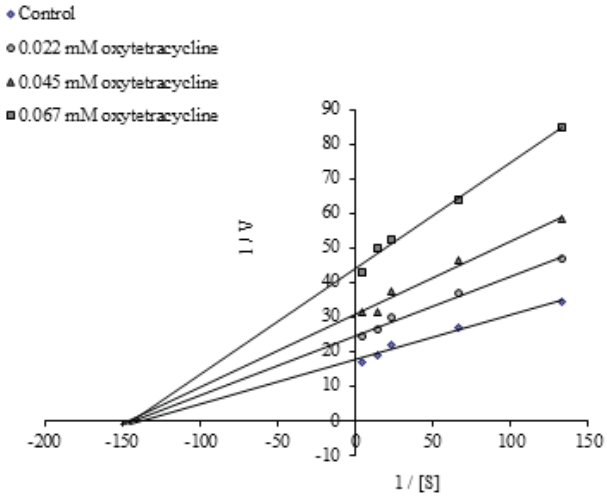

Figure 3: Lineweaver-Burk graph for 5 different substrate concentrations and 3 different oxytetracycline concentrations for determination of Ki

lethal effects in cancer patients. Therefore, it is crucially important to know the inhibitors and activators of this enzyme (8).

In order to determine the activators and inhibitors of this enzyme, it is necessary to investigate the effects of those drugs on *the enzyme's* activity. For this purpose, the enzyme was purified using affinity chromatography on a 2', 5'-ADP-Sepharose column. Some important modifications had been made during the purification process. The protein fractions within the solution were precipitated with ammonium sulphate and loaded onto an affinity column. However, high DPD enzyme activity was observed in the washing fractions. We suspected that G6PD, 6PGD, and GR enzymes have higher affinities than DPD to the used column, DPD enzyme was unable to bind to the column; thus, it was present at very high levels within washing fractions. To solve this problem, firstly G6PD, 6PGD and GR enzymes were removed from the column by using their own eluting solutions. Then, these elutes, which are free of G6PD, 6PGD, and GR enzymes, were reloaded onto the same column, which had been washed and regenerated. The washing solution was collected and subsequently analysed. No DPD activity was detected, indicating that that DPD enzyme was bound to the column. Then, elution of the DPD enzyme was carried out with buffer A containing 0.5 mM DTT, 0.2 mM NADPH, and 1 M KCl. Thus, the DPD enzyme was then purified 1660-fold and with a 12.5% yield.

The effects of many drugs such as antibiotics, analgesics, and anaesthetics on human G6PD, 6PGD, GR and CA have been investigated (12, 13, 14, 15). Another study shows that anaesthetics inhibited Na^+, K^+-ATPase, Mg^{+2}-ATPase and acetylcholinesterase activity from rat cerebral cortexes (16).

However, to the best of our knowledge, the inhibitory effects of the drugs examined here on DPD in the bovine liver have not been studied. In order to show inhibitory effects, while the most suitable parameter is the Ki constant, some researchers use the IC_{50} value (17, 18). Therefore, in this study, both the Ki and IC_{50} parameters of these drugs for DPD were determined. Whole drugs showed non-competitive inhibition. In this study, the drugs inhibited DPD activity in comparison with the control group. The drugs can cause non-competitive inhibition by binding to other sites affecting the three-dimensional structure of the enzyme (19).

Ki values show that oxytetracycline had the highest inhibitory effect, followed by ciprofloxacin, ceftazidime, cefoperazone, amikacin, ornidazole, metronidazole, cefuroxime, cefepime, ampicillin, and amoxicillin. IC_{50} values showed the same trend. According to Ki constants and IC_{50} values, *the enzyme is mostly inhibited by* oxytetracycline, ciprofloxacin, ceftazidime, and cefoperazone drugs. The chemical structures of all these drugs contain an active group of carbonyl, hydroxyl, and nitrogen.

Ki and IC_{50} parameters show that the inhibitory effect of oxytetracycline, ciprofloxacin, ceftazidime, and cefoperazone are highly potent. Therefore, it is thought that if these drugs inhibit DPD activity, they would cause significant problems in metabolism. Pyrimidine catabolism is obstructed, and β-alanine cannot be synthesized. Patients using antibiotics that inhibit DPD activity together with chemotherapy drugs, such as 5-fluorouracil, might experience poisoning and a lethal effect because of the accumulation of 5FU. In conclusion, DPD is the initial and the rate-limiting enzyme of pyrimidine catabolism. Moreover, it functions in the synthesis of β-alanine and also removes the toxic effects of 5FU from the system. Therefore, the dosage of antibiotics which inhibit DPD activity might be of great significance in cancer therapy.

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VPLIVI NEKATERIH ZDRAVIL NA ENCIM DIHIDROPIRIMIDIN DEHIDROGENAZO, PRIDOBLJEN IZ GOVEJIH JETER

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Povzetek: Proučevani so bili zaviralni učinki nekaterih zdravil na encim dihidropirimidin dehidrogenazo, pridobljen iz govejih jeter. Encim dihidropirimidin dehidrogenaza [5, 6-dihydrouracil: NADP + oksidoreduktaza, ES 1.3.1.2; DPD] so pridobili iz govejih jeter s pripravo homogenata, obarjanjem z amonijevim sulfatom in afinitetno kromatografijo. Poleg tega so bile v postopek prečiščevanja vnešene nekatere dodatne manjše prilagoditve. S postopkom prečiščevanja je bilo iz govejih jeter pridobljeno 12,5 % encima DPD. Po čiščenju encima je bila opravljena poliakrilamidna gelska elektroforeza z SDS. Raziskani so bili tudi učinki nekaterih zdravil na encimsko aktivnost dihidropirimidin dehidrogenaze v govejih jetrih. Kot zdravila so bili uporabljeni oksitetraciklin, ciprofloksacin, ceftazidim, cefoperazon, amikacin, ornidazol, metronidazol, cefuroksim, cefepim, ampicilin in amoksicilin. Vsa zdravila so na encim delovala zaviralno. Vrednosti IC_{50} proučevanih zdravil so bile določene z grafičnim modelom, pri katerem je prikazan odstotek aktivnosti v odvisnosti od [i]. IC_{50} vrednosti oksitetraciklina, ciprofloksacina, ceftazidima, cefoperazona, amikacina, ornidazola, metronidazola, cefuroksima, cefepima, ampicilina in amoksicilina so bile 0,030, 0,046, 0,140, 0,610, 1.820, 2.500, 3.600, 4.330, 4.370, 4.920 in 6.300 mM; konstante Ki pa 0,050 ± 0.01, 0.090 ± 0,06 0,130 ± 0,045, 0,185 ± 0,057, 2.010 ± 0,55, 2.096 ± 1.06, 2.115 ± 1,00, 2.700 ± 0,56, 3.730 ± 1,48, 5.240 ± 1.04 in 9.570 ± 2,84 mM za DPD govejih jeter. Konstante Ki so bile za dihidropirimidin dehidrogenazo določene z grafom Lineweaver-Burk. Vsa zdravila so pokazala nekompetitivne zaviralne vzorce delovanja.

Ključne besede: dihidroprimidin dehidrogenaza; goveja jetra; zdravila; zaviranje

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Original Research Article

EVALUATION OF ANTIMICROBIAL EFFECT OF IRANIAN SUMAC ON *Bacillus cereus* IN A COMMERCIAL BARLEY SOUP

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Abstract: The antimicrobial effect of different concentrations of water extract of sumac (*Rhus coriaria* L.) was studied on the growth of *Bacillus cereus* (ATCC 11778) by using sterilized samples and in a single incubation temperature (30 °C) during 6 days. After obtaining and powdering sumac, its water extract was prepared. After inoculation of bacteria and adding different concentrations of water extract, the antimicrobial effect of sumac was studied on *Bacillus cereus* at several concentrations. The results showed that concentrations of 0.3%, 0.5%, 1% and 2.5% had an inhibition effect on *Bacillus cereus*, and only concentration of 0.1% could not inhibit the growth of *Bacillus cereus*. According to the finding of this research, water extract of sumac (*Rhus coriaria* L.) has a retention effect against *Bacillus cereus* in soup and can be considered as a natural preservative in some food.

Key words: antimicrobial effect; sumac; *Bacillus cereus*; commercial barley soup

Introduction

Food antimicrobials are mostly synthetic chemicals, so they are limited to use in foods, because they may cause adverse effects on the health of consumers (Wetherilt and Pala, 1994). Therefore, much attention in recent years has been focused on extracts from herbs and spices, which have been used for many centuries to improve the sensory characteristics and to extend the shelf life of foods (Chung et al, 1998). Various tanniniferous plants, including sumac (*Rhus coriaria* L.), have been known to contain naturally occurring compounds with antimicrobial activities (Nasar-Abbas and Halkman , 2004).

The word of "Sumac" is derived from Aramaic word "Sumaqa" which means red (Zargari, 1996). Sumac *(Rhus coraria* L.*)* is a member of the Anacardiaceae family and it has many applications in different countries. Sumac is a shrub with a long history application in traditional medicine and Iranian cuisine. It is used as medical herb and spices as a condiment and sprinkled over kebobs, grilled meats, soups, and some salads. It is used in traditional medicine for treatment of indigestion, anorexia, diarrhea, hemorrhages, hyperglycemia,

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ocular trachoma and ear infection (Wetherilt and Pala, 1994; Chung et al, 1998; Nasar-Abbas and Halkman, 2004). It is grown wild in the region from the Canary Island over the Mediterranean area to Iran and Afghanistan (Rayne et al., 2007). In Iran sumac is grown in Mazandaran, Azarbayegan, Khorasan, Shiraz, Ghazvin, Ghom and Hamedan. Sumac is used as an antioxidant and food preservative. Sumac reduces blood sugar and uric acid; these studies have shown that sumac dramatically inhibits the alpha-amylase enzyme. Alpha-amylase is responsible for the breakdown of starch to simpler sugars. Inhibition of this enzyme by sumac increases glucose-tolerance in diabetic patients (Cowan, 1999; Chung, 1998). Sumac in powder form commonly used in a variety of foods in Iran. Since *Bacillus cereus* is one of the common foodborne pathogen that cause diarrhea and vomiting in Iran, so sumac as one of the inhibitors of the growth of *Bacillus cereus* has a considerable role.

B. cereus-associated foodborne illness occurs as two distinct intoxication syndromes: emetic and diarrhoeal. Recovery is rapid for both syndromes, usually within 12-24 hours. There are usually no long-term effects, but severe consequences, including fatalities, can occasionally occur (Ahani and Alipour Eskandani, 2013). Transmission is predominantly foodborne. Most raw foods will contain *B. cereus* spores, as do many dried herbs, spices and dehydrated foods. Emetic illness is frequently linked with raw starchy foods of plant origin (such as rice, pasta, potatoes, pastries and noodles) (Kosar et al, 2007). In 95% of emetic cases, fried or cooked rice is implicated (Razavilar, 2008). Diarrhoeal illness is often associated with meat products, soups, vegetables, sauces and milk/milk products. Dairy products may spoil through the growth of spores (including the spores of psychrotrophs) that survive pasteurization (Jenson and Moir, 2003).

According to the lack of available studies on the antimicrobial effects of Iranian Sumac against *Bacillus cereus* with considering the common use of this plant as food additives in Iran, the antimicrobial effect of water extract of this medicinal plant against *Bacillus cereus* was investigated.

Material and methods

Sumac preparation

Sumac shrub fruits were collected from the orchards of the city of Ahar, Ahar Arsban, Hrand region and Rahim Bigelow village in East Azerbaijan province of Iran.

Preparations of water extracts of sumac

Crushed dried fruit after the well was crushed with a porcelain mortar to a powder income, then 5 grams of sumac powder was soaked in 95 ml of distilled water for 1 hour at room temperature with occasional stirring followed by gentle boiling for 2 min on a plate heater (the mixture was stirred intervals for one hour). Finally, the extract was obtained by cooling and filtration (Nasar-Abbasa and Kadir Halkman, 2004).

Preparations of food model (commercial barley soup)

Mahnam commercial barley soup (Mahnam Co., Karaj, Iran). to commodity number D891 was used in this study. According to the manufacturer's protocol, he contents of a packet (about 75 grams of powdered soup) dissolved in a liter of distilled water and to prepare for use were exposed in the boiling heat for 20 minutes. After cooling to 4 °C and pass the soup through a colander, in volumes of 80 ml were distributed in autoclavable glass containers and a magnet was added to each container (Alipour- Eskandani, 2009).

Microorganism and growth conditions

Bacillus cereus ATCC 11778, was donated from food microbiology laboratory of the Faculty of Veterinary Medicine of Tehran University, were used as test organisms. This bacteria in the lyophilized form were transferred into 10 ml Brain-Heart Infusion broth (BHI) and were prepared by culturing bacteria twice consecutively in BHI broth and incubating at 37 °C for 18 hours. Then the second 24 hour culture was mixed with 50% sterile glycerin and were distributed in the micro eppendorf and were stored inside the freezer (Alipour- Eskandani, 2009).

Preparation of samples to start incubation

To investigate the effect of water extract of sumac, the required quantities of water extract of sumac to produce the concentrations of 0%, 0.1%, 0.3%, 0.5%, 1% and 2.5% , was added to the glasses that contain 80 ml soup. After adding water extracts, samples were sterilized in an autoclave at 121 °C for 15 minutes. Then after autoclaved samples were cooled to ambient temperature, 10^3 *Bacillus cereus* ATCC in per ml soup (per 80 ml soup, 8×10^4 bacteria) were inoculated into the glasses that contain samples under sterile conditions under the hood. It should be noted, as mentioned bacteria in the Eppendorf microtubes - were stored inside the freezer, therefore, to use a solution containing bacteria after being thawed was transferred to a BHI broth and was stored at 37 °C for 18 hours. Again, second culture of this 18 hour culture was prepared in broth BHI (for 18 hours at 37 °C). After bacteria inoculation, the samples were transferred to a 30 °C incubator (Alipour- Eskandani, 2009).

Evaluation of samples for growth of bacteria

The growth of bacteria was assessed on day zero, 1, 3 and 6; in each of the days, 6 different dilutions (from 1-10 to 6-10) were made from each sample, then from each dilution on plates BHI agar surface culture (SPC) was performed. The amount of growth of bacteria in samples, by counting the number of colonies that is grown on the plate was seen and recorded by Colony Counter.

Statistical analysis

Because of the growth of bacteria is exponential, and over time the distribution of the number of bacteria in different samples is ousted from normal distribution. In order to normalize the data and avoid working with very large means and standard deviations, the logarithm of the number of bacteria on the basis of 10 was used in the analysis. To compare treatment means, Analysis of Variance was used in every day. To compare means changes on different days of tests Repeated measures was used. Significance level was considered P-Value>0/05. SPSS 18 statistical software was used to analyze the data.

Results and discussion

In Figure 1, the logarithm of the number of bacteria in 6 samples with different concentrations of sumac shown during testing. As this graph shows, sumac extracts have a bactericidal

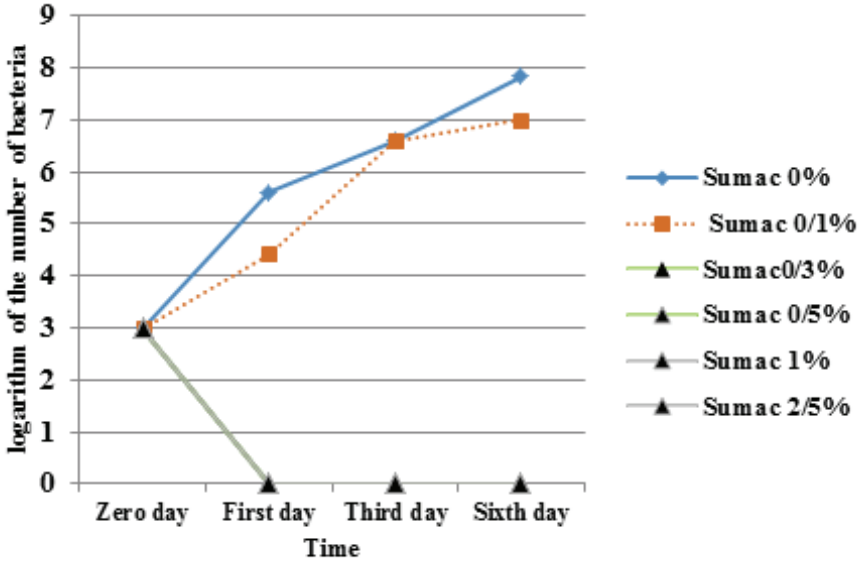

Figure 1: Comparison of effect of the different concentration of water extract of sumac on the logarithm of the number of *Bacillus cereus* during the storage period at 30 °C

effect in concentrations of 0/3%, 0/5%, 1% and 2/5%, but the bacteria have increasing growth only in concentration of 0/1% and there is not inhibitory effect. Analysis of Variance (ANOVA) showed that on the first, third and sixth, the logarithm of the number of bacteria in different treatments was statistically significant (in every three days the amount of P Value was less than 0/001), also Repeated Measures test showed logarithm changes of the number of bacteria in significant during the experiment (P Value <0.001).

Nasar-Abbas and Kadir Halkmanb in 2004 studied the antimicrobial effect of water extracts of sumac *(Rhus coriaria* L.*)* at different concentrations on the growth of 12 bacterial spp. (six Gram positive and six Gram negative bacteria), mostly food borne including pathogens. They found to be effective against all the test organisms with Gram positive bacteria being more sensitive than Gram negative bacteria. Among the Gram positive organisms, *Bacillus* species (*Bacillus cereus, Bacillus megaterium, Bacillus subtilis*, and *Bacillus thuringiensis*) were found to be the most sensitive followed by *Staphylococcus aureus*, while *Listeria monocytogenes* was found to be the least sensitive Of the Gram negative organisms, *Salmonella enteritidis* was found to be the most resistant. Bacteriostatic/bactericidal effects of sumac, as studied by enumerating survival by the viable count technique after 1 h direct contacts of each microorganism with various concentrations of sumac extract, revealed a 4–5 log cycle reduction in *Bacillus spp*. and 2–3 log cycle reduction in other bacteria tested with 1.0% sumac extract.

Moshtaghi *et al.*, 2013 investigated the antibacterial effect of ethanolic extract of Sumac *(Rhus coriaria* L.*)* on *Escherichia coli*, quantitatively and qualitatively. The results of well diffusion test showed that extracts of Sumac in a concentration of 0.5%, 1%, 2.5% and 5% could inhibit *E. coli*. The results from the evaluation of the antibacterial effects of the Sumac revealed that at 4 and 15°C, the growth of *E. coli* in test tubes containing meat extracts has increased throughout the 48 h incubation period. Results showed that the growth of this bacteria in different concentration of Sumac extract as decreased in the both tested temperatures in comparison to time zero ($p<0.05$). Furthermore, there was a significant difference in the number of microorganisms at various times between control and experimental groups in both tested temperatures ($p<0.05$). Gabr *et al.*, in 2014 studied the evaluation of *Rhus coriaria* (sumac) extracts as potential new sources of antimicrobial and antioxidant activity. The active constituents like, alkaloids, glycosides, phenol and terpenoids of sumac were extracted and separated using GC-MS. *R. coriaria* extract showed a higher content of Phenols compared to the other active constituents (glycosides, alkaloids and terpenoids). Total *R. coriaria* extract and its active constituent's phenol and glycosides were the most effective as antioxidant and antibacterial agents compared to alkaloids and terpenoids. The antibacterial activity of these compounds may relate to its total antioxidant activity. Therefore, *R. coriaria* extract and its constituents could act as bactericidal agents against bacterial infection and as a natural preservative in food against food borne diseases.

In this study antimicrobial effect of different concentrations (0%, 0.1%, 0.3%, 0.5%, 1% and 2.5%) of *Rhus coriaria* L. water extract on *Bacillus cereus* (ATCC 11778) was evaluated. The results showed this extract had an inhibition effect on the growth of *Bacillus cereus*. According to the finding of this research, water extract of sumac (*Rhus coriaria* L.) has a retention effect against *Bacillus cereus* in soup and can be considered as a natural preservative in some food.

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UGOTAVLJANJE PROTIMIKROBNEGA VPLIVA IZVLEČKA IRANSKEGA RUJA NA RAST BAKTERIJ *Bacillus cereus* V INDUSTRIJSKO PRIPRAVLJENI JEŠPRENJEVI JUHI

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Povzetek: Proučevan je bil protimikrobni učinek različnih koncentracij vodnega izvlečka ruja (*Rhus coriaria* L.) na rast bakterij *Bacillus cereus* (ATCC 11778) v industrijsko pripravljeni ješprenjevi juhi (ričet) z uporabo steriliziranih vzorcev pri inkubacijski temperaturi 30 °C v času 6 dni. Po pridobitvi in uprašitvi ruja je bil pripravljen njegov vodni izvleček. Po inokulaciji bakterij in dodajanju različnih koncentracij vodnega izvlečka je bil proučevan protimikrobni učinek različnih koncentracij ruja na *Bacillus cereus*. Rezultati so pokazali, da je imelo dodajanje izvlečka ruja ješprenjevi juhi v koncentracijah 0,3 %, 0,5 %, 1 % in 2,5 % zaviralni vpliv na rast bakterij *Bacillus cereus*, pri koncentraciji 0,1 % pa izvleček ruja rasti bakterije *Bacillus cereus* ni zaviral. Glede na ugotovitve raziskave ima vodni izvleček ruja (*Rhus coriaria* L.) učinek zadrževanja rasti bakterije *Bacillus cereus* v juhi in bi ga lahko uporabili kot naravni konzervans v različnih vrstah hrane.

Ključne besede: protimikrobni učinek; ruj; *Bacillus cereus*; ješprenjeva juha; ričet

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Original Research Article

BIOCHEMICAL AND CHEMICAL PARAMETERS CHANGES IN THE BLOOD OF CHICKENS FOLLOWING TREATMENTS WITH MADURAMYCIN, MONENSIN AND DICLAZURIL

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Abstract: The aim of this study was to monitor the biochemical and chemical parameters of the blood of commercial chickens following treatment with one of three coccidiostats: maduramycin, monensin or diclazuril. Chickens received feed treated with maduramycin at concentrations of 5, 10 and 15 mg kg^{-1}, monensin at 125, 225 and 325 mg kg^{-1} or diclazuril at 1, 5 and 10 mg kg^{-1}. A control group of chickens consumed feed without the addition of coccidiostats. Following treatment, blood was sampled for 11 days and analysed for the following biochemical and chemical parameters: aspartate aminotransferase (AST), bile acid (BA), creatine kinase (CK), uric acid (UA), glucose (GLU), cholic acid (CA), total protein (TP), albumin (ALB), globulin (GLOB) and phosphorus (PHOS). Administration of different concentrations of maduramycin, monensin and diclazuril did not affect the concentration of the parameters AST, UA, GLU, BA, TP, ALB, GLOB and PHOS in experimental groups of broilers in relation to the control group. However, significant differences were observed in the concentrations of CK and CA between the experimental and control groups. Significant differences were also found in the concentrations of AST, CA and UA between experimental groups.

Key words: maduramycin; monensin; diclazuril; chickens; biochemical and chemical parameters

Introduction

Coccidiosis in chickens, caused by *coccidia* protozoan parasites, is one of the most significant issues in poultry production today. Nine species of *Eimeria spp*. have been described in chickens: *E. acervulina, E. brunetti, E. maxima, E. mitis, E. mívat, E. necatrix, E. praecox, E. tenella* and *E. hagani* (1). The disease often affects birds between 3 and 8 weeks of age. Previously, coccidiosis was treated with chemotherapy upon observation of the initial symptoms, though this was soon ceased as the treatment was found to cause more harm than good. Therefore, for virtually all poultry broilers, preventive treatment is applied by adding a coccidiostat in feed to control the growth of *coccidia* in the intestinal tract (2).

Coccidiostats can be classified into two main groups: ionophores and synthetic products that are non-natural ionophores (3). Polyether ionophores represent a large group of natural and biologically active substances. Several polyether ionophores are widely used as growth promoters in the veterinary field, and exhibit antibacterial, antifungal, antiparasitic, antiviral, and cytotoxic activity. It was recently proven that some of these substances can kill tumour cells and have been

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identified as potential new anticancer drugs. The biological activity of ionophores is strictly connected with their molecular structure (4). In general, ionophores are defined as lipophilic chelating agents that transport cations across cell membranes, including the plasma membrane cell and subcellular structures. Genuine ionophores are highly selective for specific cations and can be monovalent (monensin, narasin, salinomycin, maduramycin and semduramycin) or divalent (lasalocid) (5). Unlike antibiotics, synthetic (chemical) ionophore coccidiostats have a completely different effect. Ionophores are focused on sporozoites and act on them before they enter the host cell, while chemical coccidiostats destroy them after they invade the host cell (6). In broilers, coccidiostats are used throughout the lifetime, while their use is prohibited for laying hens (7).

All substances from the ionophore group of coccidiostats show slight differences between the therapeutic and toxic doses. Some, such as salinomycin, narasin, maduramycin, and to a lesser extent monensin, can be toxic to animals. The scale of toxicity can be represented as follows: salinomycin < lasalocid < narasin < monensin < maduramycin (8). Maduramycin is an aminoglycoside polyether derived from the bacterium *Actinomadura rubra* (9). It occurs in two structural forms, α- and β-maduramycin, whereby α -maduramycin has a greater affinity for monovalent cations and is mainly used as an additive in animal feed or for therapeutic purposes (10). A study on chickens fed with maduramycin in a concentration of 5 and 10 mg kg^{-1} for 21 days found growth disorders among chickens (9). Clinical signs included watery diarrhoea, depression, inertia, and macrocytic anaemia (increased volume of red blood cells). Leukopenia or lymphopenia was observed in the group fed 10 mg kg^{-1} maduramycin after 21 days (9). Provisional maximum residue limits (MRLs) for maduramycin residues defined for tissues of chickens for fattening are (μg kg^{-1}): 150 in liver, skin and fat; 100 in kidney; 30 in muscle (11).

Monensin is an antibiotic product formed as a by-product of the fermentation of *Streptomyces cinnamonensis*. There are two forms A and B, which are produced in approximately equal amounts. Monensin A shows stronger biological activity against *Bacillus subtilis* than monensin B and is used as a coccidiostat. Oral administration of monensin leads to absorption, metabolism and excretion through bile, and elimination via faeces. The optimum (therapeutic) dose of monensin in chickens is 100–125 mg kg^{-1} and the lowest level at which the first toxic effects may occur in chickens is 121–150 mg kg^{-1} (12). MRLs for monensin residues in the chicken tissues are (μg kg^{-1}): 25 in skin and fat, 8 in liver, kidney and muscle (13).

Diclazuril is a derivative of benzeneacetonitrile, a broad spectrum synthetic compound against *E. acervulina, E. maxima, E. necatrix*, *E. brunetti* and *E. tenella*. It may be added to feed for the treatment of coccidiosis in broiler chickens and laying hens as of the age of 16 weeks in maximal authorised concentration of 1 mg/kg (14). Diclazuril has a very low acute toxicity and is not mutagenic, genotoxic, carcinogenic, embryotoxic, foetotoxic or teratogenic (5). MRLs for diclazuril in the chicken tissues are (μg/kg): 500 in muscle, skin and fat; 1 500 μg/kg in liver; 1 000 in kidney (15).

Chemical analysis of blood serum is used in the diagnosis and characterization of diseases, especially those diseases with poorly known pathogenesis, and to assess the general health of humans and animals (16, 17). However, due to the relatively low economic value of poultry animals, these techniques are used less often. Therefore, there are few studies reporting the concentrations of these parameters in the blood of healthy broilers or hens. The interpretation of laboratory results of these parameters is performed according to reference intervals and normal (reference) values set out for healthy animals of a given species (18). The reference values are dependent on various parameters, such as age, sex, species and natural diet, which greatly affect biochemical parameters (19).

There are few studies reporting the influence of coccidiostat application on changes in biochemical parameters, and these primarily use the therapeutic dose. The aim of this study was to examine the impact of the coccidiostats maduramycin, monensin and diclazuril at different concentrations in broiler chickens on the biochemical and chemical parameters of serum.

Material and methods

Experiment and blood sampling

The experimental chickens, a total of 315 healthy broiler chickens (*Gallus gallus*) of both sex

were purchased from commercial breeding age one day. They were housed in cages, supplied with water *ad libitum* and fed on a balanced ration free from any anticoccidial for 30 days. Chickens aged 30 days were divided into experimental groups. The control group consisted of 25 chickens and was not treated with coccidiostats. A total of 315 chickens were divided into 9 experimental groups of 35 animals each. Broiler groups Mad1, Mad2 and Mad3 received feed enriched with maduramycin in concentrations of 5, 10 and 15 mg kg^{-1}, respectively. The groups Mon1, Mon2 and Mon3 received feed with monensin at concentrations of 125, 225 and 325 mg kg^{-1}, respectively. The groups Dicl1, Dicl2 and Dicl3 received feed enriched with diclazuril at levels of 1, 5 and 10 mg kg^{-1}, respectively. All experimental groups received the treated feed for 21 days. The prescribed concentrations of coccidiostats for chickens are: 5 mg kg^{-1} for maduramycin (11), 60-125 mg kg^{-1} for monensin (13) and 1 mg kg^{-1} for diclazuril (14). Therefore, in the present design of the experiments the first experimental group of chickens received standard dose of coccidiostat while other 2 groups received overdose concentrations. Defined withdrawal periods before slaughter of chickens for fattening for three coccidiostats are: maduramycin at least 3 days (11); monensin 1 day (13); diclazuril 5 days (20).

After completion of the 21-day treatment period, on post-treatment days 1, 3, 5, 7, 9 and 11, three chickens in each group were sacrificed and their blood sampled. Serum was extracted by centrifugation at 3000 rpm for 10 min and then transferred to Eppendorf tubes and stored at -20°C until analysis.

The protocol of this study was approved by the Ministry of Agriculture of the Republic of Croatia.

Analysis of biochemical and chemical parameters

Biochemical and chemical parameters in the blood serum of chickens were determined using the biochemical analyser VetScan VS2 (ABAXIS, Union City, CA, USA). The following parameters were determined: aspartate aminotransferase (AST), creatinine kinase (CK), bile acid (BA), cholic acid (CA), uric acid (UA), total protein (TP), albumin (ALB), globulin (GLOB), glucose (GLU) and phosphorus (PHOS). The analyser performed photometric measurements on the principle of absorption of laser light. Measurement were performed using commercial rotors Avian/ Reptilian Profile Plus (ABAXIS, Union City, CA, USA), which contain all the necessary reagents. A total of 0.1 mL serum was pipetted and added to the rotor, which was then inserted into the analyser to automatically read the "rotor" default parameters.

Statistical analysis

Statistical analyses were performed using STATA® 13.1 (StataCorp LP®, USA). The concentrations of biochemical and chemical parameters were expressed as mean ± standard deviation (SD). The Shapiro-Wilk test was applied to determine the distribution of the data. One-way ANOVA test and the Kruskal-Wallis test were used to test differences in the concentrations of parameters between experimental groups. Differences were compared for statistical significance at the level $P < 0.05$ and $p < 0.01$.

Results and discussion

Enzymes AST and CK

Aspartate aminotransferase (AST) belongs to the class of enzymes present in the cytoplasm and mitochondria and various types of tissue, though high concentrations are primarily found in liver and muscles (21, 22). Creatine kinase (CK) is an enzyme that is also present in high concentrations in skeletal and cardiac muscle, smooth muscle and brain, and in smaller amounts in organs such as the intestine, liver and spleen. It is also found free in the cytoplasm of muscle cells. Four isoenzymes have been identified: brain, heart, muscle and mitochondrial isoforms. The enzymes AST and CK are used to assess injuries, as the post-injury muscle activity of AST increases at a significantly slower rate than that of CK. An increase in CK activity suggests an acute muscle injury, while increase in activity of both AST and CK indicates an active or current injury, while growth of only AST indicates muscle injury (22). These two enzymes are localized in the cytoplasm and are released during cell damage (23).

The concentrations of AST and CK in the experimental and control groups of chickens

are shown in Figure 1. Concentrations were determined in the ranges (U L^{-1}): control group AST 173–307 and CK 2023–6033; experimental groups AST 146.5–362.5 and CK 1502–6929. AST concentrations determined were in the range of the reference values 131–486 IU L^{-1} (24) and similar to previously presented values for healthy control group of broilers of 176.5 and 227.17 U L^{-1} (17, 25). However, CK values were significantly higher than defined normal plasma CK activity ranges for the most bird species from 100 to 500 IU/L (22). It has been concluded that increased plasma CK activity can result from muscle cell injury. Severe skeletal muscle injury often results in marked increases of the plasma CK activity and moderate increases of the plasma AST activity (22).

In this study AST concentrations in the experimental groups were not significantly different compared to the control group throughout the observation period. Also, there were no differences in AST and CK concentrations between the first experimental group chickens received so called standard dose (Mad1) and other 2 groups received overdose concentrations (Mad2 and 3). In the experiment with maduramycin, significantly lower concentrations were found in the group treated with a concentration of 10 mg k^{-1} (Mad2) than in the group treated with 15 mg kg^{-1} (Mad3) on post-treatment days 3 and 9 ($p<0.01$, both). Significant differences in CK concentrations were found between the Mad1 and Mad2 groups and control group on day 11 ($p<0.05$ and $p<0.001$).

A recent study using maduramycin in a concentration of 5 mg kg^{-1} determined increased activity of the enzyme AST (25). A similar study on broilers treated with maduramycin at a concentration of 5 to 8 mg kg^{-1} for 6 weeks found that animals treated with 8 mg kg^{-1} of maduramycin also had increased AST levels (26). On the other hand, the group treated with 5 mg kg^{-1} showed no significant changes in AST concentrations. Research conducted in cattle fed with hen litter with the addition of maduramycin in the concentration of 4.8 and 12 mg kg^{-1} showed elevated AST and CK levels (28). Increased AST activity in animals has been explained as the general degeneration of liver, muscle and soft tissue (25). In birds, increases of plasma AST activity are suggested when it is greater than 275 IU/L and is result from either hepatic or muscle injury. Markedly increased AST activity is considered when is above 800 IU/L (22).

In this study with monensin, AST concentrations showed variations between groups on days 7 and 9. On day 7, broilers treated with authorised monensin level of 125 mg kg^{-1} (Mon1) had significantly lower concentrations of AST (173 U L^{-1}) than those treated with 225 (Mon2) and 325 mg kg^{-1} (Mon3) of monensin (270 and 274 U L^{-1}, respectively) ($p<0.01$, both). Also, the Mon3 group had significantly lower CK levels than the control group on day 7 ($p<0.05$). However, the Mon2 and Mon3 groups showed significantly higher CK values than the control group on days 9 and 11 ($p<0.01$, all). Studies at higher doses to investigate the effects of monensin in chickens and turkeys showed increased activity of AST, indicating its undesirable effects in the body. Visibly increased activity of CK, and to a lesser extent of AST in serum indicated progressive structural muscle damage (27).

In the diclazuril experiments, on post-treatment day 9, first standard group received dose of diclazuril at level of 1 mg kg^{-1}, Dicl1 had a significantly lower AST concentration of 146.5 U L^{-1} than other two overdose groups Dicl3 and Dicl2, with values of 262.0 and 223.5 U L^{-1}, respectively ($p<0.01$, both). Significantly lower CK concentrations compared to the control group were found only for the Dicl1 group on post-treatment day 7 ($p<0.05$). However, significantly higher values were determined for group Dicl2 than the control group on days 9 and 11 ($p<0.01$, both).

Uric acid, bile acid, cholic acid and glucose

Bile acid (BA) is the group of water-soluble steroids produced during the catabolism of cholesterol, synthesized in hepatocytes in the liver. The products of primary bile acids are cholic acid (CA) and chenodeoxycholic acid (29). In healthy birds, bile acid is present in small amounts in the peripheral blood stream (22). Uric acid (UA) is the main end product of nitrogen metabolism in birds and is excreted via the faeces. It is relatively inert and substantially less toxic in comparison to ammonia and urea. Uric acid (the oxidized form of purine - hypoxanthine) is mainly synthesized in the liver by the metabolism of purine (21). The main metabolite of animal metabolism is glucose (GLU), which is the primary metabolic fuel and is stored as glycogen in the liver (1–5% of wet matter) and muscles (~1% of wet matter). It is also a major energy substrate used by the brain (30).

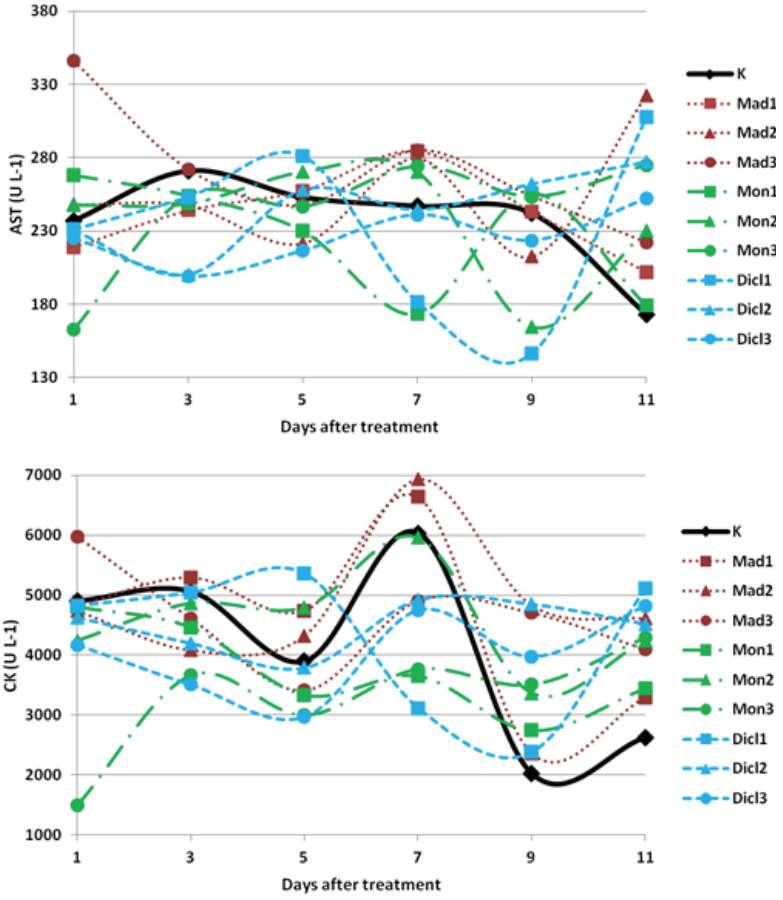

Figure 1: Concentrations of CK and AST (U L^{-1}) of the control group (K) and the experimental groups treated with maduramycin (Mad1: 5 mg kg^{-1}, Mad2: 10 mg kg^{-1}, Mad3: 15 mg kg^{-1}), monensin (Mon1: 125 mg kg^{-1}, Mon2: 225 mg kg^{-1}, Mon3: 325 mg kg^{-1}) and diclazuril (Dicl1: 1 mg kg^{-1}, Dicl2: 5 mg kg^{-1}, Dicl3: 10 mg kg^{-1}) after administration of coccidiostats

Significant differences between groups for **AST**:
day 3 and 9: Mad2-Mad3 $p<0.01$, both
day 7: Mon1-Mon2 and Mon1-Mon3 $p<0.01$, both
day 9: Dicl1-Dicl2 and Dicl1-Dicl3 $p<0.01$, both

Significant differences between groups for **CK**:
day 11: Mad1- C $p<0.05$
day 11: Mad2- C $p<0.01$
day 7: Mon3-C $p<0.05$
day 9 and day 11: Mon2-C and Mon3-C $p<0.01$, all
day 7: Dicl1-C $p<0.05$
day 9 and day 11: Dicl2-C $p<0.01$, both

In this study, the concentration of UA, CA and GLU in chicken serum were measured in the ranges (mg dL^{-1}): control group UA 6–13.7, CA 8.35–12.5 and GLU 72.5–248; experimental groups UA 3.1–22.7; CA 8.2–14.2 and GLU 20.5–301 (Figure 2). UA and GLU levels obtained for control group in this study were similar to previously obtained values for healthy control group of broilers (mg dL^{-1}): UA 4.99 and 5.31; GLU 111.41 and 242.95 (17, 25). In general, the blood GLU concentration in normal birds ranges from 200 to 500 mg dL^{-1} (22). There were no literature reference values for CA. The BA concentration for the control and all experimental groups of chickens was less than 35 µmol L^{-1} which is in line with reference BA concentration values determined by the enzymatic method and which are generally less than 75 µmol L^{-1} (22).

In maduramycin experiment, significantly higher CA level were determined in standard dose group Mad1 compared to the control group on day 9 ($p<0.05$). In treatment with monensin significant differences were found between experimental groups. Significantly higher CA concentrations were measured for the group Mon2 compared to the groups Mon1 and Mon3 in the initial days

following treatment with monensin ($p<0.01$, both). In diclazuril treatment, significantly lower CA were found in the group Dicl1 than in the control group on day 9 ($p<0.05$). Significant differences were determined on day 9 between the group Dicl2 and the groups Dicl1 and Dicl3 ($p<0.01$ and $p<0.05$).

For the parameters UA and GLU, there were no significant differences between the experimental groups and control group of broilers. Also, no significant differences were found between experimental groups for GLU. In the maduramycin experiment, significantly lower UA values were determined in the group Mad3 compared to groups Mad1 and Mad2 on post-treatment day 9 ($p<0.05$, both). On the same day after diclazuril experiments, significantly different concentrations of UA were found between the groups Dicl3 and Dicl2 ($p<0.05$).

A previous study with maduramycin at a concentration of 5 mg kg^{-1} during 6 weeks in broilers showed increased UA levels of 9.31 mg dL^{-1} in comparison with 4.99 mg dL^{-1} measured for control group a day after the end of treatment (25). In experiments in this study, serum UA changes were determined on day 9 after the end of treatments with maduramycin and also diclazuril. Uric acid is secreted in the proximal tubules of the cortical nephrons and approximately 90% of blood uric acid is removed by the kidneys. Therefore, serum or plasma UA levels has been widely used in the detection of kidney disease and damages. In general, UA greater than 13 mg dL^{-1} suggested impaired renal function in birds (22).

Proteins - total, albumin and globulins

There are over a thousand proteins (total protein, TP) in the body, and each has one or more functions (21). Two main types of plasma proteins are albumin and globulins. Albumin (ALB) is classified as a small protein and plays a major role in the transport of free fatty acids, bile acids, bilirubin, calcium, hormones and drugs. It is synthesized in the liver before entering the bloodstream, and is catabolized in different tissues (2). It is also found in plasma, mostly outside the vascular body fluids, cerebrospinal fluid and urine. The basic function is the regulation of osmotic pressure within and outside vascular areas. Albumin values were lower in broilers than in hens (21). Globulins (GLOB) are a heterogeneous group of proteins of varying size but are generally larger than albumin. In plasma, there are over a thousand different types of globulins. Most GLOB are synthesized in the liver, with the exception of immunoglobulins (antibodies), which are produced in the lymphoid tissues. GLOB are classified as alpha (α1, α2), beta (β) or gamma (γ) according to their electrophoretic mobility (22). In birds, any plasma that is not albumin or transthyretin is classified as GLOB (21), and therefore, in this study, GLOB concentrations were calculated by subtracting the concentration of the albumin concentration from total protein.

The concentrations of TP, ALB and GLOB in the experimental and control groups of broilers are shown in Figure 3. Concentrations were determined in the ranges (g dL^{-1}): control group TP 2.45–4.65, ALB 1.71–3.15 and GLOB 0.75–1.5; experimental groups TP 2.75–4.95, ALB 1.90–3.90 and GLOB 0.25–1.75. The concentrations obtained for TP were in line with the reference values for poultry and birds, and ranged from 2.5 to 4.9 g dL^{-1} (18, 22). Also, ALB values were within the reference values from 0.8 to 2.0 g dL^{-1} (22) and previously obtained values for healthy control broilers 2.3 to 3.3 g dL^{-1} (17, 31).

In this study, there were no significant differences in the concentrations of TP, ALB and GLOB between the experimental and control groups, or between experimental groups treated with different concentrations of coccidiostats. The values of all three parameters followed the trend of the control group. Somewhat higher values were determined on post-treatment day 11 for all three parameters in the experimental groups compared to the control group. ALB concentrations were slightly higher on days 7–11 for the groups Mad2 and Mad3, and were in the ranges of 1.9–3.1 and 2.7–3.1 g dL^{-1} compared to the control group (1.70–2.25 g dL^{-1}). GLOB concentrations were slightly lower in all experimental groups than in control broilers, especially in the period of 5 to 9 days after treatment.

In previous study with the maduramycin in broilers at a concentration of 8 mg kg^{-1} for 6 weeks decrease of TP, ALB and GLOB concentrations were found (26). However, there were no changes in TP and ALB levels in broilers treated with maduramycin feed concentrations of 5 mg kg^{-1} (25). However, a recent study with the application of monensin at a concentration of 13 mg kg^{-1} in goats for 5 days showed no effect on TP and ALB levels (23).

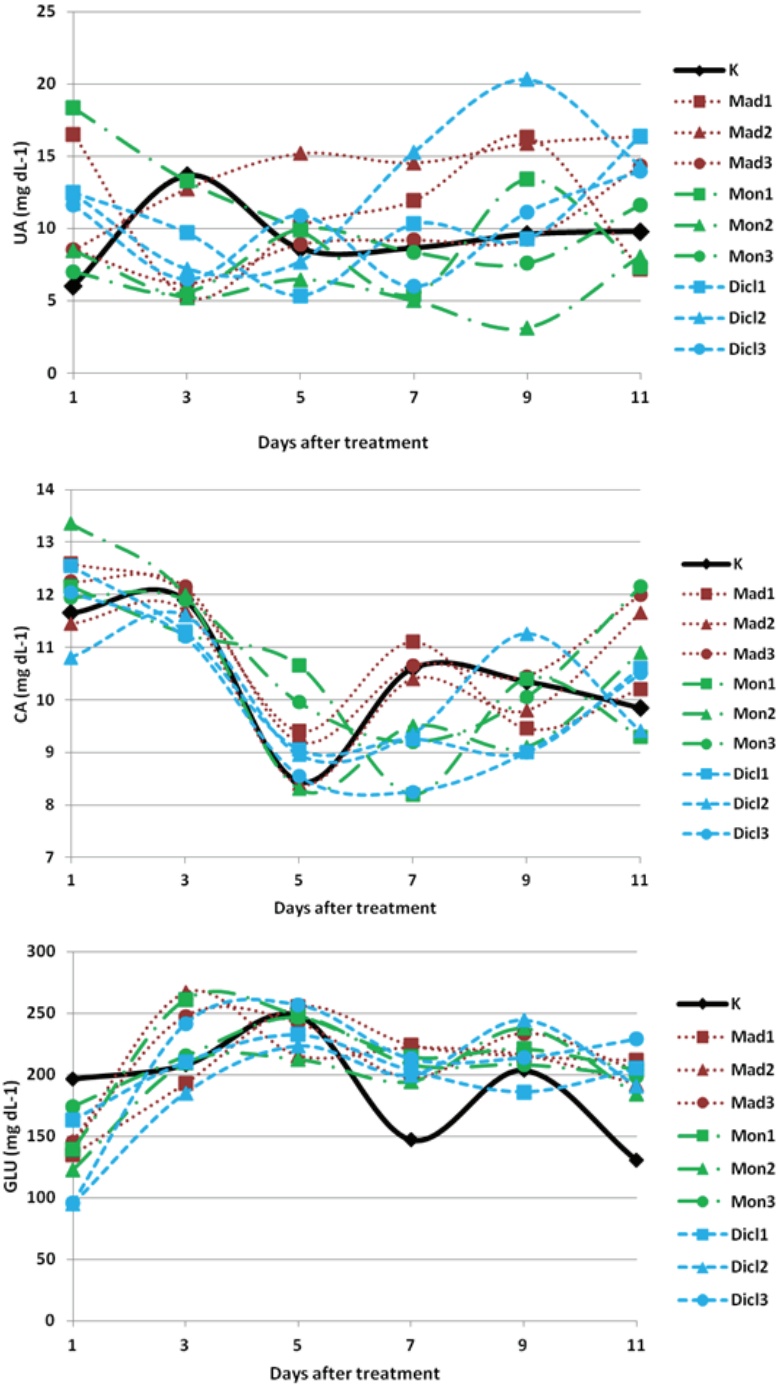

Figure 2: Concentrations of UA, CA i GLU (mg dL^{-1}) of the control group (K) and the experimental groups treated with maduramycin (Mad1: 5 mg kg^{-1}, Mad2: 10 mg kg^{-1}, Mad3: 15 mg kg^{-1}), monensin (Mon1: 125 mg kg^{-1}, Mon2: 225 mg kg^{-1}, Mon3: 325 mg kg^{-1}) and diclazuril (Dicl1: 1 mg kg^{-1}, Dicl2: 5 mg kg^{-1}, Dicl3: 10 mg kg^{-1}) after administration of coccidiostats

Significant differences between groups for CA:
day 9: Mad1-C $p<0.05$
day 1: Mon2-Mon1 and Mon2-Mon3 $p<0.01$, both
day 9: Dicl1-C $p<0.05$
day 9: Dicl1-Dicl2 and Dicl2-Dicl3 $p<0.01$ and $p<0.05$

Significant differences between groups for UA:
day 9: Mad1- Mad3 and Mad2- Mad3 $p<0.05$, both
day 1: Dicl2-Dicl3 $p<0.05$

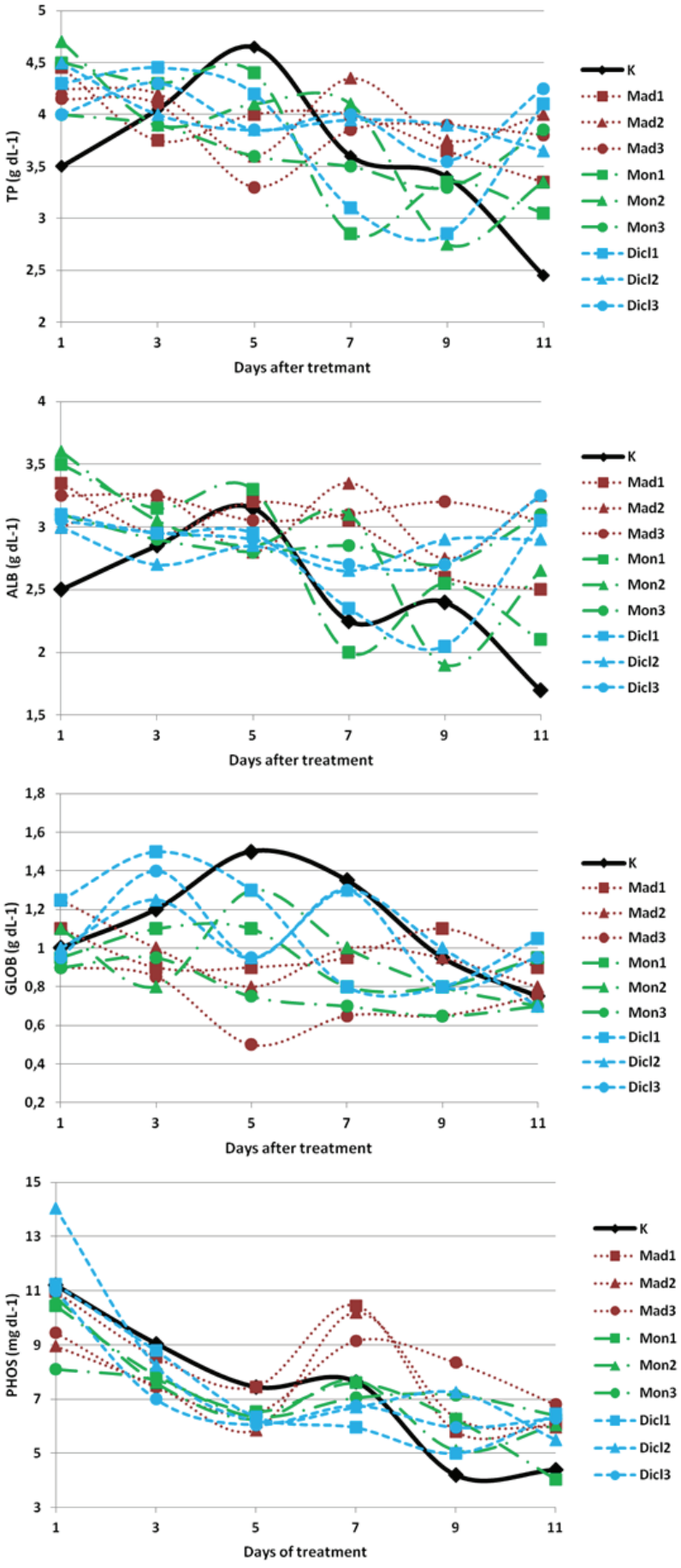

Figure 3: Concentrations of TP, ALB and GLOB (g dL^{-1}) of the control group (K) and the experimental groups treated with maduramycin (Mad1: 5 mg kg^{-1}, Mad2: 10 mg kg^{-1}, Mad3: 15 mg kg^{-1}), monensin (Mon1: 125 mg kg^{-1}, Mon2: 225 mg kg^{-1}, Mon3: 325 mg kg^{-1}) and diclazuril (Dicl1: 1 mg kg^{-1}, Dicl2: 5 mg kg^{-1}, Dicl3: 10 mg kg^{-1}) after administration of coccidiostats

Figure 4: Concentrations of PHOS (mg dL^{-1}) of the control group (K) and the experimental groups treated with maduramycin (Mad1: 5 mg kg^{-1}, Mad2: 10 mg kg^{-1}, Mad3: 15 mg kg^{-1}), monensin (Mon1: 125 mg kg^{-1}, Mon2: 225 mg kg^{-1}, Mon3: 325 mg kg^{-1}) and diclazuril (Dicl1: 1 mg kg^{-1}, Dicl2: 5 mg kg^{-1}, Dicl3: 10 mg kg^{-1}) after administration of coccidiostats

Phosphorus

Electrolytes are present in all inner- and extracellular body fluids, though their concentrations are only measured only in the blood, plasma or serum (22). Phosphorus (PHOS), alongside potassium and calcium, is the most abundant mineral in animals. It is an essential nutrient present in bones, where together with calcium it forms hydroxyapatite in the ratio Ca : P, 2 : 1. In the body, the phosphorus composition is about 70% organic phosphate and 30% inorganic (30). Unlike inorganic phosphorus, organic phosphate is an essential component found in membrane phospholipids and nucleic acids (21). Phosphorus plays a major role in the storage, release and transfer of energy, and is part of the acid-base metabolism (32).

PHOS concentrations in broiler serum after treatments with coccidiostats are presented in Figure 4. Concentrations were determined in the ranges (mmol L^{-1}): control group 4.2–11.2; experimental groups 4.05–14.05. There were no significant differences for PHOS levels in the experimental groups compared to the control, or between experimental groups. In the literature, PHOS levels in broiler chickens were measured in ranges from 3.0 to 6.8 mg dl^{-1} (31, 33).

In a previous study, higher PHOS values were found in heifers following the administration of maduramycin at concentrations of 4, 8 and 12 mg kg^{-1}. However, the increase was significant only in the third week after the treatment. Ultimately, an increase was observed in both groups treated with maduramycin (28). It was concluded that the application of ionophore coccidiostats in high concentrations caused the release of neurotransmitters, mainly norepinephrine. Consequently, ions such as sodium and calcium accumulate in the cell, accelerating intracellular oxidative processes and ultimately degenerative disorders in tissue and organs. The generation of active oxygen groups can lead to necrosis (25). The effect of ionophores on serum calcium levels and inorganic phosphorus can be attributed to the previous conclusion that carboxylic ionophores, such as monensin, lasalocid and salinomycin, react with certain cations to alter mineral metabolism (34, 35).

In conclusion, the administration of different concentrations of maduramycin, monensin and diclazuril did not affect the concentration of the parameters AST, UA, GLU, BA, TP, ALB, GLOB and PHOS in experimental groups of broilers in relation to the control group. Significant differences were observed only in the concentrations of CK and CA in experimental groups in comparison to the control. Significant differences were also determined in the concentrations of AST, CA and UA between experimental groups.

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SPREMEMBE BIOKEMIJSKIH IN KEMIJSKIH PARAMETROV V KRVI KOKOŠI PO ZDRAVLJENJU Z MADURAMICINOM, MONENZINOM IN DIKLAZURILOM

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Povzetek: Namen raziskave je bil spremljanje biokemijskih in kemijskih parametrov krvi industrijsko gojenih piščancev po zdravljenju z enim od treh kokcidiostatikov: maduramicinom, monenzinom ali diklazurilom. Piščanci so dobivali krmo z maduramicinom v koncentracijah 5, 10 in 15 mg/kg, monenzin v koncentracijah 125, 225 in 325 mg/kg ali diklazuril v koncentracijah 1, 5 in 10 mg/kg. Kontrolna skupina piščancev je dobila krmo brez dodatka kokcidiostatikov. Enajsti dan po zdravljenju je bila piščancem odvzeta kri in analizirani so bili naslednji biokemijski in kemijski parametri: koncentracija aspartatne aminotransferaze (AST), žolčnih kislin (BA), kreatininske kinaze (CK), sečne kisline (UA), glukoze (Glu), skupnih beljakovin (TP), albuminov (ALB), globulinov (GLOB) in fosforja (Phos). Dodajanje različnih koncentracij maduramicina, monenzina in diklazurila ni vplivalo na koncentracijo parametrov AST, UA, Glu, BA, TP, ALB, GLOB in Phos v poskusnih skupinah pitovnih piščancev v primerjavi s kontrolno skupino. Statistično značilne razlike so bile ugotovljene v koncentracijah CK in CA med poskusnimi in kontrolno skupino. Značilne razlike so bile ugotovljene tudi v koncentracijah AST, CA in UA med posameznimi poskusnimi skupinami.

Ključne besede: maduramicin; monenzin; diklazuril; piščanci; biokemijski in kemijski parametri

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Original Research Article

TELENCEPHALON VASCULARITY IN COMMON FOX (*Vulpes vulpes*)

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Abstract: The studies of the vascularization of the cerebrum in common fox were performed on 80 cerebral hemispheres. It was found that the middle cerebral artery is the strongest vessel supplying blood to the cerebrum. The artery gets divided into ten permanent branches. Two olfactory arteries supply the region of the cerebrum located on the border between the old and the new cortex. The other eight are divided into three branches heading towards the frontal lobe of the brain, two branches heading towards parietal lobe and three temporal branches heading towards the temporal part, that supply the region of the new cortex. The frontal, parietal and temporal branches descended independently from the main trunk of the middle cerebral artery or formed a common trunk. Common trunks for respective groups of branches have been described as the rostral, dorsal and caudal middle cerebral artery. In 7.5% of cases there were two independent branches of the middle cerebral artery extending from the rostral cerebral artery.

Key words: brain arteries; common fox

Introduction

A review of the literature shows that the first information on the construction of the middle cerebral artery in various mammalian species can be found in the publication of Hofmann (1900). More detailed information on the construction of the middle cerebral artery and its branches in the dog were reported by Hebermehl (1973). In other predatory species similar studies were performed in mink (Brown, 1968) and in the raccoon dog (Brudnicki et al., 1994). So far, in the literature on brain blood flow in fox one may find publications on the construction of the brain base arteries (Wiland, 1967; Ozudogru et al., 2012). These authors mention that the middle cerebral artery is one of the vessels extending from the arterial circle of the brain.

There are publications that discuss in detail cortical branches of the middle cerebral artery. These issues were described in cat by Chadzypanagiotis (1975), the author gives nomenclature for the various branches of this artery. Structured descriptions of the construction and the course of the cortical branches of the middle cerebral artery in some predatory species were presented by Wiland (1991).

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In recent years there have been numerous studies that discuss the construction of the middle cerebral artery in various animal species. This applies to vessels that expand as a single branch, e.g. in porcupine (Aydin et al., 2005), red squirrel (Aydin, 2008), otter (Skoczylas et al., 2012) and multiple arteries occurring in the wild boar (Skoczylas and Wiland, 1999) and in the domestic pig (Skoczylas, 2000). These publications stated that the cortical branches of the middle cerebral artery come to the same areas of the telencephalon. The differences occur in the pattern of descent and division of respective cortical branches of the middle cerebral artery. The pattern of division of the middle cerebral artery is affected by how the species has been classified and the pattern of groove-coverage of the cortex. In mammals on the surface of the cortex there is a different pattern of sulci, which can affect the structure of the cortical branches of the middle cerebral artery (Brauer and Schober, 1970). Considering the discrepancy resulting from respective descriptions and considering new studies, one has decided to investigate the pattern, the division and variation of cortical branches of the middle cerebral artery in common fox and to compare the results with the data reported by other authors.

Material and methods

The research was performed on 40 brains in common fox, namely a total of 80 cerebral hemispheres received as a result of hunting in the region of Pomerania and Kujawy. The animal heads were cut off at the height of the 3rd – 4th cervical vertebrae. The arteries were filled with latex introduced with medical syringe into the common carotid artery. The heads were fixed in a 5% formalin solution and then decalcified in hydrochloric acid, the skull cavity was opened and brains were taken out. The cerebral hemispheres were photographed and the following were being described: the anatomy, the division pattern and the course of cortical branches of the middle cerebral artery.

Results

In common fox the blood is supplied to the brain with internal carotid arteries (Fig. 1-a) and vertebral arteries.

The internal carotid artery, having entered the skull cavity and penetrated the dura mater, bifurcates into the rostral cerebral artery (Fig. 1-b) and caudal communicating artery (Fig. 1-c) which, together with their symmetrical vessels form an arterial circle of the brain.

From the initial section of the rostral cerebral artery towards the cortex there separates the middle cerebral artery.

The middle cerebral artery is the strongest vessel supplying blood to the cerebrum. The initial section of the main trunk of the middle cerebral artery goes along the dorsal surface of the optic tract. Then the section gets bended around the piriform lobe and goes through its rostral margin. Further on it runs to the lateral olfactory sulcus and, having passed it, it gets divided. From the initial section of the main trunk of the middle cerebral artery there descend minor central branches supplying blood to olfactory tracts and the piriform lobe. The main trunk of the middle cerebral artery gets divided into a number of cortical branches which run to the specific region of the cerebral hemisphere, supplying blood to specific regions of the brain.

The first permanent branches of the middle cerebral artery which supply both the old and the new cortex are olfactory arteries.

The rostral olfactory artery (Fig. 1-1), having separated from the main trunk of the middle cerebral artery, runs to the rostral part of the lateral olfactory sulcus it can ascend into in various places. Its terminal branches can also appear again from under the lateral olfactory sulcus and then ascend under the cortex surface.

The caudal olfactory artery (Fig. 1-2) ascends into the caudal part of the lateral olfactory sulcus and its terminal branches supply the area of the cortex found under the sulcus.

The other branches of the middle cerebral artery supply the areas of the cortex over the lateral olfactory sulcus. On the cortex towards the frontal lobe there spread three thick branches. As the first one there separates the orbital branch (Fig. 1-3) which is located lowest and it goes towards the region of the Presylvian sulcus where its terminal branches reach the coronary sulcus.

The ventral frontal branch (Fig. 1-4) vascularizes the middle part of the frontal lobe. The vessel goes through the rostral external Sylvian sulcus and the rostral Suprasylvian sulcus towards the coronary sulcus it passes towards the fornix.

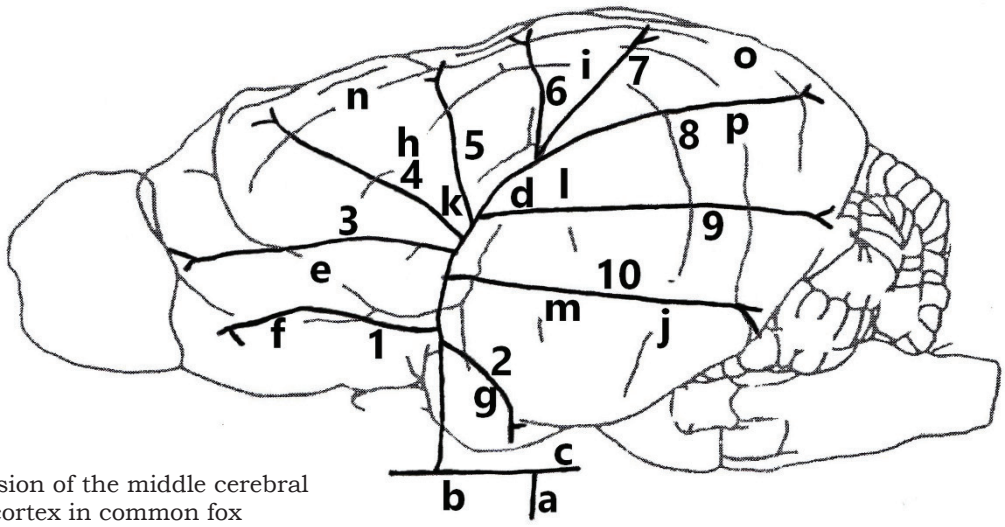

Figure 1: Diagram of the division of the middle cerebral artery on the surface of the cortex in common fox

1 – rostral olfactory artery, 2 – caudal olfactory artery, 3 – orbital branch, 4 – ventral frontal branch, 5 – dorsal frontal branch, 6 – rostral parietal branch, 7 – caudal parietal branch, 8 – dorsal temporal branch, 9 – middle temporal branch, 10 – ventral temporal branch, a – internal carotid artery, b – rostral cerebral artery, c- caudal communicating artery, d – Sylvian fissure, e – Presylvian sulcus, f – rostral lateral olfactory sulcus, g – caudal lateral olfactory sulcus, h – rostral Suprasylvian sulcus, i – middle Suprasylvian sulcus, j – caudal Suprasylvian sulcus, k – rostral external Sylvian sulcus, l – middle external Sylvian sulcus, m – caudal external Sylvian sulcus, n – coronary sulcus, o – marginal sulcus, p – external marginal sulcus

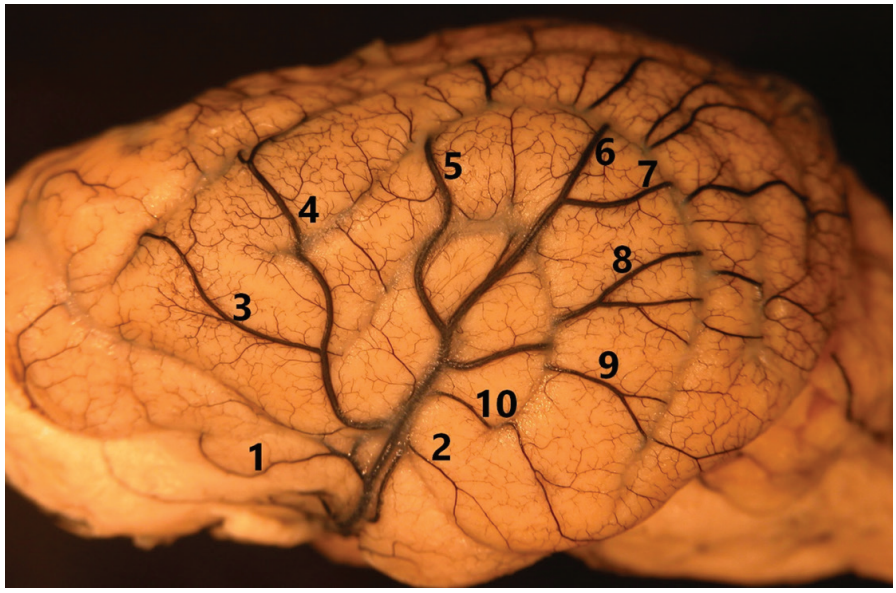

Figure 2: Independent departure of the caudal olfactory artery and the main trunk of the middle cerebral artery from the rostral cerebral artery

1 - rostral olfactory artery, 2 - caudal olfactory artery, 3 - orbital branch, 4 - ventral frontal branch, 5 - dorsal frontal branch, 6 - rostral parietal branch, 7 - caudal parietal branch, 8 - dorsal temporal branch, 9- middle temporal branch, 10 - ventral temporal branch

The dorsal frontal branch (Fig. 1-5), having separated from the middle cerebral artery at the height of the rostral external Sylvian sulcus, goes up to the region of the cruciate sulcus. The vessel supplies blood to the upper part of the medial surface of the frontal lobe.

The next vessel which runs towards the parietal lobe bifurcates into two branches.

The rostral parietal branch (Fig. 1-6) runs towards the middle external Sylvian sulcus to the marginal sulcus. The terminal twigs of that vessel supply blood to the area of the cortex found under the ansiform sulcus and run towards the middle edge of the cerebral hemisphere.

The caudal parietal branch (Fig. 1-7) also runs to the region of the marginal sulcus and further on it branches out into smaller vessels. Some of them ascend into the medial Suprasylvian sulcus and penetrate the hemisphere.

The lateral-caudal surface of the cerebral

hemisphere is supplied by the branches of the middle cerebral artery which descend from at various heights and they are referred to as temporal branches.

The dorsal temporal branch (Fig. 1-8) is usually the strongest cortical branch of the middle cerebral artery. Having left the Sylvian fissure, it runs towards the middle Suprasylvian sulcus and further to the upper margin of the cerebral hemisphere. The branch supplies blood to the upper part of the cortex.

The middle temporal branch (Fig. 1-9) descends a small distance away from the previous branch. The branches of that vessel spread towards the external marginal sulcus. Its terminal branches go onto the surface of the occipital lobe.

The ventral temporal branch (Fig. 1-10) runs to the end of the caudal external Sylvian sulcus. Having passed the caudal part of the sulcus, its branches spread towards the caudal Suprasylvian sulcus. Its terminal branches take part in the supply of a part of the occipital lobe.

Considering the general pattern of the spread the cortical branches of the middle cerebral artery in common fox, one shall note that respective sections of those branches can run inside respective sulci, always running towards the cortex areas described.

Analysing the pattern of descent of the cortical branches of the middle cerebral artery in the common fox individuals investigated, it was found that from the rostral cerebral artery on 76 (95%) cerebral hemispheres there descended a single independent vessel; the middle cerebral artery. Among them on 14 (17.5%) hemispheres from the main trunk there descended rostrally the independent rostral olfactory artery, then a common descend for orbital branch and the ventral and dorsal frontal branches. The main trunk, having ascended into the Sylvian fissure, on the surface of the cortex it showed a common trunk for rostral and caudal parietal branches as well as for the dorsal temporal branches.

In another 12 (15%) cases there descended rostrally from the main trunk an independent rostral olfactory artery and a common departure for the orbital, ventral and dorsal frontal branches. The main trunk got onto the surface of the cerebral cortex from the Sylvian fissure and formed a common descent for rostral and caudal parietal branches. Caudally from the main trunk of the middle cerebral artery, with a common trunk there separated the dorsal, middle and ventral temporal branches, whereas the caudal olfactory artery got separated independently from the main trunk of the middle cerebral artery.

On another 10 (12.5%) hemispheres from the main trunk the following separated rostrally with a common trunk: the orbital branch, the ventral frontal branch and the rostral olfactory artery. The main trunk, having ascended into the Sylvian fissure, on the surface of the cortex it showed a common trunk for dorsal frontal branch, rostral and caudal parietal branches. Caudally from the main trunk of the middle cerebral artery, with a common trunk there separated the dorsal, middle and ventral temporal branches and independent caudal olfactory artery.

On another 10 (12.5%) cerebral hemispheres from the main trunk the following separated rostrally with a common trunk: the orbital branch, the ventral and dorsal frontal branch and the rostral olfactory artery. Caudally from the main trunk the following separated with a common descent: the rostral and caudal parietal branches; dorsal, middle and caudal temporal branches as well as an independent caudal olfactory artery.

On another 8 (10%) cerebral hemispheres from the main trunk departed rostrally the common departure for the rostral olfactory artery and for the orbital branch, then a common departure for the ventral and dorsal frontal branches. Caudally from the main trunk there descended the common trunk for the ventral temporal branch and the caudal olfactory artery. The main trunk, having descended into the Sylvian fissure, got onto the surface of the cortex with a common descent for the dorsal frontal branch, rostral and caudal parietal branches as well as the middle and dorsal temporal branch.

On the other 8 (10%) hemispheres it was found that from the main trunk of the middle cerebral artery departed rostrally the rostral olfactory artery, the main trunk for the orbital branch and for the ventral frontal branch as well as the independent supeior frontal branch. The main trunk gave rostrally the independent caudal olfactory artery and having passed the lateral olfactory sulcus, it provided the common trunk for the ventral and the middle temporal branches. Having ascended into the Sylvian fissure, on the surface of the cortex it showed a common trunk for parietal branches and dorsal temporal branches.

On another 8 (10%) hemispheres from the main trunk of the middle cerebral artery separated

rostrally a common trunk for the olfactory artery and the orbital branch and the common decent for the interior and dorsal frontal branch. The main trunk gave caudally the caudal olfactory artery with a common departure for the ventral temporal branch. Having ascended into the Sylvian fissure, on the surface of the cortex it showed a common trunk for rostral and caudal parietal branches as well as the middle and dorsal temporal branch.

On yet another 6 (7.5%) cerebral hemispheres from the main trunk of the middle cerebral artery rostrally there separated, independently, the rostral olfactory artery, the orbital branch and the common departure for the ventral and dorsal frontal branch. Caudally from the main trunk the following separated with a common descent: the caudal olfactory artery and the ventral and the middle temporal branch. The main trunk of the middle cerebral artery, having got into the surface of the cortex, separated a common descent for rostral and caudal parietal branches and the dorsal temporal branch.

On the other 6 (7.5%) hemispheres it was found that from the rostral cerebral artery there bifurcated two independent branches of the middle cerebral artery. Among them in 2 (2.5%) cases the first independent branch from the rostral cerebral artery was the rostral olfactory artery, while the second branch from the rostral cerebral artery – the main trunk of the middle cerebral artery from which there descended rostrally independently: the orbital branch, the ventral and dorsal frontal branch. Caudally from the main trunk there separated an independent caudal olfactory artery and the common descent for the ventral and the middle temporal branch. The main trunk, having descended into the Sylvian fissure, got onto the surface of the cortex with a common descent for rostral and caudal parietal branches as well as the middle and dorsal temporal branch.

On yet another 2 (2.5%) cerebral hemispheres from the rostral cerebral artery there descended independently the caudal olfactory artery. Rostrally from the main trunk there separated an independent rostral olfactory artery and the common descent for the orbital branch and for ventral frontal branch and the independent dorsal frontal branch. Caudally from the main trunk there separated the independent ventral temporal branch. The main trunk, having ascended into the Sylvian fissure, got onto the cortex surface with the common descent for the middle and dorsal temporal branch and the common trunk for the rostral and caudal parietal branches (Fig.2).

Discussion

The middle cerebral artery supplies blood to the greatest region of the cerebrum. In the common fox the middle cerebral artery supplies the same areas of the brain as in the mammalian species studied so far. The discrepancies concern mostly its division into respective branches. Chadzypanagiotis (1975), describing the cortical branches in cat, differentiated between the branches supplying the old cortex, the branches on the border of the old and the new cortex as well as the branches for the new cortex. In the common fox the arteries supplying the old cortex are minor branches onto the piriform lobe and olfactory tracts. On the border of the old and the new cortex there are found the rostral and caudal olfactory arteries. In the common fox the rostral olfactory artery in 2.5% of the cases was a vessel which descended independently from the rostral cerebral artery. On the other cerebral hemispheres it was a vessel which got separated independently from the main trunk of the middle cerebral artery in 52.5% of the cases. In the other 22.5% cases the rostral olfactory artery was the vessel descending from the common trunk of the middle cerebral artery that gave rise to orbital branch and the ventral frontal branch. In another 12.5% of the cases the rostral olfactory artery descended with a common departure with the orbital branch, the ventral and dorsal frontal branch.

The caudal olfactory artery, on the other hand, in 5.0% of the cases was a vessel which descended independently from the rostral cerebral artery. On 50% hemispheres it was the vessel descending independently from the main trunk of the middle cerebral artery. In 20% of the cases the caudal olfactory artery separated with a common descent with the ventral temporal branch. In the other 25% hemispheres the caudal olfactory artery was one of the branches of a common trunk for the middle and ventral temporal branch.

The other cortical branches of the middle cerebral artery can be divided into a group of frontal, parietal and temporal branches. In the common fox, similarly as in other Carnivora species there occur eight main vessels which supply blood to the area of the new cortex of the cerebrum.

Besides, respective cortical branches can descend from the main trunk of the middle cerebral artery with a common descent. Such cases of descent were reported by Wiland (1991), Skoczylas et al. (2012) as the rostral, dorsal and caudal middle cerebral artery. In common fox the rostral middle cerebral artery has been presented as a common trunk for frontal branches and it occurred in 32.5% of the cases investigated, the dorsal middle cerebral artery was described as a common trunk for parietal branches, which was observed in 20% of the cases. The caudal middle cerebral artery as a common trunk for temporal branches was found in 27.5% of the cases.

In common fox the dorsal middle cerebral artery occurred as the lowest percentage of the cases, however, here the rostral middle cerebral artery dominated. Making a comparison of the present results with those reported by Wiland (1991), one can state that also in American otter the rostral middle cerebral artery was reported as the highest percentage of the cases. In common fox, similarly as in the other animal species studied, the parietal branches have developed poorest. On the surface of the cerebrum the best developed are the frontal branches of the middle cerebral artery.

From the description of the structure of the middle cerebral artery in the publications by Aydin et al. (2005), Aydin (2008), Skoczylas et al. (2012) in the porcupine, red squirrel and otter one can see that it is usually a single vessel descending from the rostral cerebral artery. The vessel, having passed the lateral olfactory sulcus, gets divided along its course into respective cortical branches. In the material investigated such a pattern of division of the middle cerebral artery was found in 92.5% of the cases. In common fox there were identified the cases of descent from the rostral cerebral artery of two independent arterial trunks in 7.5% of the cases. The second independent branch from the rostral cerebral artery was the rostral olfactory artery in 2.5% of the cases, the caudal olfactory artery –5 % of the cases and a common trunk of the rostral olfactory artery with the orbital branch in 2.5% of the cases. In other mammalian species the presence of two independent descents of the branches of the middle cerebral artery was found in wild rabbit (Brudnicki et al., 2012) in 36.5% of the cases, in raccoon dog (Brudnicki et al. 1994) in 18.5% of cases.

The present research show that observed in common fox the division of the middle cerebral artery into the same branches like in the other species investigated so far is a result of genetic limitations.

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PREKRVAVLJENOST TELENCEFALONA PRI LISICI (*Vulpes vulpes*)

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Povzetek: Študije o prekrvavitvi možganov so bile opravljene na hemisferah 40 lisic. Ugotovljeno je bilo, da je srednja možganska arterija najmočnejša žila za dovod krvi v možgane. Arterija se razdeli na deset stalnih vej. Dve vohalni arteriji oskrbujeta področje v možganih, na meji med staro in novo skorjo. Drugih osem vej oskrbuje območje nove skorje: tri se razvejajo v smeri čelnega režnja, dve v smeri temenskega in tri v smeri senčnega režnja možganov. Čelne, temenske in senčne veje se spuščajo neodvisno od glavnega debla srednje možganske arterije ali pa oblikujejo skupno deblo. Skupna debla za posamezne skupine vej so bila opisana kot rostralna, dorzalna in kavdalna sredinska arterija. V 7,5 % primerov sta bili opaženi dve neodvisni veji srednje možganske arterije, ki se nadaljujeta od rostralne možganske arterije.

Ključne besede: možganske arterije; lisica

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Original Research Article

ASSOCIATION OF NUMBER OF ARTIFICIAL INSEMINATIONS PER PREGNANCY IN HOLSTEIN DAIRY COWS WITH POLYMORPHISM IN LUTEINIZING HORMONE RECEPTOR AND FOLLICLE STIMULATING HORMONE RECEPTOR GENES

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Abstract: Failure to become pregnant is the primary reason for a dairy cow to be culled from the production herd. A cow that is cycling normally, with no reproductive abnormalities, but has failed to conceive after at least three successive inseminations may cause economic losses in dairy farms. The present study aimed to examine the association between follicle-stimulating hormone receptor (*FSHR*) and luteinizing hormone receptor (*LHCGR*) genes polymorphisms and number of artificial inseminations in the Holstein cattle breed, raised in Turkey. A total of 264 Holstein cows were included in this study, consisting of 222 cows which had undergone a low number of artificial inseminations (two or less inseminations; LI) and 42 cows with a high number of artificial inseminations (three or more inseminations; HI). The polymerase chain reaction followed by restriction fragment length polymorphism method was used to determine the *FSHR-Alu*I and *LHCGR-Hha*I DNA variants. Three genotypes (CC, CG and GG) were observed for the *FSHR* gene in LI and HI cows. No statistical difference was found among LI and HI animals for the *FSHR* genotypes (P=0.934). However, only the CC genotype was detected in LI cows whereas the CC, CT and TT genotypes were detected in HI cows for the *LHCGR* gene. The genotype frequency of CC was found to be highest (93%) in the HI animals and an association between LHCGR genotypes and the number of artificial inseminations per pregnancy was identified (P<0.001). This is the first report to describes an association between *FSHR* and *LHCGR* polymorphisms and number of artificial inseminations in cows.

Key words: candidate genes; pregnancy; cow; number of artificial inseminations; polymorphism

Introduction

Many parameters such as calving interval, average days open, heat detection efficiency, number of inseminations per pregnancy, and pregnancy rates are important for determining the reproductive performance of dairy herds (1, 2, 3). Reproductive problems cause important economic losses due to reduced fertility as a result of prolonged calving intervals (CI) (4), increased artificial insemination (AI) costs, reduced number of calves and higher replacement costs (5). Reproductive problems in dairy cattle breeding are the most common cause for the culling of animals (6). In France, the culling of cows due to low fertility rate has been reported to account for 25% of disposed animals (7). Depending on the yield of offspring, the prolonged calving interval costs dairy farms about 1.8 million US dollars in Ireland, without accounting for the costs of higher selection due to failures to conceive (8). In different studies, 1.9 (1), 1.7-2.2 (2) and <2 (3) were given as the average number of inseminations required to

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maintain a successful pregnancy. In Turkey, cows that were inseminated more than twice, financial losses due to extra service and to extended calving interval were estimated to be 3-fold and approximately 2-fold higher than in cows which were inseminated only a few times (9).

Fertility traits such as calving interval and the number of inseminations per pregnancy have been neglected in dairy cattle genetic improvement studies globally, mainly because fertility traits are known to have low heritability (10). There are many reasons for fertility decline in dairy cows, such as genetics, physiology, nutrition and management (11). Furthermore, dairy cows that have high genetic merit for milk production traits were reported to have reduced fertility (12) and subsequently required a higher number of inseminations per pregnancy in recent years (13).

The degree of heritability for fertility traits in dairy cattle was estimated to be low (14). On the other hand, an association between fertility traits and some genes in different dairy cattle was reported (15). Due to their roles in the physiological and biochemical processes for reproductive traits, some candidate genes were identified in different studies (15, 16). The analysis of candidate genes from hormones and hormone receptors, which affect fertility, has been considered a favourable tools (17, 18) and, for this purpose, the genes encoding the follicle-stimulating hormone receptor gene (*FSHR)* and luteinizing hormone/choriogonadotropin receptor (*LHCGR)* have been selected as candidate reproductive markers for livestock.

FSHR encodes the transmembrane receptor that interacts with the follicle-stimulating hormone (FSH) (19). FSHR activation is necessary for the hormonal functioning of FSH and is found at high levels in the ovaries and testes of mammals (20). Mayorga *et al.* (21) showed that the *FSHR* gene is a main determinant of ovarian responsiveness to FSH for the induction of ovulation in female. The *FSHR* gene is located on bovine chromosome 11 (BTA 11) and consists of 10 exons (22).

Another candidate gene is *LHCGR* which is located on BTA 11 and encodes the transmembrane receptor. *LHCGR is* found predominantly in the ovaries and testes and the receptor interacts with luteinizing hormone (LH). LH is critical for follicular development, ovulation, corpus luteum formation, and preimplantation embryonic development (23). Previous studies showed that *LHCGR* gene variants are associated with polycystic ovary syndrome in humans (24) and superovulation traits in cattle (23).

Although the association between *FSHR* and *LHCGR* gene variants with different fertility traits was studied in various mammal species, no study has been conducted to investigate the effects of these gene variants with the number of artificial inseminations per pregnancy in dairy cattle. Therefore, the objective of this study was to investigate the effects of two single nucleotide polymorphisms (SNPs) in the *FSHR* and *LHCGR* genes in dairy cattle requiring either low or high numbers of artificial-inseminations per pregnancy.

Material and methods

The study was conducted in a large commercial dairy herd in Kayseri province of Turkey. HI and LI cows were housed in semi-covered sheds and fed according to (25) requirements with appropriate amount of forage and concentrated feed. The voluntary waiting period was 60 days in the herd. Cows were observed for estrus activity. Estrous signs were comfirmed by rectal and ultrasonographic examinations (such as preovulatory follicle, tonic uterus, echogen endometrium). In this study 264 Holstein cows, varying in age of 4 to 7 years and having birth twice, were examined for *FSHR* and *LHCGR* genes. All of animals have no problems had affected reproductive performance such as cystic ovaries, anoestrous, suboestrus, endometritis and pyometra. Artificial insemination is only the method applied for reproduction and the average milk yield was 27 kg in LI and 26.2 kg in HI in studied herd. The body condition score was 3.25-3.5 among all animals in the herd. No animals were culled for reproductive failure due to nutritional problem in the herd but culling rate for reproductive failures were 8% among all breeders. The blood samples were collected in heparinized tubes from Vena coccygea A total of 264 Holstein cows were examined for *FSHR* and *LHCGR* genes. The cows were divided into two groups according to their number of inseminations to become pregnant. The first group (low insemination number-LI) represented pregnant cows (n= 222) that were inseminated once or twice (average 1.6), The average age was 5.5 years. In the second group (high insemination number-HI) (n= 42), three or more inseminations (average 3.1) were

Table 1: Primer sequence and PCR product size

Gene	Accession number	Restriction Enzymes		Primers	PCR product size
FSHR	L22319.1	*Alu*I	F	5'- CTG CCT CCC TCA AGG TGC CCC TC-3'	306 bp
			R	5'- AGT TCT TGG CTA AAT GTC TTA GGG GG-3'	
LHCGR	U20504	*Hha*I	F	5'- CAA ACT GAC AGT CCC CCG CTT T- 3'	303 bp
			R	5'-CCT CCG AGC ATG ACT GGA ATG GC- 3'	

F: Forward; R: Reverse

necessary for two previous pregnancies. The postpartum periods ranged from 45 to 60 days in this group and the average age was 5.8 years. Average intercalving period was 405 and 480 days in LI and HI groups, respectively.

Genomic DNA was isolated from blood using a phenol chloroform protocol (26). Genotyping for the *FSHR-Alu*I and *LHCGR-Hha*I polymorphism were performed by PCR-RFLP according to the method proposed by Houde *et al.* (20). PCR analysis of *FSHR* and *LHCGR* genes were performed in thermal cycler (Bio-Rad-T100, USA). The nucleotide sequences and accession numbers of the specific PCR primers used for standard PCR amplification are shown in Table 1.

The PCR amplification reaction was carried out in a total volume of 25 µl consisting of ddH_2O, 1.5 mM $MgCl_2$ 50 µM dNTP, primers (5 pmol), 1X buffer, Taq DNA polymerase 1U/ µL and DNA 100 ng. The PCR protocol started with an initial denaturing step of 94°C for 5 min, followed by 35 cycles of 94°C for 45 sec, 57°C for 45 sec for *FSHR* and 60°C for 45 sec for *LHCGR*, 72°C for 45 sec and a final cycle at 72°C for 10 min. The PCR product of *FSHR* and *LHCGR* genes were digested with the enzymes *Alu*I (Fermentas, Vilnius, Lithuania) and *Hha*I (Fermentas, Vilnius, Lithuania) respectively. Restriction products were electrophoresed on 3% agarose gel and then displayed under a UV-transilluminator (Kodak-Gel Logic 200, USA).

Direct counting was used to estimate the genotype and allele frequencies of the *FSHR* and *LHCGR* genes. Differences among the genotype groups were examined by simple logistic regression. Significance was considered to be $P<0.05$. Goodness-of-fit of genotype distribution to Hardy–Weinberg equilibrium was examined by Pearson's x2-test. All calculations were performed using the computer program SPSS version 14.0 (SPSS Inc., Chicago, IL, USA).

Results

The amplified PCR product of *FSHR* and *LHCGR* genes produced 306 bp and 303 bp fragments using *Alu*I and *Hha*I restriction enzymes, respectively. After the digestion of *Alu*I, two fragments were expected to be seen for the CC genotype (243 and 63 bp), four fragments were expected to be seen for the CG genotype (243, 193, 63 and 50 bp) and three fragments were expected to be seen for the GG genotype (193, 63 and 50 bp). However, since 243 and 193 bp bands were clearly seen together and separately, it can be assumed that genotyping was successfully fulfilled without observing the 63 and 50 bp bands. Three genotypes (CC, CG and GG) were identified for the *FSHR* gene both of LI and HI cows (Figure 1).

After the digestion of the PCR products of the *LHCGR* gene with *Hha*I restriction enzyme, a polymorphism with two alleles was detected. The *LHCGR* site had three genotypes: TT (303 bp), TC (303 bp, 155 and 148 bp) and CC (155 and 148 bp) (Figure 2).

The allelic and genotypic frequencies of the *FSHR* and *LHCGR* genes and polymorphisms for the LI and HI Holstein cows are given in Table 2. Deviation between observed genotypic frequencies and this expected under HWE was significant in LI and HI cows for the *FSHR* gene (Hardy-Weinberg Equilibrium-HWE, P=0.029). Also, a significant deviation from HWE was observed in the HI cows on the *LHCGR* gene (HWE, P=0.06). For the *LHCGR* gene, the CC genotype was found in LI and HI cows; additionally, the CC genotype was found to be highest in the HI cows (92.86%). The CC genotype frequency (69.23%) was observed to be higher than CG and GG frequency in the HI and LI (61.26%) cows for the *FSHR* gene (Table 2). 7

No statistical differences between groups (LI and HI) were observed for genotypes of the *FSHR*

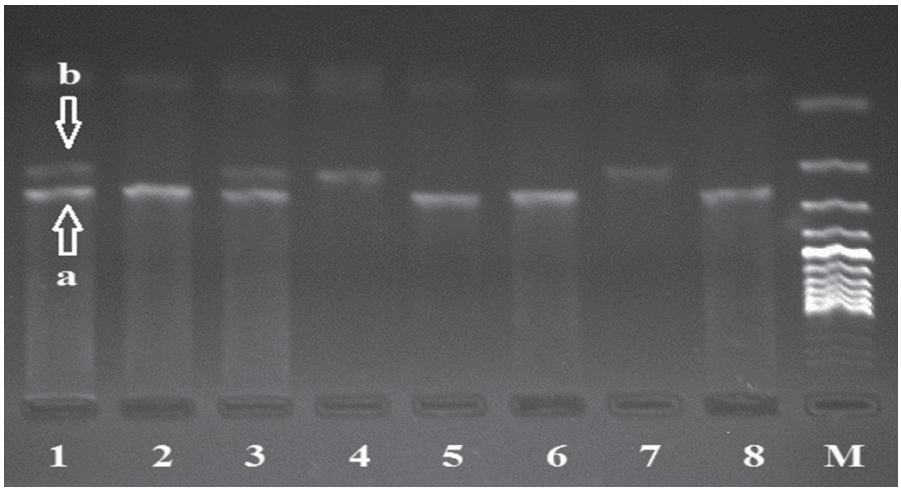

Figure 1: *AluI* enzyme digestion products of different *FSHR* genotypes. a: 243bp, b:193bp, M: 100 bp DNA ladder; 2, 6 and 8 CC individuals genotyped; 1 and 3 CG individuals genotyped; 4, 7 GG individuals genotyped

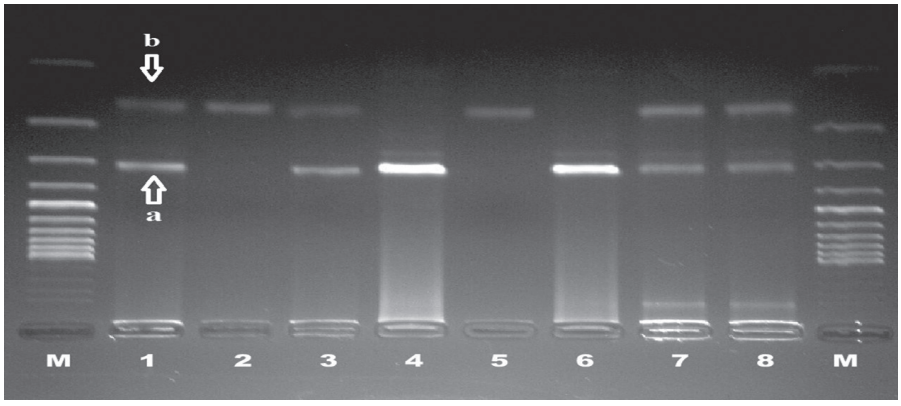

Figure 2: *HhaI* enzyme digestion products of different *LHCGR* genotypes. a: 303 bp, b: 155, 148 bp, M: 100 bp DNA ladder; 4, 6 TT individuals genotyped; 1 and 3,7,8 TC individuals genotyped; 2, 5 CC individuals genotyped

Table 2: Allelic and genotypic frequencies of *FSHR* and *LHR* genes in LI and HI cows

Gene	Groups	Frequency	Genotypes Frequency (%)			Allele Frequency (%)		Statistical Significant (Chi-squared HWE)
			CC	CG	GG	C	G	
FSHR	LI	Observed	61.26	30.63	8.11	76.6	23.4	x2 = 4.74 P=0.029 (df=1)
		Expected	58.64	35.87	5.49			
	HI	Observed	69.23	23.08	7.69	80.8	19.2	x2 = 3.44 P=0.06(df=1)
		Expected	65.24	31.07	3.70			
			CC	CT	TT	C	T	
LHCGR	LI	Observed	100	0	0	100	0	-
		Expected	100	0	0			
	HI	Observed	92.86	4.76	2.38	95.2	4.8	x2 = 9.48 P=0.002(df=1)
		Expected	90.70	9.07	0.23			

HWE: Hardy-Weinberg Equilibrium; x2: Chi-Square value; df: Degree of freedom

Table 3: Relative distribution of *FSHR* and *LHCGR* genotypes on LI and HI cows and test of statistical significancy

Gene	Genotypes	Groups		Total	Statistical Significant (Chi-Square Tests)
		LI	HI		
FSHR	CC	136 (84.0%)	26 (16.0%)	162	$x^2 = 0.137$ P=0.934
	CG	68 (85.0%)	12 (15.0%)	80	
	GG	18 (81.8%)	4(18.2%)	22	
LHCGR	CC	222 (85.1%)	39 (14.9%)$_a$	261	$x^2 = 16.039$ P<0.001
	CT	0 (0%)	2(100%)$_b$	2	
	TT	0 (0%)	1 (100%)$_b$	1	
Total		222 (84.1%)	42 (15.9%)	264	

a,b : Different subscripts within the same column demonstrate significant differences at 0.05 level for each loci

gene (P=0.934). For the *LHCGR* gene,-only the CC genotype was seen in LI cows. Additionally, the CC genotype was found quite often in HI cows (39/42; 93%). Therefore, the genotypes between LI and HI cows were significantly different (P<0.001). The lack of CT and TT genotypes was observed in LI (Table 3).

Discussion

The selection of animals, which is aimed at improving quantitative traits, is a complex process, which may cause adverse effects on other traits. Genetic selection for milk yield has enabled increase in the amount of milk obtained per dairy cow. However, due to the antagonistic genetic correlation between milk yield and fertility, dairy cows with high milk yield have lower fertility (24). In dairy cattle, the production of one calf a year is a prerequisite for both the maintenance of the herd size and its profitability. Previous studies have demonstrated that candidate gene analysis is a good option for increasing the reproductive performance of cattle (16, 28).

The *FSHR* gene has been reported to be polymorphic in various cattle breeds (29, 30). In Zebu × *Bos taurus* hybrids raised in Brazil, the *FSHR* gene was reported to have three genotypes, including GG, CG and CC, and it was found that the CG genotype had the highest frequency and that the frequencies of the C and G alleles were similar (29). In the same study it was reported that in purebred Nellore cattle (*Bos indicus*) raised in Brazil, unlike in hybrids, the GG genotype displayed the highest frequency (0.490), the genotype CC did not exist, and the frequency of the G allele (0.745) was higher than that of the C allele (0.255) (30). In an investigation on European dairy cattle breeds, (Holstein Jersey) and European beef breeds (Angus and Charolais), the frequency of the *FSHR*-G allele was found to be higher in both beef breeds (Angus:0.53,Charolais:0.41) than in dairy cows (Holstein:0.28, Jersey:0.17) (31). Similarly, in the present study carried out in Holstein cattle, in both group LI and group HI, the frequency of the C allele was found to be higher than that of the G allele (Table 2).

Although the correlation between the *FSHR* gene and fertility has been investigated extensively in humans showing significant dependencies (32, 33), research on this topic in livestock is scarce. Only a few studies have focussed on the correlation between the *FSHR* gene and fertility in cattle. In a study carried out in Zebu × *Bos taurus* hybrids raised in Brazil, the investigation of the correlation between *FSHR* genotypes and pregnancy rate demonstrated that, although no significant difference existed between the genotypes for pregnancy rate, the pregnancy rate of the heifers with a CG genotype (66%) was higher than that of heifers with a CC genotype (64%) and GG genotype (58%) (30). In another study conducted in Holstein cattle raised in China, a new SNP of G-278A was detected in the 5′-upstream region of the *FSHR* gene. The number of ova obtained for this SNP from cattle with a CC genotype was found to be higher than that obtained from cattle with a genotype of CD and DD. Thus, it was reported that a higher number of transferable embryos were produced

by heifers with a CC genotype, in comparison to heifers with a CD and DD genotype (28). Cory *et al.* (31) reported that the c.337C>G, c.871A>G and c.1973C>G mutations were correlated with the percentages of viable embryos and unfertilized ova obtained after superovulation.

It was also found that the LH peak was delayed in "repeat breeder syndrome" cases. This delay caused prolonged lifespan of the preovulatory follicle, and a late postovulatory rise of plasma progesterone (34). Considering the central role of LH in ovulation, common genetic variation in the luteinizing hormone/choriogonadotropin receptor (*LHCGR*) may have functional consequences in reproduction performance (28). The *LHCGR* gene has been investigated to a limited extent in livestock. In one of these few studies, carried out in the *Bos indicus* × European *Bos taurus* beef hybrids of six different breed compositions, the digestion of the *LHCGR* gene with *Hha*I enzyme produced three genotypes, namely, TT, CT and CC. The frequency of the TT genotype was low and ranged from 0 to 0.091, whilst the frequencies of the CT and CC genotypes ranged between 0.366-0.849 and 0.151-0.574, respectively. The frequency of the C allele was higher than that of the T allele (29). Furthermore, it was reported that in purebred Nellore cattle (*Bos indicus*) raised in Brazil, the frequency of the TT genotype (0.540) was high, the frequency of the CC genotype (0.030) was low and the frequency of the T allele (0.755) was higher than that of the C allele (0.245) (35). In the present study only LI cows carried the *LHCGR*-CC genotype. HI cows included animals of all three genotypes and the frequency of the CC genotype was the highest among those tested (92.86%). These findings are in agreement with the reports of Marson *et al.* (29) and Milazzotto (35). While Milazzotto (35) reported that the frequency of the *LHCGR*-TT genotype (0.540) was high in purebred Nellore cattle (*Bos indicus*), Marson *et al.* (29) reported that the frequency of the *LHCGR*-TT genotype was lower than that of the other genotypes in *Bos indicus* × *Bos taurus* hybrids. In the present study, it was ascertained that in Holstein cattle, the *LHCGR*-TT genotype did not exist in group LI and had the lowest frequency in HI cows (1; 2.38%). On the other hand, while Milazzotto (35) reported that the frequency of the *LHCGR*-CC genotype was lowest in purebred *Bos indicus* cattle, in the present study, the frequency of the *LHCGR*-CC genotype was determined to be the highest in Holstein cattle (*Bos taurus*).

Very few studies are available on the correlation of the *LHCGR* gene with reproductive traits in different cattle breeds. A significant QTL was identified for inseminations per conception trait between 32.6-32.7 Mbp where the *LHCGR* gene is found on BTA 11 according to *Bos taurus* genome assembly (36). Since no QTL for male fertility, non-return rate and sire conception rate have been identified on BTA 11 in this region (37), this mutation may be responsible for female reproduction, especially inseminations per conception. Marson *et al.* (30) reported that no statistically significant correlation exists between the *LHCGR* genotype and conception rate in *Bos indicus* x *Bos taurus* hybrids. However, the authors indicated that, although not significant, the conception rate was higher in heifers with a CT genotype (67%), in comparison to those with a TT genotype (65%) and CC genotype (58%). Furthermore, in another study, it was suggested that the *LHCGR* genotype was not associated with early development (before 14 months) or prolonged development, in purebred Nellore cows (*Bos indicus*) raised in Brazil (35). In the present study, the heifers included in group LI were only of the CC genotype, whilst heifers included in group HI were of all three genotypes. In group HI, the frequency of the CC genotype (92.86%) was the highest.

In this report we investigated the relations between the *FSHR* and *LHCGR* gene polymorphisms and insemination number to obtain pregnancies in Holstein cows. No difference between LI and HI groups was found for the *FSHR* gene. However, it is noteworthy that all LI cows were of the CC genotype in the *LHCGR* gene. Further association studies with larger numbers of samples and artificial insemination number on these polymorphisms is required.

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POVEZAVA MED ŠTEVILOM UMETNIH OSEMENITEV IN POLIMORFIZMOM GENOV ZA RECEPTOR ZA LUTEINIZIRAJOČI HORMON IN RECEPTOR ZA FOLIKLE STIMULIRAJOČI HORMON PRI KRAVAH MOLZNICAH PASME HOLSTEIN

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Povzetek: Težave z obrejitvijo so glavni razlog za izločitev krav molznic iz proizvodne črede. Krave z normalnim ciklusom, brez reproduktivnih motenj, ki se ne obrejijo po vsaj treh zaporednih osemenitvah, so vzrok za velike ekonomske izgube na mlečnih farmah. V opisani raziskavi smo proučevali povezavo med genskimi polimorfizmi v genih za receptorje za folikle stimulirajoči hormon (FSH-R), genih za receptorje za luteinizirajoči hormon (LH-R) ter številom umetnih osemenitev pri kravah pasme hlstein, vzrejenih v Turčiji. V raziskavo je bilo vključeno skupno 264 krav holstein, od katerih jih je bilo 22, osemenjenih največ dvakrat do obrejitve (skupina LI), in 42 krav, ki so bile osemenjene trikrat ali večkrat (skupina HI). Preiskovane gene smo pomnožili v verižni reakciji s polimerazo in nato izvedli pregled dolžine razrezanih odsekov DNK (metoda RFLP) z namenom, da bi določili prisotnost različic genov *FSHR*-AluI in *LHR*-HhaI pri preiskovanih živalih. Tri različice genotipa (CC, CG in GG) so bile ugotovljene pri genu za FHSR pri kravah v skupinah LI in HI, med skupinama pa ni bilo statistično značilnih razlik v pogostnosti posameznih genotipov ($p = 0,934$). Pri genu za LHR je bil ugotovljen genotip CC le pri kravah iz skupine LI, ostale tri variante genotipa (CC, CT in TT) pa so bile ugotovljene pri kravah iz skupine HI. Pogostnost genotipa CC je bila najvišja (93 %) pri živalih iz skupine HI, pri statistični analizi pa smo ugotovili povezavo med genotipi *LH-R* in številom umetnih osemenitev ($p < 0,001$).

Ključne besede: FSH-R, LH-R; obrejitev; krava; število umetnih osemenitev; polimorfizem

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