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Research Article

Sero-Prevalence Survey of Small Ruminant Lentivirus (SRLV) Infections in Kosovo

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Abstract

The objectives of this cross-sectional study were to detect the presence of small ruminant lentiviral infections in Kosovo and estimate the serological prevalence for the year of 2016 . A total of 5.272 sheep and 435 goats were tested using a commercially available indirect enzyme-linked immunosorbent assay (ELISA) for Maedi-Visna/Caprine Arthritis-Encephalitis, giving an overall individual sero-prevalence in sheep of 34.8% (95% confidence interval 31.8% to 38.0%), and a flock prevalence of 85%, and in goats an overall individual sero-prevalence of 15.6% (95% confidence interval 7.2% to 25.6%) and flock prevalence of 35%. Sero-prevalence in sheep was higher in the South and West of Kosovo, whereas in goats was higher in the East and South. There were no statistically significant differences in sero-prevalence between sheep in different age groups <2 year to ≥4 year. There were statistically significant differences between the age groups in goats: chi square 25.74 (3d.f.) with P-value <0.0001. Increasing sero-prevalence observed in goats up to 4 years old with a sharp drop in goats older than four years highlights the need for further investigation based on clinical impact and genotype characterization. While retained sero-positive sheep in the population beyond 4 years old may suggest mild clinical impact of small ruminant lentiviral infections in Kosovo sheep.

Keywords: *Small ruminant lentivirus, Maedi-Visna, Caprine Arthritis-Encephalitis, ELISA, Kosovo*

Kosova'daki Küçük Ruminant Lentivirus (SRLV) Enfeksiyonlarının Sero-prevalansının Araştırması

Öz

Bu kesitsel çalışmanın amacı, Kosova'da küçük ruminant lentiviral enfeksiyonların varlığını saptamak ve 2016 yılı için serolojik prevalansı tahmin etmekti. Toplam 5.272 koyun ve 435 keçi ticari olarak temin edilebilen dolaylı enzim bağlı immünosorbent test (ELISA) kullanılarak Maedi-Visna/Keçi Artritis-Ensefalitis hastalığı yönünden kontrol edildi. Koyunlarda bireysel sero-prevalans %34.8 (%31.8 ila %38.0; %95 güven aralığı), sürü prevalansı % 85 bulunurken keçilerde %15.6'lık bireysel sero- prevalans (%7.2 ila %25.6; %95 güven aralığı) ve %35'lik sürü prevalansı belirlendi. Hastalık sero-prevalansı koyunlarda Kosova'nın güneyinde ve batısında, keçilerde ise doğusu ve güneyinde daha yüksekti. Koyunlarda <2 ila ≥4 yaş aralığındaki farklı yaş gruplarının sero prevalansında istatistiksel olarak anlamlı bir fark yoktu. Keçilerdeki yaş grupları arasında ise istatistiksel olarak anlamlı farklılıklar vardı: ki-kare 25.74 (3d.f.) P değeri <0.0001. Dört yaşına kadar olan keçilerde sero prevalansının artması ancak 4 yaşından büyük keçilerde görülen keskin düşüş, klinik etki ve genotip karakterizasyonuna dayanarak daha fazla araştırma yapılması gerektiğini vurgulamaktadır. Populasyondaki dört yaşından büyük sero-pozitif koyunların sürüde tutulması, küçükbaş hayvan lentiviral enfeksiyonlarının Kosova koyunlarında hafif klinik etkisi olduğu yönünde değerlendirilebilir.

Anahtar sözcükler: *Küçük ruminant lentivirüs, Maedi-Visna, Keçi Artritis-Ensefalitis, ELISA, Kosova*

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INTRODUCTION

Maedi-Visna (MV) in sheep and Caprine Arthritis-Encephalitis (CAE) in goats are caused by two closely related viruses, commonly referred as small ruminant lentiviruses (SRLVs). SRLVs share many features, but are genetically heterogeneous as shown by phylogenetic analysis [1,2]. Cross-infection between small ruminant species is possible [3,4]. The main routes of transmission are via colostrum/milk and horizontal transmission through aerosol via the natural close contact between the dam and her progeny, especially under intensive housing or grazing conditions. Intrauterine transmission occurs infrequently [5,6]. Some resistance to lentivirus infection may be related to host genetics [7]. Once established, infection is lifelong and persistent. Infected animals are a constant reservoir of infection [8]. Incubation period is long and highly variable. Most infected animals will remain asymptomatic during their productive lifespan. Often it takes years before clinical infection becomes apparent and 30% of infected animals will develop slow progressive multi-systemic chronic disease [1,5]. MV is clinically manifested as chronic progressive interstitial pneumonia (maedi) and/or progressive neurologic form of the disease (visna) usually in adult sheep, although it has been reported in older lamb and encephalitis in 4-6 months old lambs [9-13]. The most common manifestation of CAE infection in goats is polysynovitis-arthritis in adult goats and encephalomyelitis, which is generally seen in kids 2-4 months old but has been described in older kids and adult goats [14,15]. Chronic indurative mastitis is seen in both species [16,17].

Small ruminant lentivirus infection causes underestimated substantial losses in the small ruminant industry due to reduced animal production and increased replacement rates [5]. There are no specific treatments for any of the clinical syndromes associated with MV or CAE virus infection and to date, no vaccines are available [18]. Diagnosis is based on clinical signs, flock history and confirmed by serological tests, PCR, western blot, radio-immunoassay and radioimmuno-precipitation assay. Both agar gel immune-diffusion (AGID) and enzyme-linked immuno-sorbent assay (ELISA) are considered referent tests, according to the 2008 OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* [18,19]. However, delayed and intermittent seroconversion, maternal antibodies, high antigenic and genotype variations represent major drawbacks for use of serological tests in control strategies [1,19-21]. SRLV infections are reported in neighbouring and other countries around Kosovo, including Macedonia [22-28]. There is record of samples from a goat flock in Kosovo testing positive (ELISA) for CAE in 2009 [29]. Because there was no recent evidence of existing SRLV infection in Kosovo the initial aim of the surveys reported here was to detect evidence of infection and to perform the first structured and country based serological survey for SRLV in Kosovo.

MATERIAL and METHODS

Kosovo is a landlocked country in South-eastern Europe with a humid continental climate. The total area is 10.908 km^2, divided into 37 municipalities that for convenience can be grouped into five geographical areas: North, east, central, west, and south *(Fig. 1)*.

Survey Design and Implementation of Sampling Activity

Because there was no recent evidence of existing SRLV infection in Kosovo the initial aim of the surveys reported here was detection of evidence of infection. The survey sample was determined for multiple purposes which were to detect the presence of SRLV and to estimate the sero-prevalence of brucellosis in small ruminants in Kosovo. The survey design set out to collect 8.000 samples from sheep and goats on 500 premises. This number of samples, divided between sheep and goats in similar proportion to that in the whole population (slightly above 10% goats), was calculated to provide at least 95% probability of detecting the both infection if present at a minimum individual prevalence of 0.1% in sheep and 0.5% in goats. In the final analysis, having established the presence of both infections, the results obtained were sufficient to provide estimates of sero-prevalence of SRLV infection with 95% confidence intervals of ±3% in sheep and ±10%

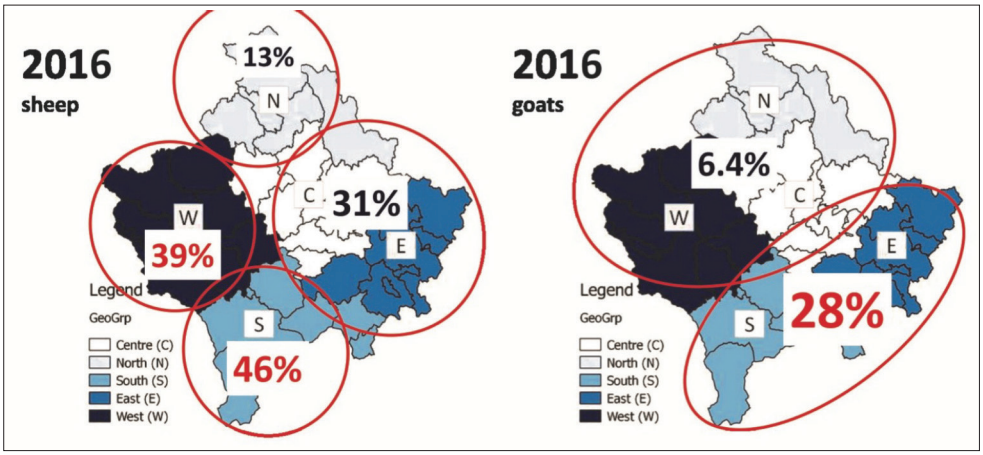

Fig 1. Representation of the spatial patterns of MVV/CAEV antibodies sero-prevalence in sheep and goat across Kosovo in 2016

in goats. The sampling frame of premises was derived from the official identification and registration (I&R) database maintained by Kosovo Food and Veterinary Agency (KFVA). A list of 2.666 premises with recently registered sheep or goats was extracted. Premises with fewer than 10 registered sheep and goats recorded were omitted from the sampling frame because these flocks would not have sufficient numbers of eligible animals to make sampling viable. These smaller flocks were 22% of all flocks but contained less than 1% of the registered population so their exclusion will have had little impact on the representative nature of the sample. Based on the registrations in the I&R database, the resulting sampling frame included about 270.000 sheep and 28.000 goats held on 2.078 premises (297 goats only, 1.202 sheep only and 579 mixed species). Distribution of samples according to geographic area are shown in *Table 1*.

Sampling was conducted in two stages. At the first stage, a random selection of premises was made from the sampling frame. The selection of premises from the sampling frame was stratified by flock size and intentionally biased towards larger flocks to ensure that the resulting sample of individual animals matched the whole population in terms of the flock sizes in which individuals exist.

At the second stage a pre-defined number of animals was set for sampling by the field staff during the visit to each selected premises. The target animals for sampling were adult animals, 12 months old or over (almost all were female). The number of animals to be sampled per premises was defined according to the total number of small ruminants registered in the premises (5 samples in flocks of 10-39, 10 samples in flocks of 40-99 and 20 samples in flocks of 100 or more). The sampling instructions for field teams included a breakdown of samples to take by species, based approximately on the proportions of sheep and goats registered in each selected premises.

Field sampling was organised by a single contracted private veterinary company. Sample collection for the survey was carried out between May and September 2016. Bleeding of animals in the field was carried out by locally sub-contracted licensed private veterinary practitioners (PVPs). Blood samples were taken into plain vacutainer tubes. Each sample was given a unique ID and the species, sex and estimated age in years of each sampled animal was also recorded. Instructions were issued that samples were kept cool in transit. Samples were gathered by the contracted veterinary company in Pristina, checked and recorded and then forwarded to the Kosovo Food and Veterinary Laboratory (KVFL) for storage and testing.

Laboratory Diagnostic Testing

On receipt at KFVL the serum was separated and stored at -20°C. Samples were tested using an indirect ELISA based on the use of an immunogenic peptide of transmembrane protein (TM, ENV gene) and of the recombinant p28 protein which enters into the composition of the viral capsid (GaG gene) (Maedi-Visna/CAEV Antibody Test Kit REF: P00303-10 IDEXX MVV/CAEV p28 Ab Screening). Anti-p28 antibodies can appear slightly later than the antiviral envelope protein antibodies. The use of this stable protein allows the serological detection of a wide spectrum of serological variants. The cut-off point was calculated according to the manufacturer's instructions. Samples with sp% ≥120 of the control positive were classified positive and samples with sp% <120 negative. Samples with sp% >110 but <120 could be classified as 'inconclusive'.

Statistical Analysis

Asymmetric Wilson score confidence intervals taking into

Table 1. *Distribution of samples received and tested by geo-spatial group*

Geographic Area	Total Samples Tested (MV/CAE ELISA)	Registered Population	% of Registered Population Sampled
SHEEP SAMPLES			
Centre	918	46660	2.0%
East	1137	54198	2.1%
North	503	32195	1.6%
South	1339	72196	1.9%
West	1375	68452	2.0%
Overall	**5272**	**273701**	**1.9%**
GOAT SAMPLES			
Centre	104	7091	1.5%
East	124	8661	1.4%
North	47	2820	1.7%
South	61	3987	1.5%
West	99	7087	1.4%
Overall	**435**	**29646**	**1.5%**

account the sample size and the total population (sampling fraction) were calculated for prevalence estimates using the online statistical toolbox at *OpenEpi.com* [30]. This method provides exact, non-symmetrical confidence intervals for estimates based on simple random samples that are robust even when sample size is small and/or the prevalence is close to 0% or 100% [31-33]. The data were analysed using the method described by Bennett et al.[34] so that the effect of two-stage sampling could be taken into account, using premises as the unit of sample clustering. This analysis included a calculation of the overall sampling design effect. To take account of the two-stage sampling design, the lower and upper bounds of the calculated intervals were inflated by a factor of the square root of the design effect.

Calculation of the overall sero-prevalence estimates also took into account the distribution of samples by geographic area. The proportions of the registered sheep or goat population contained within each geographic area are used as weighting factors to adjust the overall prevalence estimate for Kosovo according to the relative population in each geographic area. Sero-prevalences for different age groups (by year cohort) where information on estimated age was provided were calculated using Wilson score 95% CI as for simple random sample (no accounting for possible design effect).

Differences in sero-prevalence between groups were assessed for statistical significance using the chi-square statistic where more than two groups were involved and the Fisher exact test for 2-way comparisons only *(Table 2)*.

RESULTS

Of the planned 8.000 samples from 500 premises, 6.013 were collected from 356 premises. This difference in sample size reflects the degree of uncertainty in the I&R database which was expected. The total planned sample size had been increased to take this problem into account. Out of 6013 samples collected, 306 samples were classified as haemolysed or did not fulfil other criteria to be tested. The total number of sheep samples tested was 5.272 from 318 premises (average just over 16 per premises) and the total number of goat samples tested was 435 from 54 premises (average just under 8 per premises). These samples represent an overall 1.9% of sheep population and 1.5% of goat population.

Table 2. *Maedi-Visna sero-prevalence (ELISA) in sheep and Caprine Arthritis-Encephalitis sero-prevalence (ELISA) in goats across Kosovo in 2016, by age*

SHEEP SAMPLES			
Estimated Age (years)	**n Samples Tested**	**n MV ELISA Positive**	**%MV Positive With 95%CI**
1 to <2	899	332	36.93% (33.84% to 40.13%)
2 to <3	1718	567	33.00% (30.82% to 35.26%)
3 to <4	1311	467	35.62% (33.08% to 38.25%)
≥4	1052	352	33.46% (30.67% to 36.37%)
not specified	292	137	46.92% (41.27% to 52.64%)
GOAT SAMPLES			
Estimated Age (years)	**n Samples Tested**	**n CAE ELISA Positive**	**%CAE Positive With 95%CI**
1 to <2	116	12	10.34% (6.02% to 17.21%)
2 to <3	86	17	19.77% (12.72% to 29.40%)
3 to <4	105	30	28.57% (20.81% to 37.85%)
≥4	128	8	6.25% (3.20% to 11.85%)

Table 3. *Maedi-Visna sero-prevalence (ELISA) in sheep across Kosovo in 2016, by geographic area*

Geographic Area	**Number of Premises Sampled (and Number of Samples Tested)**	**Sero-prevalence (%) with 95% CI**
Centre	53 (918)	30.83% (24.87% to 37.13%)
East	68 (1.137)	31.05% (24.96% to 37.43%)
North	33 (503)	12.92% (7.95% to 18.95%)
South	84 (1.339)	45.93% (40.89% to 51.01%)
West	80 (1.375)	39.20% (32.10% to 46.47%)
Overall	318 (5.272)	35.19% (32.13% to 38.29%)
Kosovo (Weighted for geographic area)		34.84% (31.79% to 37.94%)

Table 4. *Caprine Arthritis-Encephalitis sero-prevalence (ELISA) in goats across Kosovo in 2016, by geographic area*

Geographic Area	Number of Premises Sampled (and Number of Samples Tested)	Sero-prevalence (%) with 95% CI
Centre	12 (104)	4.81% (2.10% to 10.73%)
East	20 (124)	30.65% (8.05% to 56.79%)
North	4 (47)	4.26% (0.58% to 16.30%)
South	7 (61)	21.31% (8.00% to 40.06%)
West	11 (99)	9.09% (4.12% to 17.67%)
Overall	54 (435)	15.40% (7.05% to 25.40%)
Kosovo (Weighted for geographic area)		15.55% (7.20% to 25.55%)

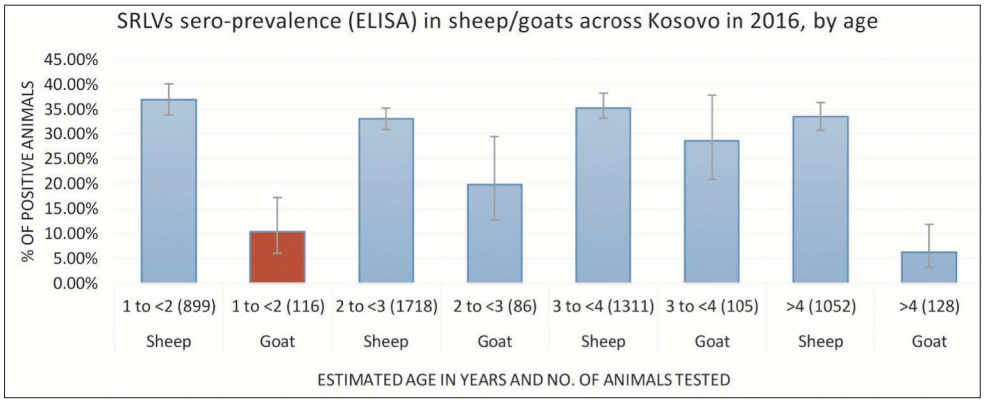

Fig 2. MVV/CAEV antibodies sero-prevalence (ELISA) in sheep and goats across Kosovo in 2016, by age. Estimated age in years and no. of animal tested (in brackets) are shown. The error bars indicate the Wilson score 95% CI calculated as for a simple random sample

The percentage sampling was similar in all geographic areas except the North, where a slightly lower percentage of sheep and higher percentage of goats were sampled. Any possible bias in the overall sero-prevalence estimate as a result of this was accounted for by using a weighted estimate.

Overall individual prevalence in sheep was 35% and flock prevalence was 85%. Average within flock prevalence for sheep was 40%. In total, 67 goat samples were positive giving an overall individual prevalence of 15% and flock prevalence of 35%. Average within flock prevalence for goats was 29%. *Table 3* and *Table 4* show the sero-prevalences calculated for each geographic area and the overall sero-prevalence both unweighted and weighted to account for differences between geographic distribution of the sample and the target population.

Sero-prevalence for SRLV antibodies in sheep is highest in the South and West geographic areas. Sero-prevalence for SRLV antibodies in goats is highest in the East and South geographic areas. There are statistically significant differences in SRLV sero-prevalence in sheep between geographic areas as indicated by an overall chi-square value of 203 (4d.f.) with a P-value of <0.00001. In pairwise comparisons, all geographic areas are significantly different from one another except the Centre and East *(Fig. 1)*. There are statistically significant differences in SRLV sero-prevalence in goats between geographic areas as indicated by an overall chi-square value of 40.2 (4d.f.) with a P-value of <0.00001. Not all geographic areas are significantly different from one another. The distinct spatial groupings are the South and East together, with an aggregated prevalence of 27.6% and the Centre, North and West together, with an aggregated prevalence of 6.4%. The difference between these two aggregated prevalences is statistically significant (chi-square value of 34.95 (1d.f.) with a P-value of <0.00001), while differences within these two groupings are not significant (Centre vs North vs West: chi-square p-value 0.3683; South vs East: chi-square P-value 0.2465) *(Fig. 1)*.

The sero-prevalences for different age groups, where information on estimated age was provided with the sample are shown in *Table 2* and *Fig. 2*, with Wilson score 95% CI calculated as for a simple random sample (not accounting for possible design effect).

There are no statistically significant differences between the four age groups in sheep (from <2 year to ≥4 year): chi square 5.285 (3d.f.) with P-value =0.1521. There are statistically significant differences between the age groups in goats: chi square 25.74 (3d.f.) with P-value =0.00001082. The sero-prevalence of the age group 1 to <2 years is significantly lower than the sero-prevalence of the age group 3 to <4 years. Also, the sero-prevalence of the age group ≥4 years is significantly lower than the sero-prevalence of the age groups 2 to <3 years and 3 to <4 years.

DISCUSSION

Although KFVL has previously tested samples from small ruminants for SRLV and found positive results, this had

not been as part of a formal survey and these results had not been reported internationally. The results reported in this paper are resulting from the first structured and country-based survey assessing the SRLV infection across Kosovo. Sero positive sheep and goats were found in all five geographic areas, which means that the headline result of the survey is that there is clear evidence that SRLV infection is present in sheep and goats throughout Kosovo. It is difficult to compare and elaborate the results with neighboring countries due to the extended period of time in reports and differences in study design. Nevertheless, the present data as well as those from neighboring countries suggest that SRLV infection at least in sheep must be endemic in the region. Although, the survey was not designed to estimate flock prevalence with any specified accuracy or precision, flock-level statistics were calculated and reported in the results but only succinctly even though the sample sizes are small. In particular, when analysing the sheep and goat samples separately the number of goat samples available per flock tended to be much fewer than the number of sheep samples. The probability of detecting goat flocks affected by SRLV would therefore be quite low. The flock-level estimates should be viewed cautiously, with the possibility in mind that the true percentage of flocks affected, particularly goat flocks affected by SRLV, could be higher than the percentage apparent from the survey results.

In order to avoid interference of maternal antibodies, only adult >1 year old sheep and goats were included in this study. It is recommended that serologic or molecular testing of lambs and kids occur at least 4-6 months following weaning [18]. Previous studies have shown that if lambs are allowed to suckle naturally from positive dams and weaned at 8 months, maternal SRLV antibody is detectable starting the first day after suckling and up to 52 weeks of age in some lambs [29]. In addition, under the same conditions, the SRLV provirus may be detectable in the peripheral blood mononuclear cell of lambs up to 24 weeks old [29]. Present data suggest that different epidemiological scenarios might apply for sheep and goats. There are no statistically significant differences in SRLV sero-prevalence between sheep in different age groups from <2year to ≥4 year. The fact that sero-prevalent sheep appear to be retained in the population beyond 4 years old may suggest that the clinical impact of SRLV infection is mild in Kosovo sheep. This is in accordance with the fact that mortality due to SRLV infection in sheep may be low in enzootic areas [5]. In contrast, for SRLV sero-prevalence in goats, there are statistically significant differences between the age groups. There is a steady increase in sero-prevalence from age group 1 to <2 years to age group 3 to <4 years, followed by a sharp decrease, with sero-prevalence in the age group ≥4 years being significantly lower than the sero-prevalence of the age groups 2 to <3 years and 3 to <4 years. This highlights the need for further investigation based on clinical impact of infection and genotype characterization. However, this could also be explained as an artefact of the lower sample size per flock. This should be further explored through investigations with farmers and PVPs which can be focussed on those flocks where antibodies have been detected.

Kosovo has a very long tradition of sheep production. Sheep production is one of the sectors within Kosovo agriculture that suffered the most severe decline in the post-war period (after 1999). By November 2001, sheep populations were at 56% of their pre-war levels [35]. Contrary, in former Yugoslavia, the number of goats dramatically dropped after the law of banning goats from fields had been passed in 1954 and lasted till 1989. Since then goat production was never important in Kosovo. After post-war (1999) small ruminant population bottleneck, there was a steady increase of sheep and goat population with a replacement from imported animals as a donation, and from other different sources (frequently uncontrolled). Therefore, multiple-source infections should be expected.

SRLV are heterogeneous, the strains circulating in different areas may differ from each other and thus the performance of diagnostic tests in these areas might vary accordingly. Clear differences have been found among the different ELISA tests in analytical and diagnostic sensitivity and overall diagnostic performance, whereas no significant differences in specificity were found [19,36,37]. Interestingly, genotype A derived antigens seem more suitable than genotype B antigens to detect heterologous infection [19,38]. On the other hand, sero-prevalence against genotype E may be underestimated using commercially available ELISAs [39]. Although, IDEXX MV/CAE p28 Ab screening Kit used in this study is designed to detect a wide range of serological variants, some sensitivity issues might be expected [37]. Therefore, before any strategy planning, further genotyping of the SRLVs is pivotal. Parturition in small ruminants in Kosovo is exclusively during winter months. Bleeding of animals was done during summer. Therefore, false negative results due to the fluctuation of antibodies during periparturient period [20] are ruled out. In the absence of vaccination, test and slaughter is the only currently available strategy for control and elimination of these infections. However, this is not financially feasible under current conditions in Kosovo.

Moreover, any attempts of control strategies on a large scale will be hampered by uncontrolled and illegal movements of animals. Small ruminants are confined for long periods due to the harsh Balkan winter and that increases the risk of horizontal transmission. Some control of infection is possible with MV and CAE by separating lambs/kids from infected dams at the time of birth and running separate herds/flocks of uninfected and infected animals but this is complicated and probably not practical for many livestock keepers under conditions in Kosovo. However, at present, it is the only applicable measure to control the prevalence of SRLV infections in Kosovo. Owners are usually unaware

of the role of SRLV infections in animal welfare and economics of sheep and goat farming. Existing authorities should pursue and encourage an active information policy through pre-existing animal health information channels and private veterinarians. Regional cooperation is a must in order to ensure a successful control program.

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Research Article

Effect of *Myrtus communis* L. Plant Extract as a Drinking Water Supplement on Performance, Some Blood Parameters, Egg Quality and Immune Response of Older Laying Hens

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Abstract

The aim of the present study was to evaluate the effects of myrtle plant extract (MPE) on performance, some blood parameters, egg quality and immune response of older laying hens. A total of 192 laying hens (67 weeks old; initial body weight 1.63±0.17 kg) were used in this study. The MPE was added to the water of the experimental groups (0%, 2.5%, 5.0%, or 10%) for 8 weeks. Liquid chromatography analyses showed that myricetin was the predominant active ingredient (15.34 mg/L) in MPE. In treatment groups, feed consumption, egg mass (P<0.05), egg production (P<0.01) increased (10% MPE) and water consumption ascended (5% MPE) compared to the control birds. Feed conversion ratio did not change. In 2.5% MPE group, greater egg weight (P<0.001) and darker egg yolk (P<0.01) were observed than the control birds. Regarding immunity, the 5% Myrtus group produced higher neutrophil and large amounts of IgG (P<0.05) indicating that significant results were also observed in the immunological response of laying hens vaccinated against the Newcastle virus. Serum alanine aminotransferase level increased (P<0.05) in treatment groups whereas serum glucose levels decreased significantly (P<0.01). Other than that, none of the blood parameters changed. It is concluded that *Myrtus communis* L. plant extract showed positive effects on the egg yolk, some performance parameters and immune system of older laying hens without any adverse effects on egg traits.

Keywords: *Common myrtle, Antibody response, Immunonutrition, Serum profile, Aged hens*

İçme Suyu Katkısı Olarak Kullanılan *Myrtus communis* L. Bitki Özütünün Yaşlı Yumurtacı Tavuklarda Performans, Bazı Kan Parametreleri, Yumurta Kalitesi ve Bağışıklık Tepkisi Üzerine Etkileri

Öz

Bu çalışmanın amacı, mersin bitki özütünün (MPE) yaşlı yumurtacı tavukların içme sularına ilavesinin performans, bazı kan parametreleri, yumurta kalitesi ve bağışıklık tepkisine olan etkilerini değerlendirmektir. Çalışmada 67 haftalık yaşta 192 yumurtacı tavuk kullanılmıştır (Başlangıç ağırlıkları 1.63±0.17 kg). Özüt, deneme gruplarının içme sularına 8 hafta boyunca %0; %2.5; %5 ve %10 düzeylerinde katılmıştır. Sıvı kromatografi analizleri, MPE içerisindeki en baskın aktif bileşenin mirisetin olduğunu göstermiştir (15.34 mg/L). Uygulama gruplarında; yem tüketimi, yumurta kütlesi (P<0.05) ve yumurta verimi (P<0.01) artmış (%10 MPE), su tüketimi de yükselmiştir (%5 MPE). Yemden yararlanma oranı değişmemiştir. Kontrol grubuna göre %2.5 MPE grubunda daha yüksek yumurta ağırlığı (P<0.001) ve daha koyu yumurta sarısı (P<0.01) gözlenmiştir. Newcastle virusuna karşı yapılan aşılamaya verilen immun yanıt açısından değerlendirildiğinde; %5 Myrtus grubunda daha yüksek düzeyde nötrofil sayısı ve IgG düzeyi belirlenmiştir. Serum Alanin Aminotransferaz düzeyi deneme gruplarında yükselirken (P<0.05); serum glikoz düzeyleri düşmüştür (P<0.01). Bunlar haricindeki diğer hiçbir kan parametresi değişmemiştir. Sonuç olarak, *Myrtus communis* L. bitki özütü yaşlı yumurtacı tavukların yumurta kalite özellikleri üzerine herhangi bir yan etki oluşturmadan yumurta sarı rengini, bazı performans parametrelerini ve bağışıklık sistemini olumlu yönde etkilemiştir.

Anahtar sözcükler: *Mersin, Antikor yanıt, Immun besleme, Serum profili, Yaşlı yumurtacı tavuklar*

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INTRODUCTION

Poultry sector plays an important role in the economy of any country and also poultry is one of the most economical and easily available sources in terms of protein. Currently, many studies are being conducted to keep poultry products safer for human health because humans of all ages consume poultry products such as eggs and meat. In the past, many synthetic antibiotics were used to maintain poultry health and production, however, nowadays herbal extracts are explored for their potential as a replacement for synthetic antibiotics without affecting the profitability of poultry farms [1].

Many dietary herbal products (powder, extracts, and essential oils) showed good effects on the performance parameters (eggs production, internal and external egg quality) of laying hens [2]. The chemical components of many plant extracts have antioxidant properties which help to eliminate free radicals in the body to cover stress-related problems and therefore improve the health status of hens [3].

Myrtle *(Myrtus communis* L.) is a medicinal plant and largely used for therapeutic purpose. Myrtle belongs to the family of *Myrtaceae*, which contains more than 5500 species [4]. Its leaves are traditionally used as a disinfectant, hypoglycemic and antiseptic agent [5]. Myrtle is among natural herbal products, which is mostly used in skin lotions for different skin disorders such as sunburn, acute wounds and to accelerate the healing process of the skin. Myrtus plant extract (MPE) is a pure natural, nontoxic, non-steroidal and commonly available herbal extract which is used for different medical treatments such as rashes, allergies, healing wounds, pimples [6]. Researchers observed that MPE has high phenolic content which activates the plants' defense mechanism. Moreover, MPE also contains antioxidants which further avoids oxidative damage in the body [7,8].

Myrtus plant extract contains many active components such as gallic acid, protocatechuic acid (PCA), catechin, myricetin, salicylic acid, rosmarinic acid, quercetin, genistein, and octanoate [9-11]. Even though research on the effects of the dietary MPE is limited in poultry, there are some studies about the aforementioned active ingredients in avian models. For instance, Samuel et al.[12] observed that supplementation of dietary gallic acid improved plasma antioxidant activity in broilers. In another study, it was observed that the survival rate of chickens in the group treated with PCA was 90% against infectious bursal disease virus, therefore, indicating that PCA could improve the immune organ index (spleen, bursa) in chickens [13]. Research shows that the dietary supplementation of broiler feed with genistein overcomes the deleterious effects of heat stress in persistent summer stress. Moreover, genistein also increases the feed conversion ratio (FCR), feed consumption, and body weight of broilers in extremely hot weather [14]. Similarly, positive results have been observed in the sensory score, breast meat quality and oxidative stress of broiler chickens when their diets are supplemented with hesperidin and genistein [14].

Previous evidence suggests that provision of any nutrients or additives as dietary or water supplement may have different effects on birds. Noy and Sklan [15] concluded that carbohydrates supplied with drinking water in the early stages of chicks' life were more effective than carbohydrates provided by diet for body weight gain. Similarly, Ritzi et al.[16] observed that probiotics as a water supplement had a better effect on broiler performance than the same probiotics provided by the diet. Gültepe et al.[17] observed some positive effects of lemon juice as a water supplement on egg production during the late phase production cycle of laying hens. Furthermore, Çetingül et al.[18] reported that supplementation of pomegranate molasses with drinking water to laying hens may affect some quality parameters in eggs after 30 days of storage.

This study was carried out to determine the effect of MPE on production parameters, egg quality, and blood physiology in laying hens. In our knowledge, the novelty of this study is that MPE was used first time in drinking water of older laying hens. The bioavailability of water is very high and also its metabolism is very fast. Therefore, we chose the water route for the supplementation of MPE to the laying hens.

MATERIAL and METHODS

This study was conducted to investigate the effects of Myrtus plant extract (Biyoderm®, ArsArthro Biotechnologies Inc, Ankara, Turkey) on egg production, egg quality and some blood parameters in older laying hens. All experimental procedures were performed at the Animal Research Center of Afyon Kocatepe University, Turkey after the approval of the ethics committee under approval No: 49533702/91; dated: 30/05/2017.

Production and Validation Processes of The Extract

Myrtus plant extract was done with a process containing collection, size reduction, drying, authentication of plant material, filtration, extraction, drying, and reconstitution [19]. Briefly, the dried leaves and barks of plants were used for the extraction. The extraction was water-based and no solvent was used except distilled water (1% MPE, 99% pure water as a carrier). LC-MS/QTOF (Agilent Technologies, Santa Clara, CA, USA) and Orbitrap (Thermo Electron, Bremen, Germany) were used. Moreover, Agilent Poroshell 120 EC-C18 3.0x100 mm, 2.7 µm colon were also used by 10mM Amonyum Format 0.1%, Formic Acid and water with MFB 0.1% Formic Acid MeOH (Gas Temperature 325°C, Gas Flow 10 L/min, Nebuliser 45 psig) for active ingredients determination (Agilent Technologies, Santa Clara, CA, USA).

All the ingredients were reported by negative and positive polarization with both instruments.

Poultry, Management and Experimental Design

A total of 192 Babcock white laying hens (67 weeks old; initial body weight 1.63±0.17 kg) were divided into 4 groups (n=48) with 8 subgroups containing 6 hens in each subgroup. MPE was added to the drinking water of the experimental groups with 0%, 2.5%, 5.0%, and 10% respectively during 8 weeks. For lighting, 16 h light and 8 h dark were applied and feed and water were supplied ad-libitum. All MPE treatment groups including the control group were fed a basal diet prepared to meet the needs of laying hens *(Table 1)* as reported in the NRC [20].

Automatic nipple drinking system was used and each group has separated water tank where the different concentration of *Myrtus communis* L. plant extract (MPE) was added in their water tank. Graduated cylinder glass was used for scaling of Myrtus plant extract. Then, MPE was mixed with water at the ratio of 0 mL/L, 2.5 mL/L, 5 mL/L and 10 mL/L for control, 2.5%, 5%, and 10% groups respectively in 20-liter water box between 1 p.m. to 2 p.m. every day. The product could be solved easily in the water and homogeneity was confirmed visually. During the study, water consumption (l) was measured by total water consumption per each group after 24 h interval.

Data Collection and Analyses

Hens were weighed at the beginning and end of the study to determine their live weights. Egg production and mortality were recorded daily while feed consumption was recorded weekly. Eggs were weighed once per week. Egg mass was calculated as follows:

Egg mass = Percent egg production × average egg weight in grams

Feed conversion ratio (FCR) values were calculated as follows:

FCR = feed consumption (g)/egg mass (g)

Eggs delivered to the laboratory at the end of the 4th week and also at the end of the 8th week to determine egg quality parameters. Eggs were kept for 24 h at room temperature before the egg trait analyses. Egg weight, egg yolk color index, breaking strength, eggshell thickness and Haugh unit were determined. Egg breaking strength was measured by using ORKA Egg Force Reader, EF 0468-2011 (Orka Feed Tech. Ltd., Hong Kong, China) and Haugh Unit were calculated by measuring albumen height (Digital Caliper, CD-15CP, Mitutoya Ltd., UK) according to the method devised by Haugh [21]. Egg yolk color was determined by using Roche Improved Yolk Color Fan and comparing the color of yolks with 15 bands of the color fan (YolkFan™, DSM Nutritional Products AG, Kaiseraugst, Switzerland). Albumen index and yolk index were calculated as follows [22]:

Table 1. *Composition and nutrient ingredients of the basal diet*

Ingredients	**%, As-fed Basis**
Corn grain	54.90
Vegetable oil	0.34
Sunflower meal (32% CP)[1]	16.93
Full fat soybean	10.00
Soybean meal (44% CP)[1]	7.39
Limestone	7.87
Dicalcium phosphate	1.73
Common salt	0.40
Vitamin-mineral premix[2]	0.25
L-lysine hydrochloride	0.10
DL-methionine	0.10
Chemical components[3]	
CP[1],%	17.00
ME[4],kcal/kg	2750
Calcium,%	3.71
Available P,%	0.38
Sodium,%	0.20
Methionine + Cystine, %	0.71
Lysine,%	0.83
Treonin,%	0.61
Triptophane,%	0.20
Linoleic acid,%	2.36
Active ingredients of MPE[5]	**mg/L**
Myricetin	15.34
Catechin	4.80
Quercetin	0.19
Gallic acid	0.13
Salicylic acid	0.06
Rosmarinic acid	0.01

[1] CP: Crude Protein; [2] Provided per kg of diet: Vitamin A: 12.000.000 IU, Vitamin D_3: 3.000.000 IU, Vitamin E: 35.000 IU, Vitamin K_3: 3.500 IU, Vitamin B_1: 2.750 IU, Vitamin B_2: 5.500 IU, Nicotinamide: 30.000 IU, Ca-D-Panthotenate: 10.000 IU, Vitamin B_6: 4.000 IU, Vitamin B_{12}: 15 IU, Folic acid: 1.000 IU, D-Biotin: 50 IU,Choline chloride: 150.000 IU, Manganese: 80.000 mg, Iron: 60.000 mg, Zinc: 60.000 mg, Copper: 5.000 mg, Iodine: 2.000 mg, Cobalt: 500 mg, Selenium: 150 mg, Antioxidant: 15.000 mg; [3] Calculated according to NRC [20]; [4] ME: Metabolisable energy; [5] LC-MS/QTOF analyse [19]

Albumen index = Albumen height (mm)/[Albumen length (mm) + Albumen width (mm)] × 100

Yolk index = Yolk height (mm)/Yolk diameter (mm) × 100

To determine the antibody level against Newcastle disease, all chickens were vaccinated with Newcastle vaccine at the beginning of the study. At the end of the trial, 3 animals were randomly selected from each subgroup and blood was collected directly from the heart. The samples were transferred into two separate tubes (vacutainer tubes without anticoagulant and with EDTA; BD Vacutainer®, Franklin Lakes, NJ, USA). Blood samples immediately

arrived in the laboratory under a cold chain. Samples with anticoagulant were used for the full blood count. The blood count was performed by a compact blood analyzer (BC 2800 Vet, Mindray Medical International Ltd., Shenzhen, China) for determining the numbers of total leukocyte (TLC), lymphocyte (LC), neutrophil (NC), monocyte (MC), and red blood cell (RBC); the levels of Hemoglobin (He), mean corpuscular hemoglobin (MCH), and platelet (PLT); the concentration of mean corpuscular hemoglobin concentration (MCHC); the volume of mean corpuscular (MCV) and mean platelet (MPV). The samples in vacutainer tubes without anticoagulant were centrifuged for 10 min (5 000 g). The supernatants were stored at -20°C till analyses in 2 mL microcentrifuge tubes. The levels of an automated analyzer (Elisys Uno, Human mbH, Wiesbaden, Germany) was used for serum biochemical assays (glucose, total cholesterol - CHO, high density- and low density-lipoprotein - HDL & LDL, aspartate and alanine aminotransferase - AST & ALT), gamma-glutamyl transpeptidase - GGT, phosphorus, calcium and Immunoglobulin G - IgG.

Statistical Analysis

Firstly, Shapiro-Wilk and Levene tests were performed for determining the normal distribution of data and variance homogeneity. One-way analysis of variance was used for comparison of mean between the group and Tukey-Kramer test was chosen for *post-hoc*. The following model was used for data to analyze:

$$Y_{ij} = \mu + \alpha_i + e_{ij}$$

where Y_{ij} = the response variable, μ = the general mean, α_i = the effect of water supplements and e_{ij} = the random error. The significance level was determined as $P<0.05$. All data were expressed as mean±SEM in tables. The environment of MedCalc Software (v.18, Oostend, Belgium) was used all data analysis.

RESULTS

All active ingredients of MPE were determined as follow: Gallic acid 0.13 mg/L, Catechin 4.8 mg/L, Myricetin 15.34 mg/L, Salicylic acid 0.06 mg/L, Rosmarinic acid 0.015 mg/L, Quercetin 0.197 mg/L).

The result of the recent study revealed that hen-day egg production was significantly ($P<0.01$) increased by 10% of the MPE group as compared to the control group. Similarly, feed consumption and egg mass were also significantly ($P<0.05$) increased in 10% MPE group. For egg weight, only 2.5% MPE group showed significant ($P<0.01$) results. None of the groups manifested any impact on FCR. Moreover, water consumption significantly increased ($P<0.01$) in 5% MPE groups as compared with the control group *(Table 2)*.

The group supplemented with 2.5% of MPE showed significant ($P<0.01$) results in terms of egg yolk and produced darker yellow egg yolks. However, the results for other egg quality parameters such as egg-breaking strength, shell thickness, albumin index, yolk index, and Haugh unit remained insignificant ($P>0.05$) during the whole study period *(Table 3)*.

For serological examinations, quadratic decreases ($P<0.01$) were observed for serum glucose level in all MPE treatment groups compared to the control group. However, serum ALT level significantly ($P<0.05$) increased in the group supplemented with 2.5% MPE group. Other serological parameters such as serum CHO, HDL, LDL, AST, GGT, calcium, and phosphorus levels did not show any significant ($P>0.05$) results. Regarding immunity, serum IgG level significantly ($P<0.05$) increased in the group supplemented with 5% of MPE compared to the other treatment and control groups *(Table 4)*.

For hematological parameters, NC increased significantly ($P<0.05$) in 5% and 10% MPE treatment groups compared to the control group. However other parameters such as TLC, LC, MC, RBC, He, MCV, MCH, MCHC, PLT, and MPV did not show any positive ($P>0.05$) response *(Table 5)*.

DISCUSSION

Many studies have manifested that dietary phytogenic extracts had some positive effect on the performance of laying birds [23]. Until now, most of the phytogenic research in avian models focused on dietary supplementation route. However, the comparative studies showed that dietary supplementation or water supplementation of similar products can cause a different response to performance in laying hens and broilers [15,16]. Although the research on water supplement of phytogenic extracts and herbal

Table 2. *Effects of graded levels of Myrtus plant extract on performance parameters of laying hens for 8 weeks (Mean±SEM; n=48)*

Group	Feed Consumption (g/hen/day)	Egg Production (%/day)	FCR	Egg Weight (g/hen/day)	Egg Mass (g/hen/day)	Water Consumption (L/hen/day)
Control	108.51±1.59^{a}	83.36±0.74^{a}	2.03±0.05	64.14±0.27^{b}	53.48±0.97^{a}	8.88±0.14^{a}
2.5 %	113.65±1.76^{ab}	83.98±0.75^{a}	2.10±0.04	65.27±0.29^{a}	54.22±0.55^{ab}	8.65±0.11^{a}
5 %	113.77±2.02^{ab}	84.92±0.72^{a}	2.08±0.05	64.76±0.29^{ab}	54.74±0.79^{ab}	9.92±0.14^{b}
10 %	115.30±1.57^{b}	90.32±0.66^{b}	2.02±0.06	63.40±0.26^{b}	57.08±0.99^{b}	9.22±0.15^{ab}
P value	0.024	0.001	0.705	0.001	0.038	0.001

Values with different superscripts in the same column differ significantly ($P<0.05$); FCR Feed conversion ratio; FCR = feed consumption (g)/egg mass (g)

Table 3. *Effects of graded levels of Myrtus plant extract on egg quality parameters of laying hens for 8 weeks (Mean±SEM; n=48)*

Group	Egg Breaking Strength (kg/cm)	Egg Shell Thickness (mm)	Haugh Unit	Egg Yolk Colour	Albumen Index (%)	Yolk Index (%)
Control	32.04±1.57	0.364±0.002	81.07±0.52	11.40±0.07^{a}	5.78±0.14	42.41±0.42
2.5%	31.52±1.86	0.365±0.002	82.32±0.57	11.72±0.06^{b}	6.02±0.14	42.29±0.44
5%	29.45±2.15	0.361±0.002	82.79±1.68	11.54±0.06^{ab}	6.62±0.95	43.18±0.39
10%	30.48±2.05	0.368±0.002	80.74±0.58	11.42±0.06^{a}	5.63±0.13	43.01±0.79
P value	0.656	0.242	0.369	0.001	0.548	0.559

Values with different superscripts in the same column differ significantly (P<0.05)

Table 4. *Effects of graded levels of Myrtus plant extract on serum biochemical parameters of laying hens for 8 weeks (Mean±SEM; n=48)*

Group	Glucose (mg/dL)	CHO (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	AST (U/L)	ALT (U/L)	GGT (U/L)	P (mg/dL)	Ca (mg/dL)	IgG (mg/dL)
Control	215.06±2.85^{a}	136.00±8.42	26.5±1.33	36.50±3.14	176.63±10.10	3.50±0.74^{b}	28.69±1.07	5.66±0.30	23.91±0.96	14.21±1.23^{b}
2.5%	193.19±5.08^{b}	156.13±11.84	23.88±0.44	45.81±4.41	161.00±6.71	8.19±0.89^{a}	26.81±0.75	6.43±0.34	25.69±0.91	12.32±1.73^{b}
5%	196.38±2.48^{b}	123.19±7.61	25.81±0.84	33.44±2.89	175.44±7.11	6.50±0.88^{ab}	28.44±1.35	5.42±0.27	23.73±0.89	17.75±0.66^{a}
10%	201.06±2.58^{b}	130.19±9.47	24.69±0.57	35.44±3.41	158.31±9.35	5.44±0.97^{ab}	28.31±1.03	5.44±0.38	23.73±0.62	14.53±1.36^{b}
P value	0.001	0.141	0.169	0.186	0.215	0.002	0.568	0.125	0.402	0.048

Values with different superscripts in the same column differ significantly (P<0.05); CHO: Total cholesterol; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transpeptidase; P: Phosphorus; Ca: Calcium

Table 5. *Effects of graded levels of Myrtus plant extract on hematological parameters of laying hens for 8 weeks (Mean±SEM; n=48)*

Group	TLC (10^9/L)	LC (10^9/L)	NC (10^9/L)	MC (10^9/L)	RBC (10^{12}/L)	He (g/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	PLT (10^9/L)	MPV (pg)
Control	2.86±0.14	1.39±0.07	0.68±0.07^{a}	0.220±0.019	2.42±0.08	11.00±0.23	108.94±0.52	29.39±0.50	30.17±0.39	27.11±0.29	6.49±0.08
2.5%	2.99±0.14	1.59±0.09	0.88±0.08^{ab}	0.266±0.021	2.39±0.07	11.59±0.22	109.38±0.35	28.29±0.43	31.33±0.38	27.23±0.26	6.47±0.07
5%	2.98±0.17	1.53±0.10	1.01±0.10^{b}	0.263±0.020	2.46±0.07	11.76±0.18	109.05±0.48	29.14±0.52	31.23±0.35	26.83±0.25	6.48±0.08
10%	3.11±0.15	1.51±0.09	0.93±0.07^{b}	0.246±0.020	2.57±0.08	11.48±0.24	110.06±0.31	29.78±0.36	31.03±0.45	26.80±0.30	6.71±0.18
P	0.742	0.521	0.020	0.319	0.385	0.102	0.242	0.132	0.152	0.619	0.398

Values with different superscripts in the same column differ significantly (P<0.05); TLC: Total leukocyte count; LC: Lymphocyte count; NC: Neutrophil count; MC: Monocyte count; RBC: Red blood cell count; He: Hemoglobin; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; PLT: Platelet; MPV: Mean platelet volume

products are limited, the findings of the aforementioned studies are various. Karadağoğlu et al.[24] reported no beneficial effect of water supplementation of an herbal blend (peppermint, thyme, and anise oil) on growth performance, meat quality and intestinal development of quails. However, Gültepe et al.[17] reported a positive effect of lemon juice on the performance of older laying hens. Additionally, Çetingül et al.[18] reported that providing pomegranate molasses in drinking water to laying hens had no major effect on egg quality after 30 days storage at 4°C, although it had some minor effects in earlier storage days. In our knowledge, this is the first report on the effects of water supplementation of MPE on the performance of laying hens. Bülbül et al.[25] observed that dietary myrtle oil increased egg production in laying quails after 8 weeks in agreement with the present study. Although Çabuk et al.[26] reported a greater number of eggs in laying hens fed with an essential oil blend (myrtle leaf, oregano, laurel leaf, sage leaf, fennel seed, and citrus peel oil) than the layers in control group after 140 days, they observed no significant effect of oil blend on egg production percentage of the layers. The incompatible results of the aforementioned study from the results of the present study and Bülbül et al.[25] can be due to the potential interaction between myrtle leaf oil and the others in the blend. Also, the presence of heat stress in the experiment of Çabuk et al.[26] may have limited the potential effects of myrtle oil.

A lot of research has been conducted on the effects of dietary supplementation of herbal products on egg traits of avian models. Christaki et al.[23] observed no major effect on egg traits of the quails fed with oregano, anise and olive leaves. Additionally, Freitas et al.[27] observed no positive effect of *Syzygium cumini* which is a herb belongs the same family with myrtle *(Myrtaceae)* on egg weight and egg mass of laying hens. Yet in the present study, we found that the supplementation of laying hens with 2.5% of MPE improved egg weight. The differences in observed findings from previous reports may be caused by the purity of MPE used in the present study.

Abdel-Wareth and Lohakare [28] observed that dietary herbs and plant extracts improved some performance parameters, such as feed consumption, egg mass, and FCR, in laying hens during the late phase of the production cycle. However, other studies reported that the addition of herbal extracts had no impact on the FCR of quails [29]. In the present study, we found that feed consumption and egg mass increased significantly ($P<0.05$) in the group supplemented with 10% of MPE while no effects were seen on FCR among any of the treatment groups. Bülbül et al.[25] showed that supplementations of 5% dietary Myrtus oil did not alter the feed consumption of laying quails. Similarly, in the present study, there was also no significant effect observed on the feed consumption in control, 2.5%, and 5% groups. Recently, Liu et al.[30] observed that the taste sense of birds may be more sophisticated and developed than previously thought. Unlike the dietary supplementation, the highest dose (10% MPE) of myrtle oil in the present study may change the water flavor due to high concentrated essential oil content and fresh supplementation method. Increase in the feed and water consumption of the layers in the highest dose MPE groups may be a result of mentioned taste improvement.

Researchers observed that the increase in egg production had a direct correlation with water consumption. Increase in egg production will increase water consumption [31]. Our result was consistent with the finding of Medway and Kare [31], we also observed a significant increase in both egg production and water consumption.

Previous evidence of the research suggests that the effects of essential oils on egg quality parameters are inconsistent. Bölükbaşı et al.[32] reported that dietary thyme oil, sage oil, and rosemary oil had no effect on albumen proportion of egg while it affected Haugh unit, yolk and eggshell proportion of egg in laying hens. Çabuk et al.[26] observed that no effect of dietary essential oil blend (myrtle leaf, oregano, laurel leaf, sage leaf, fennel seed, and citrus peel oil) on egg weight of laying hens suffering from heat stress. Olgun [33] reported a quadratic response to supplementation of a dietary essential oil blend on eggshell thickness while the supplement had no effect on egg breaking strength and the specific gravity of eggs in laying hens. Also, Ding et al.[34] recently reported no effects of an essential oil blend on egg quality parameters in laying hens. In the present study, egg yolk color significantly ($P<0.01$) increase in 2.5% MPE group while other egg quality parameters such as egg-breaking strength, shell thickness, Haugh unit, albumen index, and yolk index remained unchanged ($P>0.05$). The darker egg yolks may be caused by antioxidant properties of myricetin [35], which is the predominant active ingredient in MPE according to the ingredient analyze in the present study.

In the present study, some serological parameters such as serum glucose, ALT, and IgG showed significant ($P<0.01$) results such as the decrease in glucose level in a quadratic manner compared to the control group. In contrast, serum ALT concentration significantly ($P<0.01$) increased in treatment groups. However other serological parameters such as CHO, HDL, LDL, CHO, AST, GGT, serum phosphorus, and calcium did not show any significant results ($P>0.05$). In the present study, LC-MS analyses showed that myricetin is the most abundant active ingredient in MPE. Myricetin is a hexahydroxyflavone and significant quantities of myricetin are absorbed in the gut of monogastric animals [35]. Ong and Khoo [36] investigated the effects of more than 30 bioflavonoids on lipogenesis and only myricetin had a stimulatory effect. They found that myricetin enhanced the glucose transport in rat adipocytes without any effect of insulin receptor function and Glucose Transporter Type 4 (GLUT-4) translocation. In a consecutive study, Ong and Khoo [37] concluded that treatment of diabetic rats with myricetin resulted in the lowering of hyperglycemia. In the present study, the significantly low serum glucose level in the treatment groups might be explained by the hypoglycemic effect of myricetin.

For immunity, it was observed that active components of onion juice had positive effects on lymphoid organs [38] and produced a large number of antibodies in chickens [39]. The effects of garlic and onion juice on immunoglobulin have been found to be similar to that of antibiotics [40]. In the present study, we observed that the serum IgG level increased in the group supplemented with 5% of MPE compared to the other treatment and control groups. Lee et al.[41] founded that myricetin inhibited both Cyclo-oxygenase-2 (COX-2) and nuclear factor kappa B (NF-kappaB) trans-activation in phorbol ester-treated JB6 P+ cells. The COX-2 is an inducible isoform member of cyclooxygenase enzyme family, which is regulated by growth factors and different cytokines such as IL1β, IL6, or TNFα, therefore overexpressed during inflammation [42]. The NF-kappaB is a family of inducible transcription factors that play a key role in the immune system. Transcription of genes which are responsibly regulating the inflammation, proliferation, and differentiation of immune cells are induced by primary activation of the NF-κB pathway [43]. Hence, myricetin could have anti-inflammatory as well as antioxidant effects through COX-2 inhibition pathway like nonsteroidal anti-inflammatory drugs. In the present study, the significant effect of myrtus on serum IgG levels might be explained by these complex mechanisms. Further studies are needed to evaluate both antioxidant and anti-inflammatory potential of MPE.

Unlike serum biochemical parameters, the research findings of the effects of MPE on hematology are limited in laying hens. Çetin et al.[44] reported no effect of propolis (bee glue), which is an antioxidant flavonoid, on most of the hematological parameters such as total leucocyte, hemoglobin and hematocrit although they observed improvement on serum IgG and IgM levels in laying hens. In the present study, NC increased quadratically however

other parameters such as TLC, LC, MC, RBC, He, MCV, MCH, MCHC, PLT, and MPV remained unaffected in agreement with Çetin et al.[44].

In conclusion, MPE could be used as a potential drinking water supplement and has shown positive effects on performance, egg production, egg quality and immunity without any adverse effects on the egg traits in laying hens. It is recommended to conduct more research during prolonged storage at higher temperatures to explore further effects of MPE on egg quality parameters.

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Conflict of Interest

This study was partially-funded by ArsArthro Biotechnologies Inc. (Ankara, Turkey). The funder provided financial support for expenses of serum biochemical analyses and hematology analyses, but did not have any additional role in the study design, data collection and analysis, perform the laboratory analysis, decision to publish, or preparation of the manuscript.

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Research Article

Evaluation on Shelter Medicine and Stray Animal Shelters in Turkey [1] [2]

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Abstract

In Turkey, the institutions established to serve the purpose of sheltering and rehabilitating abandoned, weakened and stray animals are known as an animal shelter, rehabilitation centre or a (temporary) animal sanctuary. In this study, we aim to explore the general structure of the care and treatment services provided in shelters in Turkey which host stray animals and to take the veterinarians' opinions on veterinary care, who work in these shelters. Twenty-eight shelters from 28 provinces, with four cities from seven regions of Turkey, were included in this study. Data was collected through the survey form (personnel status, physical condition, routine practices, duties and authorities of the personnel and functioning of the institution) prepared as a result of the legislation texts and literature reviews. In addition, 67 veterinarians working in these institutions were interviewed face to face to determine attitudes about shelter practice and their opinions and recommendations were obtained. It is identified that the number of shelter staff is generally insufficient. Routine care includes vaccination, spaying, marking with microchip/ear tag, registration and adopting etc. It is determined that there are differences between regions in terms of giving specialization training for medicine in shelters (P=0.031). In addition, almost all veterinarians reported that a veterinarian should be conscientious and patient (95.5%). There are many reasons behind the problem of stray animals in Turkey and veterinarians have critical roles to play on behalf of the solution to this problem. For solutions to the problems, proposals include providing cooperation between the institutions and sharing responsibilities; increasing the staff recruitment of veterinary and auxiliary staff; ensuring information and raising the awareness of veterinarians and assistants by training and courses; increasing the number and opportunities of shelters.

Keywords: *Animal shelter, Shelter medicine, Stray animal, Street animal*

Türkiye'de Sahipsiz Hayvan Barınakları ve Barınak Hekimliği Üzerine Bir Değerlendirme

Öz

Türkiye'de sahipsiz, güçten düşmüş, terk edilmiş hayvanların barındırılması ve rehabilitasyonu için kurulan ve bu amaçla hizmet veren kurumlar, hayvan barınağı, rehabilitasyon merkezi ya da (geçici) bakımevi olarak adlandırılmaktadır. Çalışmada, Türkiye'de sahipsiz hayvanların barındırıldığı, bakım ve tedavilerinin yapıldığı hayvan barınaklarında verilen hizmetlerin genel yapısı ve bu barınaklarda görev yapan veteriner hekimlerin barınak hekimliği konusundaki görüşlerinin araştırılması amaçlandı. Türkiye'nin yedi bölgesinde, her bölgeden dört il olmak üzere 28 ilde toplam 28 hayvan barınağı, çalışmanın kapsamına alındı. Konu ile ilgili mevzuat metinleri ve literatür taramaları sonucu hazırlanan veri formu (personel durumu, fiziki durum, rutin uygulamalar, personelin görev ve yetkileri ile kurum işleyişi) aracılığıyla veri toplandı. Ayrıca bu kurumlarda görev yapan 67 veteriner hekimle yüz yüze görüşme yöntemi ile barınak hekimliği konusundaki tutumları belirlenerek, görüş ve önerileri alındı. Barınaklarda personel sayısının genelde yetersiz olduğu; rutin yapılan uygulamaların aşılama, kısırlaştırma, mikroçip/kulak küpesi ile işaretleme, kayıt ve sahiplendirme vb şeklinde olduğu belirlendi. Barınak hekimliğine ilişkin uzmanlık eğitiminin verilmesi konusunda bölgelere göre farklılık olduğu (P=0.031) saptandı. Ayrıca barınak hekimlerinin tamamına yakını (%95.5), veteriner hekimin vicdanlı ve sabırlı olması gerektiğini bildirmişlerdir. Türkiye'de sahipsiz hayvan sorununun arkasında birçok neden yatmakta olup bu sorunun çözümü adına veteriner hekimler oldukça kritik bir role sahiptir. Sorunun çözümü için, kurumlar arasında işbirliğinin sağlanması ve sorumlulukların paylaşılması; veteriner hekim ve yardımcı personel istihdamının arttırılması; veteriner hekim ve yardımcı personelin eğitim ve kurslarla bilgilendirme ve bilinçlendirilmesinin sağlanmasının yanı sıra barınak sayısı ve imkânlarının arttırılması gerektiği söylenebilir.

Anahtar sözcükler: *Hayvan barınağı, Barınak hekimliği, Sahipsiz hayvan, Sokak hayvanları*

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INTRODUCTION

Nowadays, stray animals have become an important and a current issue among municipalities and the people in terms of modern urbanism [1,2].

Dodurga [3] defines the concept of street animal as stray animals, who live on the street and/or were born on the street or abandoned by their previous owners. The World Organisation for Animal Health (OIE) has a dog-centric definition for stray animals in three ways: 1) free-range animal that is not under control for a certain time; 2) free-range animal that does not have an owner; 3) the animal which is domestic and abandoned to the wild, returned to wild life and cannot be produced directly for humans [4].

Legal regulations regarding stray animals vary by country. It is reported that the European Union does not have a legal draft nor principles on the subject, and there is no exemplary state in the member countries, and each country takes appropriate measures according to its own conditions [4].

As a result of the efforts made for years in order to shelter and rehabilitate abandoned, weakened and stray animals in Turkey, Animal Protection Law No. 5199 entered into force in 2004[1]. The animal care facility/shelter, which refers to a facility where animals will be rehabilitated, which is also stated in the fourth article of the same law as "*It is essential that local governments establish shelter and hospitals in order to protect weakened and stray animals in cooperation with voluntary organizations to provide their care and treatment and to conduct training activities.*" has been assigned to the responsibility of municipalities in accordance with the provisions of the law [5].

In this study, our aim is to explore the general structure of care and treatment services provided in shelters in Turkey which host stray animals and to take the veterinarians' opinions on veterinary care, who work in these shelters.

[1] Animal Protection Law, No. 5199, Official Gazette no. 25509 of 01.07.2004.

MATERIAL and METHODS

In 2015, 200 municipal and private animal shelters available in Turkey according to data of the Ministry of Agriculture and Forestry (then known as the Ministry of Forestry and Water Affairs) have created the target population of the study. A total of 28 provinces (all of municipal animal shelters), each four taken from seven regions of Turkey, were included into this study *(Fig. 1)*. In determining the provinces, criteria such as the existence of a veterinary faculty and whether it is metropolitan or not are taken into consideration.

Data was collected through the survey form (personnel status, physical condition, routine practices, duties and authorities of the personnel and functioning of the institution) prepared as a result of the legislation texts and literature reviews. One on one interviews (face-to-face) with the shelter' management, which are determined within the study, were conducted. In addition, by face-to-face interview method [6] and conducting a poll with 67 veterinarians about shelter practice and medicine, their attitudes were determined and their opinions and recommendations were obtained.

The statistics packaged software SPSS 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) was used to evaluate the data of the poll. Median (IQR), percentage and frequency values of the variables were used. Categorical data was analysed by Fisher's Exact Test, Mann-whitney U and Chi-Square Test. In cases where the expected frequencies are less than 20%, *"Monte Carlo Simulation Method"* was used to include these frequencies into the analysis. For the significance level of the tests, $P<0.05$ and $P<0.01$ were accepted.

RESULTS

According to data received from the Ministry of Agriculture and Forestry during the study's data collection period (July 2015-June 2016), it was determined that there are 200 animal shelters in Turkey and the animal capacity in these

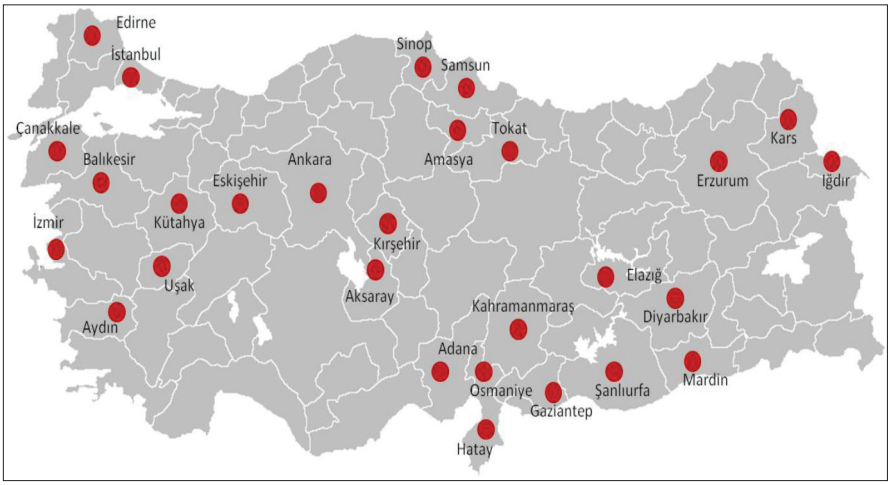

Fig 1. The distribution on the map of the visited animal shelters under the study

shelters are 65.000. As a result of the records made on the Wild Life Information System (YABİS) dated January 1, 2015, the total capacity of the shelters was determined to be 82.287. It was determined that the total capacity of the 28 animal shelters in the study was 15.760 and 9.056 stray animals (cats, dogs, poultry and wild animals) were taken care of in these shelters *(Table 1)*. A total of 80 veterinarians working under permanent, covenanted or by tender model (subcontracted) were confirmed in these 28 shelters.

The stalls are generally in the form of panel fencing, reinforced concrete and/or sheet metal in the shelters, and all of the shelters observed in the study were determined to have open spaces and huts in these areas. In the evaluation of the required areas in accordance with the legislation[2] which should be located in shelters, it is presented in *Table 2* whether they have an Administrative Section, Operation-Examination Room, Quarantine Room, Cemetery, Pension, Aquarium, Cage, Training Room or not.

[2] Regulation on The Protection of Animals. Official Gazette no. 26166 of 12.05.2006.

Routine practices in shelters were determined as follows.

1. Neutering/Spaying
2. Vaccination (rabies, mixed vaccine, *Bordetella, Echinococcus granulosus)*
3. Antiparasitic control (endo- and ecto-parasitic control)
4. Treatment
5. Adopton by contract
6. Disinfection
7. Identification (earing, microchip, collaring)
8. Procedures to the dead animals (proper embedding, calcined burial)

Some socio-demographic characteristics of the veterinarians participated into the study are presented in *Table 3*.

It was determined that the veterinarians interviewed in shelters, also provided services in the below stated work/duty area(s).

- Manager/Management
- Animal adaptation and control

Table 1. *Frequency values of animals in animal care centres*

Regions	Province	Animal Number/Species							
		Dogs		Cats		Poultry	Wild	Total	Capasity
		M	F	M	F				
Mediterranean	Adana	500	500	50	50	-	-	1100	2000
	Hatay - Antakya	120	170	7	8	4	4	403	500
	Kahramanmaraş	150	100	42	38	-	-	330	250
	Osmaniye	1	5	-	10	-	-	16	650
Eastern Anatolia	Elazığ*	-	-	-	-	-	-	-	-
	Erzurum	120	129	12	10	-	-	271	350
	Iğdır	-	-	-	-	-	-	-	-
	Kars	180	40		1	-	-	221	250
Aegean	Aydın - Söke	90	90					180	300
	İzmir - Karşıyaka	200	500	40	60	100		900	800
	Kütahya	131	93	4	11	-	-	243	350
	Uşak	50	100	20	20	-	-	190	200
Southeastern Anatolia	Diyarbakır	200	330	30	23	-	-	583	750
	Gaziantep	200	250	5	-	-	-	455	600
	Mardin	100	150	30	30	-	20	290	250
	Şanlıurfa	100	100	5	3	-	-	208	250
Central Anatolia	Aksaray	15	30	-	-	-	-	45	150
	Ankara - Merkez	510	518	3	3			1037	2500
	Eskişehir	40	60	-	-	-	-	100	200
	Kırşehir	30	40	-	-	-	-	70	150
Black Sea	Amasya	75	175	-	-	-	-	250	250
	Samsun	150	200	70	80-	-	-	500	3000
	Sinop	90	230	4	16	-	-	341	600
	Tokat	58	57	-	-	-	-	145	150
Marmara	Balıkesir	150	175	10	15	-	1	350	350
	Çanakkale	5	20	-	-	-	-	25	250
	Edirne	6	7	-	-	-	-	13	60
	İstanbul	350	200	55	40	-	-	645	600
Total								9.056	15.760

** It is stated that during the data collection period, the shelter belonging to the Elazig municipality was under construction and a couple of animals (dogs) that were rehabilitated in a shelter which is near the clinics of Veterinary Faculty, were observed. The shelter is currently active. M: Male, F: Female*

Table 2. *Some data on the physical properties of animal shelters*

Regions	Province	Sections of Shelter								
		Management	Operation Practice	Quarantine	Cemetery	Hostel	Aquarium	Cage	Training	Production
Mediterranean	Adana	+	+	+	+	-	-	-	-	-
	Hatay-Antakya	+	+	+	+	-	-	+	-	-
	Kahramanmaraş	+	+	+	-	+	-	-	-	-
	Osmaniye	+	+	+	+	-	-	-	-	-
Eastern Anatolia	Elazığ	-	-	-	-	-	-	-	-	-
	Erzurum	+	+	+	-	-	-	+	-	-
	Iğdır	-	+	-	+	-	-	-	-	-
	Kars	+	+	+	-	-	-	-	-	-
Aegean	Aydın - Söke	+	+	+	-	-	-	+	-	-
	İzmir – Karşıyaka	+	+	+	-	-	-	+	-	-
	Kütahya	+	+	+	-	-	-	+	-	-
	Uşak	+	+	+	+	-	-	-	-	-
Southeastern Anatolia	Diyarbakır	+	+	+	-	-	-	-	-	-
	Gaziantep	+	+	+	+	-	-	-	-	-
	Mardin	+	+	+	+	-	-	-	-	-
	Şanlıurfa	+	+	+	+	-	-	-	-	-
Central Anatolia	Aksaray	+	+	+	-	-	-	-	-	-
	Ankara	+	+	+	+	+	-	-	-	-
	Eskişehir	+	+	+	-	-	-	-	-	-
	Kırşehir	+	+	+	-	-	-	-	-	-
Black Sea	Amasya	-	+	-	-	-	-	-	-	-
	Samsun	+	+	+	+	-	-	-	-	-
	Sinop	+	+	+	+	-	-	-	-	-
	Tokat	+	+	+	+	-	-	-	-	-
Marmara	Balıkesir	+	+	+	+	-	-	+	-	-
	Çanakkale	+	+	+	+	-	-	-	-	-
	Edirne	+	+	+	-	-	-	-	-	-
	İstanbul	+	+	+	-	-	-	-	+	-

- Routine animal health services
- Registration system, follow-up
- Administrative procedures (paperwork, correspondence)

Participants stated that they were tasked with other works of the municipality such as food control, spraying, etc. in addition to above services.

The problems reported as a result of the interviews with the participants are given below under the headings of animal(s), shelter, volunteer-media and society, education-faculty.

Problem Related to Animals;

- Excessive number of animals (difficulty in population control)
- Insufficiency of prophylactic (preventive and protective) medicine
- Patients' coming as late cases
- Lack of information about incoming (stray) animals
- Insufficiency of treatment options in response to disease diversity
- Dropping of earrings in time

Problems Related to Shelters;

- Insufficiency of staff and lack of suitably qualified staff
- Excessive burden of work and responsibility (services such as administration, animal health and etc.)
- Political problems (There have been a number of staff members who have left)
- Difficulty in communicating with the staff
- Inadequate training of the staff on care conditions
- High risks such as infection and injury of personnel
- Lack of love for animals by the employees (unwillingly working, bad behaviour to the animals)
- Misuse by some employees (e.g. Sale of animals)
- Shelter' not being hygienic places
- Inadequate allowances
- Insufficiency of diagnostic methods (laboratory, x-ray, ultrasound, etc.)

Table 3. *Some socio-demographic characteristics of the veterinarians participated into the study*

Socio-demographic Data		Number	Percent
Gender	Female	16	23.9
	Male	51	76.1
Age	≤25	1	1.5
	26-35	30	44.8
	36-45	21	31.3
	≥46	15	22.4
Working time	1-5 years	38	56.7
	6-10 years	15	22.4
	11-15 years	5	7.5
	16-20 years	3	4.5
	≥21	6	22.4
Working before another practice	Clinic	37	55.2
	Food industry	7	10.4
	Pharmaceutical industry	4	6.0
	Poultry industry	1	1.5
	Others	5	7.5
	No	13	19.4
Membership in a non-governmental protection of animals organization for the	Yes	7	10.4
	No	60	89.6
Personal status	Municipality	46	68.7
	Contractual	10	14.9
	Other (Subcontractor)	11	16.4
Training/course on shelter medicine	No	49	73.1
	1-7 dates	14	20.9
	8-14 dates	1	1.5
	15-30 dates	2	3.0
	≥ 31 dates	1	1.5
Relations to municipality management for shelter medicine	Supported	47	70.1
	Not supported	11	16.4
	Not sure	9	13.4

- Necessity to work out of working hours
- Lack of systematically working shelters in every district
- Conflict in authority (e.g. in adoption-Having the authority of fining in different units)

Volunteer-Media - Problems Related to Society;

- Unfair pressure by media
- Difficulty/Challenge in public relations (having to deal with extra problems instead of the main job such as caring for animals and health care)
- Destructive rather than constructive criticism by animal lovers
- Involvement of volunteers in administrative processes and their attempts to participate in veterinary medicine
- Citizens' unawareness and lack of interest in stray animals
- Impulsive behaviour of animal lovers in general
- Lack of education on animal adoption and its responsibility

Problem Related to Education-Faculty;

- Inadequate training in shelter medicine, even if not in specialization level
- Not (being able to) giving training about the shelter services and its functioning
- Not having a better education in emergency medicine
- Inadequate training in zoonotic diseases that may be found in shelters
- Inadequate education in communication with the community and shelters

The opinions of the participants about shelter medicine and the qualifications required for the shelter physician are presented in *Table 4* and the categorical evaluation of these opinions is presented in *Table 5*.

In the study, the approaches of metropolitan municipalities on the organization of student visits and training-seminar studies with primary and secondary schools are presented in *Table 6*.

DISCUSSION

By the year 2012, there are, according to sources, 159 animal shelters with a capacity of approximately 38.970 animals

Table 4. *Frequency, percentage, median (IQR), min and max values of participants' views on shelter medicine*

Questions*	Number	Percent	Median	Minimum	Maximum
Special training on shelter medicine should be given	57	85.1	1 (1)	1	5
Shelter physician should have good clinical knowledge	51	76.1	2 (3)	1	5
Shelter physician should have good knowledge on management and organization as well as medical knowledge	66	98.5	1 (.00)	1	2
Shelter physician should be conscientious	64	95.5	1 (.00)	1	3
Shelter physician should be patient	66	98.5	1 (.00)	1	2
Shelter physician should be good in human relationships	65	97.0	1 (.00)	1	2

** In questions asked through five-point Likert shows as: 1: Strongly agree; 2: Agree; 3: Not sure; 4: Disagree; 5: Strongly disagree. IQR: Interquartile Range*

Table 5. *Evaluation of categorical data on shelter medicine*

Specialization Training is Required							X^2 =38.233 P=0.031*			
Region	Strongly Agree		Agree		Not Sure		Disagree		Strongly Disagree	
	n	%	n	%	n	%	n	%	n	%
Mediterranean	8_a	11.9	3_a	4.5	0_a	.0	$1_{a,b}$	1.5	2_b	3.0
Eastern Anatolia	3_a	4.5	0_a	.0	2_b	3.0	0_a	.0	$0_{a,b}$	.0
Aegean	6_a	9.0	8_b	11.9	$0_{a,b}$	.0	$2_{a,b}$	3.0	$0_{a,b}$	.0
Southeastern Anatolia	$5_{a,b}$	7.5	0_b	.0	1_a	1.5	1_a	1.5	$0_{a,b}$	.0
Central Anatolia	8_a	11.9	3_a	4.5	0_a	.0	0_a	.0	0_a	.0
Black Sea	3_a	4.5	4_a	6.0	0_a	1.5	1_a	3.0	0_a	.0
Marmara	4_a	6.0	2_a	3.0	0_a	.0	0_a	.0	0_a	.0
Total	37	55.2	20	29.9	3	4.5	5	7.5	2	3.0
Shelter Physician Should be Patient							**X^2 = 13.296 P=0.025***			
Age	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
≤25	1_a	1.5	0_a	.0	0	.0	0	.0	0_a	.0
26-35	28_a	41.8	2_b	3.0	0	.0	0	.0	$0_{a,b}$	.0
36-45	12_a	17.9	8_b	11.9	0	.0	0	.0	$1_{a,b}$	1.5
46-55	14_a	20.9	1_a	1.5	0	.0	0	.0	0_a	.0
≥56	0	.0	0	.0	0	.0	0	.0	0	.0
Total	55	82.1	11	16.4	0	.0	0	.0	1	1.5

** P<0.05*

in 81 provinces of Turkey [1]. In the first Congress of Stray Animals Welfare in 2016, it was reported that there should be a sufficient number of veterinarians and assistant personnel who would answer/face the capacity of shelters [7]. The increase in the number of shelters increases the need for veterinarians and their guidance in the fields of animal health and public health [8]. By the year 2014, 200 animal shelters were identified in the study. It can be said that from 2012 to 2014, the increase in the number of shelters was an important step for resolving the stray animal problem and the increasing of the number of shelters and veterinarians and assistants who will serve here should increase day by day. In addition, it is determined that the majority of the participants became permanent staff (68.7%; n=46) *(Table 3)*. Employment security is very important in working life; and parallel to the increase in the number of shelters, it can be said that municipalities should increase the number of veterinarians with permanent staff cadre and also, especially the current problems of veterinarians should be solved.

According to Steven and Gruen [9], shelters give lots of training opportunities for the veterinary faculty students. Because, these students have the practice chance on the control of species-typical zoonotic and contagious diseases; on behavioural assessment and its management; on primary care; on animal welfare, ethics and public policies.

It was uttered that the necessity of providing adequate training specific to the shelter medicine *(Table 4)*. When 25 veterinary faculties in Turkey which have begun to give education, at least in the shelters in their provinces, combine their facilities' potential with the facilities of shelter, it can be said that both veterinarian candidates will have the opportunity to practice on many subjects and the existing problems experienced in the shelters will be eliminated

In the veterinarian-oriented survey, that attained 37 of the 39 district municipalities in Istanbul, it found that only 37.2% of the participants were employed in temporary animal shelters, while others were employed in food control, administrative and spraying units [10]. In this study,

Table 6. *Status of organizing student visits and training-seminars in metropolitan municipal shelters*

Are student Visits Organized?					Z=297.000 P=0.004**	
Answers	Yes/Metropolitan Municipality		N/Not Metropolitan Municipality		Total	
	Number (n)	%	Number (n)	%	Number (n)	%
Yes	54	80.6	11	16.4	65	97.0
No	0	.0	2	3.0	2	3.0
Total	54	80.6	13	19.4	67	100
Is Information and/or Seminar Being Held?					**Z= 173.000 P<0.000**	
Answers	Yes/Metropolitan Municipality		No/Not Metropolitan Municipality		Total	
	n	%	n	%	n	%
Yes	44	65.7	4	6.0	48	71.6
No	10	14.9	9	13.4	19	28.4
Total	54	80.6	13	19.4	67	100

*** P<0.01*

we determined that the veterinarians were assigned in different units and in different fields. Providing veterinary medicine services in municipalities and when the conditions of nursing veterinary medicine are evaluated and can be asserted that the channelling of the people working in a single area is important for the quality and continuity of the service they will perform.

Yalçın [11] states that for the right shelter/temporary shelter design, firstly, the welfare of the animals that will live in has to be considered. In the shelter, suitable food and water for each species should be provided; an environment should be created in which animals can exhibit their typical behaviour patterns; they should be allowed to communicate with other animals and it should be organized in order to prevent unnecessary pain and suffering of animals. In addition, it has been reported that an accurate shelter design cannot be mentioned without these criteria being provided, and that structural standards, interior design, equipment and cage standards should be applied for a proper temporary shelter [11]. In the study, it was determined that shelters differed from region to region, from city to city, such as some had some units and some didn't have some parts at all (aquarium and training place) *(Table 2)*. When considered from this point of view, there is no standardization on this issue. It can be said that in current and future of shelters, the missing parts should be completed by taking animal welfare criteria into consideration and should be built standardized shelters to provide better quality services.

In shelter medicine, besides surgical practices, a comprehensive understanding, knowledge and skills such as preventive medicine, control of contagious disease, public health, animal behaviour, veterinary forensic medicine, epidemiology, developing policies relating to the solution of the problems and the design of facilities should play an important role [8]. Along with the difficulties experienced in the rehabilitation process of street animals, it is stated that various ethical problems are encountered due to the inadequate education and information studies on the subject [1]. In 1st Congress of Stray Animals Welfare held in Istanbul in 2016, it is indicated that the assistant staff working in the animal shelters should be employed after a minimum of 200 hours of training on animal care, animal behaviour, animal welfare, hygiene, catching and keeping the animals [7]. In the study, it was found that most of the veterinarians were not subject to any training (73.1%; n=49) before starting to work in shelters *(Table 3)* and they required specialization training (85.1%; n=57) *(Table 5)*. It can be said that nowadays, when information about veterinary medicine changes and updates quickly, it is quite necessary to educate veterinarians and the staff that work or will work there in certain periods.

It is an important issue whether municipalities have enough budget for treatment and care of animals due to excessive number of stray animals [2]. 70.1% of the veterinarians interviewed in the study stated that enough contribution was provided in municipal services *(Table 3)*. However, stray animal problems such as the number of animals, deficiency in staff education, seeing shelters as a place of exile, shelter management, inadequacy of community communication, the existence of chaos in authority become difficult to be solved because of the cultural and socio-economic reasons, lack of information and education, lack of communication and coordination between stakeholders and decision-makers which are the basis of the stray animal problem and also which are mentioned by FECAVA [4]. For this reason, it can be asserted that the communication and coordination of the municipalities and the relevant ministry (Ministry of Agriculture and Forestry), which is the authorized unit for the practice of animal protection legislation, is very important for the solution of the issue.

Signal and Taylor [12] stated that those who are members of a non-governmental organization related to the protection of animals have a more positive attitude towards the protection of animals than those who are not. Likewise, in Turkey, Özkul et al.[13] found that the members have

more positive attitude on the same subject. In Turkey, non-governmental organizations (NGO) for the protection of animals are perceived by society as the place that you can call only when you witness an animal abuse. Today, most of these organizations have difficulties in terms of both financial and to have qualified staff [14]. In the study, it was determined that only seven of 67 veterinarians (10.4%) were found members of NGOs` working on animal protection *(Table 3)*. It can be said that this situation may occur especially in order to avoid the increasing pressures of animal lovers/volunteers. However, it can be asserted that veterinarians should be more involved in these NGOs in order to change the perception of the society towards NGOs, to eliminate the deficiency in qualified people, in particular to create a healthy communication with the society by explaining veterinary practices appropriately.

Veterinary medicine services provided in shelters, because of their containing many features, may be said to require certain characteristics from veterinarians, who are employed and/or will be employed in these shelters. In the study, it was determined that a significant part of the participants participated in the questions about education, knowledge-skills and communication related to veterinarians working in shelters. Shelter veterinarians' being agreed on the characteristics such as education, knowledge-skills, empathy and communication can be accepted as an indicator of knowing which skills are required from themselves for the work. In addition, it can be said that by developing themselves in this direction, they can be even more beneficial to stray animals; they are generally aware of the problems which were expressed in different categories in face-to-face interviews and they are disposed to solve them.

Özkul et al.[13] indicates that discussing animal rights deliberatively in schools by teachers will built strong basis in the perspective of animal rights. Some authors have reported that there is a positive relationship animal ownership in childhood and positive behaviour towards animals in older ages [15,16]. In the study, it was determined that metropolitan municipal shelters give more importance to the activities of organizing trainings and seminars in primary and secondary schools compared with non-metropolitan cities *(Table 6)*. It can be asserted that animal love given at early ages can have a positive effect on the perspective of stray animals and also can make a contribution by raising awareness to solve stray animal problem.

In conclusion, there are many reasons behind the problem of stray animals in Turkey and veterinarians have a critical role to play on behalf of the solution of stray animal problem. In order to solve the problem, it can be said that providing cooperation between the institutions and sharing the responsibility; increasing the employment of veterinarians and auxiliary personnel, informing the veterinarians and auxiliary staff and raising awareness of them by training and courses, and increasing the number of shelters and their facilities.

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Research Article

Mitochondrial *Cytochrome-b, Cytochrome-c* and *d-loop* Region Based Phylogenetic and Diversity Analysis in Blackbuck *(Antilope cervicapra)*

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Abstract

The present study was designed to find diversity analysis of *Antilope cervicapra* family in Pakistan. Fecal samples of *Antilope cervicapra* were collected from their different habitats of Pakistan. Fecal DNA was extracted and Polymerase Chain Reaction (PCR) was performed. Sequencing was performed by Big DyeTM Terminator method. Diversity and phylogenetic analysis was performed by different Bioinformatics tools. Less genetic variability was observed within *Antilope cervicapra* population through Multi-Dimensional Scaling (MDS). However, significant genetic variation was observed among other species and *Antilope cervicapra*. Phylogenetic analysis revealed distinct clade of this specie with respect to other species of deer. This is the first report from Pakistan that could help for designing effective strategy in future conservation practices of deer species.

Keywords: *Blackbuck, Phylogeny, Mitchondrial, Cytochrome, Phylogenetic diversity*

Kara Antilop'ta *(Antilope cervicapra)* Mitokondriyal *Sitokrom-b, Sitokrom-c* ve *d-loop* Bölgelerine Dayalı Filogenetik ve Çeşitlilik Analizi

Öz

Bu çalışma Pakistan'daki *Antilope cervicapra* ailesinin çeşitlilik analizini belirlemek için tasarlandı. *Antilope cervicapra* dışkı örnekleri Pakistan'ın farklı habitatlarından toplandı. Fekal DNA ekstrakte edildi ve Polimeraz Zincir Reaksiyonu (PCR) yapıldı. Dizileme işlemi Big DyeTM Terminator metodu ile yapıldı. Çeşitlilik ve filogenetik analizlerde farklı biyoenformatik araçlar kullanıldı. *Antilope cervicapra* popülasyonunda Çok Boyutlu Ölçeklendirme (MDS) değerlendirmesinde düşük düzeyde genetik değişkenlik gözlendi. Bununla birlikte, diğer türler ve *Antilope cervicapra* arasında önemli genetik varyasyon belirlendi. Filogenetik analiz, bu türün diğer geyik türlerine göre belirgin bir tür olduğunu ortaya koydu. Bu araştırma, Pakistan'dan geyik türlerinin gelecekteki koruma uygulamalarında etkili bir strateji tasarlanmasına yardımcı olabilecek ilk rapordur.

Anahtar sözcükler: *Kara antilop, Filogenetik, Mitkondrial, Sitokrom, Filogenetik çeşitlilik*

INTRODUCTION

It is necessary to keep the information of wild animal genetic diversity for the better management and conservation of these wild species [1]. So, present day biological studies are needed to be focused on conservation of genetic diversity. The assessment of the genetic diversity is needed for maintenance of sustainable hunting, conservation and improvement of genetic resources of animals of a specific population [2].

Male and female Blackbucks *(Antilope cervicapra)* are somewhat morphologically different from each other. Females are yellow in color and most of them do not have horns. Female blackbuck has thin and slim body. Whereas, males have horns and horns grow before the change of their body color. Blackbucks can breed throughout the year, but March to May and August to October are top breeding periods.

Blackbucks originally belong to Indian subcontinent. They were found everywhere in this area from plains up to

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mountains. Nowadays, human activities have destructed their habitat so they are found only in isolated places. In our country of Pakistan, blackbucks are found in Cholistan and Thar area of Punjab and Sindh along eastern border areas. At present, there is no specific area for their living in Pakistan. Now they are found as captive in different places like Kirthar and Lal Suhanra National Parks. The antilope specie is now extinct in Sindh. However, a large population of the species exits in Khairpur's Mehrano reserve. Some other places, where blackbuck are found in Pakistan are zoos and different wildlife centers. In 2008; more than 1500 Blackbucks were found in different breeding centers of Punjab and Sindh in Pakistan. DNA barcoding has been used to find genetic diversity at species level. It is considered as standardized approach to show interspecific variations from the mitochondrial region [3].

For phylogenetic studies and species identification of different animals, *Cytochrome-b* along with other mitochondrial DNA markers has been used frequently in the recent times [4]. So, the current study was designed to identify the polymorphisms in *Antilope cervicapra* phylogenetic relationships within and among other animals.

MATERIALS and METHODS

Taxonomic Species and Sampling Strategy

Blackbucks *(Antilope cervicapra)* species were selected from their natural habitats, parks, zoos, and captive breeding centers at various places of Pakistan. Sampling areas are shown in *Table 1*. Selection was carried out on the basis of phenotypic characteristics. Fecal samples (n=25) were collected from species. Permission was taken from the competent authorities of sample collection areas.

Genome Extraction and Purification

Fecal samples were taken and preserved in 95% ethanol at room temperature. DNA extraction from fecal mass was extracted through inorganic method by the protocol of Zhang et al.[5] and with minor modifications as by Maryam et al.[6]. Briefly, 1.0-1.5 g of feces material was taken and centrifuged with 5 mL ethanol (4000 × *g*, 2 min). The washing step was repeated once using 5 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Three mL TNE buffer (10 m mol/L Tris-Cl, 0.5% SDS, 1 m mol/L $CaCl_2$) and 50 µL Proteinase K (20 mg/mL) were added to the centrifuge tube, and the whole was incubated at 55°C for 1-2 h. The lysate was centrifuged (4000 × *g*, 1 min) to pellet the fecal particle. The supernatant was centrifuged again with potato starch and pipetted into a new 2 mL centrifuge tube, to which 150 µL NaCl solution (3.5 mol/L) and 250 µL CTAB solution (0.7 M NaCl, 10% cetyl trimethyl-ammonium bromide, CTAB) were added, followed by incubation at 70°C for 10 min. The mixture was extracted twice using an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Elute was filtered and preserved with 200 µL TE, and 50 µg/mL RNase was added at 4°C. DNA quantity was measured by Nanodrop (Thermoscientific, Wilmington USA).

Table 1. *Sampling details of Antilope cervicapra samples*

Sr. #	Samples ID	Source	Google Coordinates	Number of Samples
1	AC18, AC19	Bahawalpur Zoo, Bahawalpur	29°24'8.7"N 71°40'54.5"E	2
2	AC13, AC21	Bahria Town Lahore	31°18'51.5"N 74°12'11.7"E	2
3	AC20, AC12	Bahria Town Rawalpindi	33°29'45.2"N 73°6'20.3"E	2
4	AC16	Basti Bahadurpur Multan	30°15'27.9"N 71°29'48.2"E	1
5	AC9	Changa Manga, Kasur	31°5'19.3"N 73°57'44.7"E	1
6	AC24	Charagh Abad, T T Sing	31°20'6.3"N 72°46'2.4"E	1
7	AC2	Peerowal Khanewal	30°20'22.7"N 72°2'2.4"E	1
8	AC25	Khangur Ghotki, Sindh	27°52'10.8"N 69°25'2.5"E	1
9	AC6, AC10	Kirthar National Park, Sindh	25°41'27.8"N 67°31'23.4"E	2
10	AC3	Lahore Safari Park	31°22'53.9"N 74°12'41.6"E	1
11	AC5	Lahore Zoo, Lahore	31°33'22.7"N 74°19'34.0"E	1
12	AC15, AC11	Lal Suhanra National Park	29°19'1.4"N 71°54'16.4"E	2
13	AC4	Lohi Bher Wildlife Park Rawalpindi	33°57'49.5"N 73°11'93.1"E	1
14	AC17	Mir of Khairpurs Mehrano Reserve, Sindh	27°16′47.1″N 68°40′32.6″E	1
15	AC7	New Jatoi, Nawab Shah, Sindh	26°47′37.6″N 67° 59′28.0″E	1
16	AC1, AC23	Rajoa Saadat, Chiniot	31°38′35.5″N 72°58′26.7″E	2
17	AC22, AC14	Head Balloke Raavi River	31°11'25.9"N 73°52'32.6"E	2
18	AC8	Wildlife Farms Raiwind Road Lahore	31°23'5.5"N 74°14'8.5"E	1
TOTAL				25

Primer Designing

Amplification of complete mitochondrial genome including three loci, *cytochrome-b, Cytochrome-c* and *d loop* regions of *Antilope cervicapra* was carried out. Reference sequences (Accession No.NC_020614) were taken from NCBI for primer designing and primers were designed by the primer blast of NCBI.

PCR and DNA Sequencing

PCR amplification and DNA sequencing was performed as described by the Abbas et al.[7] and Nadeem et al.[8]. PCR was carried out in four steps. Initial denaturing was performed at 95°C for 2 min in first step. Second step comprised of 30 cycles each for 30 s at 95°C for denaturation, 30 s at 51°C-54°C (as optimized per locus) for primer annealing and 2 min at 72°C for extension. Third step was final extension for 10 min at 72°C and Fourth step was maintaining the final temperature at 4°C until completion of PCR. PCR products along with 1 kb ladder were run on 1.2% agarose gel at 100 volts for 35 min. to visualize the bands of amplified products.

DNA Sequencing and Sequence Submission to NCBI

Ethanol was used for precipitation of PCR products. 40 mL of 75% ethanol was added to each 10 mL reaction to final concentration of 60%. The reaction mixtures were mixed left at room temperature for 20 min. Then, these were centrifuged at 16000 x g (14000 rpm for 20 min) at 4°C. The supernatant was discarded and pellets were washed with 100 mL of 70% ethanol. Then pellets were dissolved in 15 mL of deionized and were sequenced by dideoxy terminator sequencing by using ABI Genetic Analyzer 3130 XL (Applied Biosystem Inc., Foster city, CA, USA). The sequences (41) were submitted to GeneBank (NCBI) with accession numbers MH155269, MH181808-181822; MK051009-MK051020; MH920321- MH920333.

Bioinformatics and Statistical Analysis

Sequenced were aligned through Alignments Blast 2 and Clustal W tools [9]. Phylogenetic analysis was carried out using the MEGA2 [10]. Bayesian Phylogenetic tree and Maximum Parsimony Tree were used. Diversity score was evaluated by the R statistical package [11]. Multidimensional scaling and genetic variation plots were drawn for all three genes.

RESULTS

In *Antilope cervicapra*, 1139 bp fragment sequence of mitochondrial *Cytochrome-b* gene was analysed. A total of thirteen variable sites were observed *(Table 2)*. Out of these, six variations were found monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. The variable sites were comprised of 11 transitions and two transversions as shown in *Table 2*. Low frequency of mutant allele and no heterozygous individuals was observed.

Table 2. *Polymorphisms identified in cytochrome-b gene of Antilope cervicapra*

No.	Base Position[a]	Change in Nucleotide (Wild to Mutant)	Allele Frequencies	
			A	B
1	14174	C→A	0	1
2	14177	A→C	0	1
3	14256	G→A	0	1
4	14544	A→G	0	1
5	15220	G→A	0	1
6	15293	G→A	0	1
7	13650	G→A	0.56	0.44
8	13657	C→T	0.52	0.48
9	13660	A→G	0.80	0.20
10	13664	A→G	0.56	0.44
11	14532	T→C	0.76	0.24
12	14710	A→G	0.80	0.20
13	14735	T→C	0.80	0.20

[a] *with reference to NC_012098*

Table 3. *Polymorphisms identified in cytochrome-c gene of Antilope cervicapra*

No.	Base Position[a]	Change in Nucleotide (Wild to Mutant)	Allele Frequencies	
			A	B
1	5890	C→T	0	1
2	6577	A→G	0	1
3	5420	A→G	0.96	0.04
4	5459	G→A	0.92	0.08
5	5510	G→A	0.96	0.04
6	5536	C→T	0.88	0.12
7	5579	C→T	0.96	0.04
8	5616	T→C	0.92	0.08
9	5665	C→T	0.84	0.16
10	5811	T→C	0.96	0.04
11	5990	A→G	0.96	0.04
12	6032	G→A	0.80	0.20

[a] *with reference to NC_012098*

In the *Cytochrome-c* gene of the mitochondrial DNA, 1544 bp fragment from *Antilope cervicapra* individuals was analysed. A total of twelve variable sites were observed *(Table 3)*. Out of these, three variations were found monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. There were 12 transitions but no transversion was observed. Allele frequency of all variations was calculated, and very low frequency of mutant allele was observed. As no heterozygous individuals were found, so allelic frequency and genotypic frequency was same.

Mitochondrial *d-loop* region (997 bp fragment) of *Antilope cervicapra* individuals was sequenced and analysed. A total of sixteen variable sites were observed as shown in *Table 4*. Out of these, ten variations were monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. The variable sites were comprised of 16 transitions and no transversion. No heterozygous individuals were found.

Table 4. *Polymorphisms identified in mitochondrial d-loop region of Antilope cervicapra*

No.	Base Position[a]	Change in Nucleotide (Wild to Mutant)	Allele Frequencies	
			A	B
1	15578	A→G	0	1
2	15591	G→A	0	1
3	15644	A→G	0	1
4	15676	A→G	0	1
5	15681	G→A	0	1
6	15699	G→A	0	1
7	15725	A→G	0	1
8	15762	A→G	0	1
9	15781	T→C	0	1
10	15876	A→G	0	1
11	15446	T→C	0.84	0.16
12	15455	A→G	0.84	0.16
13	15473	A→G	0.72	0.28
14	16386	C→T	0.88	0.12
15	16387	A→G	0.92	0.08
16	16397	T→C	0.88	0.12

[a] *with reference to NC_012098*

Multidimensional scaling (MDS) plot of mitochondrial cytochrome-b, cytochrome-c and d-loop regions for *Antilope Cervicapra* was generated individually as shown in *Fig. 1*, *Fig. 2*, *Fig. 3* and collectively *(Fig. 4)*. The greater clustering of the *Antilope Cervicapra* samples indicated lower genetic variability. Overall significant genetic differences among species of deer were observed.

Pairwise evolutionary distance also showed that populations were significantly different from each other. Genetic variability and phylogenetic relationship within and between analyzed groups was evaluated, which was based on polymorphic loci from *Bovidae* and *cervidae*. The level of heterozygosity was relatively comparable in all evaluated groups of animals. The highest homozygosity was observed in all selected species of *Bovidae* and *cervidae* and differences were very low.

Phylogenetic based analysis of *Cytochrome-b, cytochrome-c* and *d-loop* of the gene sequences revealed that each species individuals comprise a distinct clade *(Fig. 5)*. This clade was evidently distinct from other species of deer. The neighbour-joining tree created from mitochondrial genes based data set from *cervidae* and *bovidae* showed clear differentiation.

DISCUSSION

In this study, significant reduction in genetic diversity of the *bovidae* and *cervidae* members were found from different regions of Pakistan. Both allelic richness and heterozygosity were lower in these populations compared to the other populations of this region, as previously reported [12-17]. This reduction in genetic diversity could be due to direct result of a combination of geographic isolation or due to small

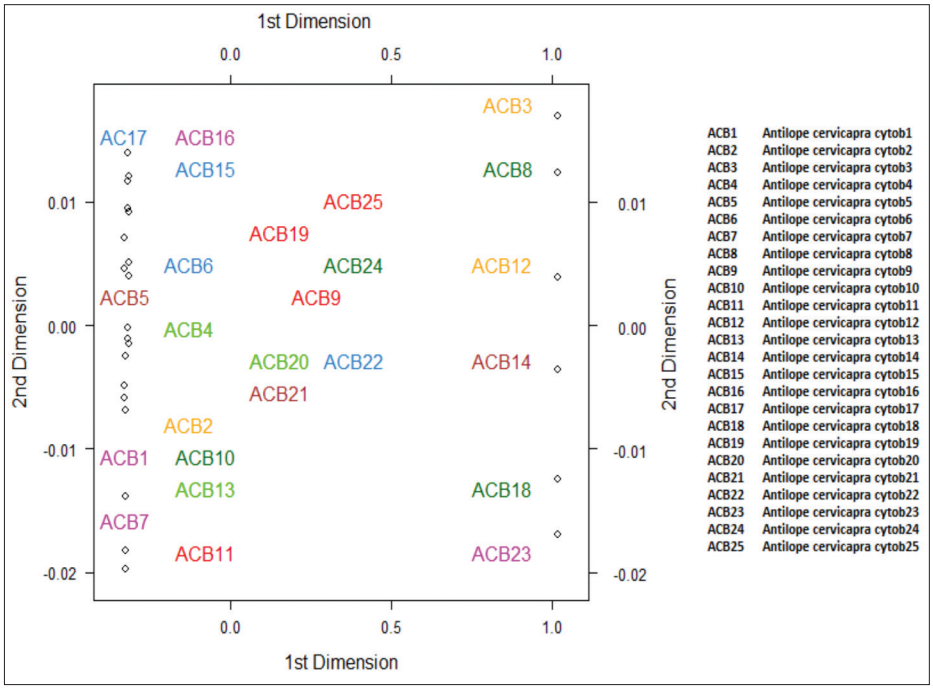


Fig 1. *cytochrome-b* based multidimensional scaling plot of *Antilope cervicapra*

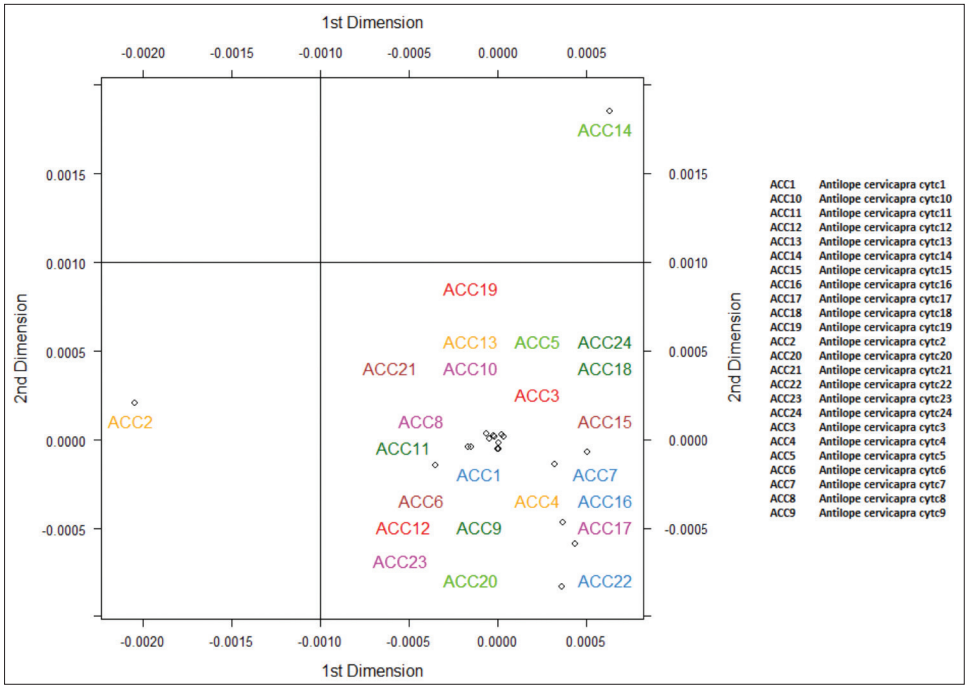
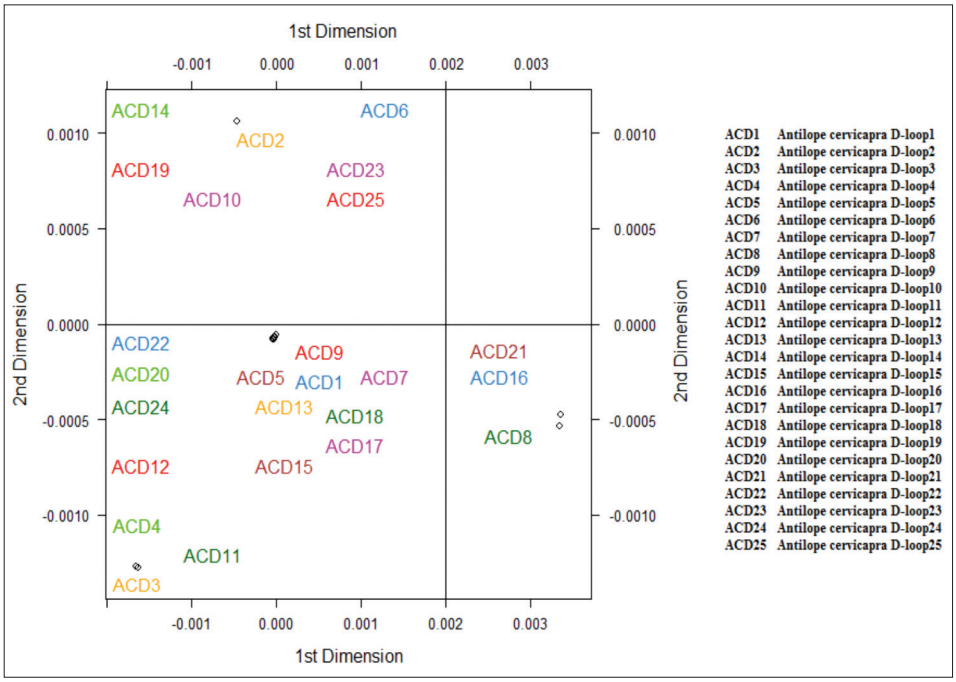


Fig 2. Multidimensional scaling plot of *cytochrome-c* gene for *Antilope cervicapra*

Fig 3. Multidimensional scaling plot of *d-loop region* for *Antilope cervicapra*



population sizes in the last decade. Similar observations have also been reported by Dellicour et al.[18] and Irwin et al.[19]. Phylogenetic analysis of Pakistani livestock breeds such as buffalo, goat, sheep, camel [4,20-24] have previously been reported but wildlife species data is scarce.

High mitochondrial DNA based genetic diversity was observed in Pakistani domestic goat's Indian blackbuck was successfully identified from other wildlife species of that region through DNA barcoding, which supports the findings of the current study. In another study on Japanese Sika deer *(Cervusnippon)*, demonstrated significant genetic diversity among other deer species through tandem repeats in D-loop of mitochondrial genome [23]. Phylogenetic analysis based upon a single gene is considered untrustworthy as far as topology is concerned, particularly in situations when larger classifications between species, such as interorder or interfamily, was under consideration. The reason was different evolutionary rates between different mitochondrial genes. So, phylogenetic analysis of *Cervidae* and *Bovidae* using more mitochondrial genes is needed. The phylogenetic analysis of this study supports other cladistics studies conducted on *antilopes* and *Bovidae* using DNA barcoding genes [24]. So, phylogenetic analysis

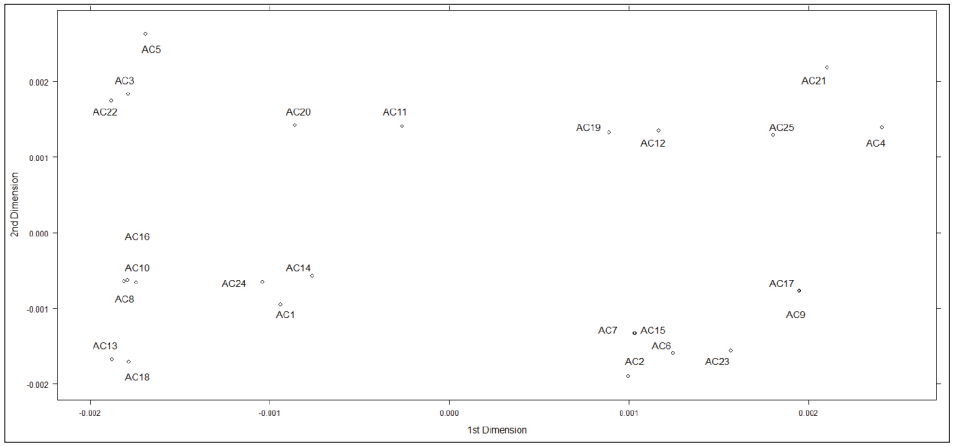


Fig 4. Multidimensional scaling plot of mitochondrial genomic *cytochrome-b, cytochrome-c* and *d-loop region (collectively)* for *Antilope cervicapra*

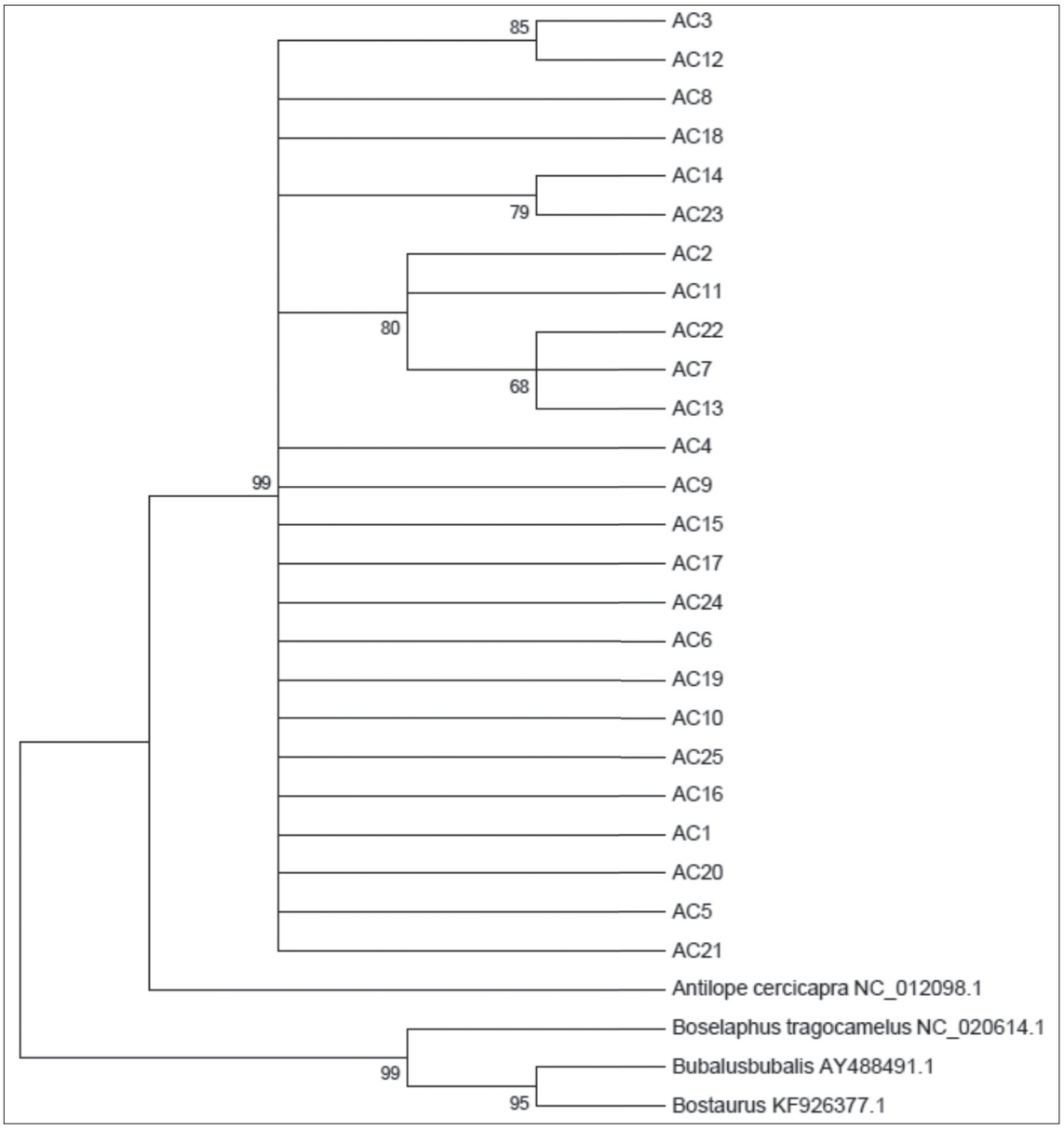


Fig. 5. Phylogenetic tree (rectangular) of *cytochrome-b, cytochrome-c and d-loop* region (collectively) of *Antilope cervicapra*

of *Cervidae* and *Bovidae* using more mitochondrial genes is needed. Another study regarding phylogeny analysis was conducted using *cytochrome b* gene, in different orders of eutherian animals including *Cervidae* and *Bovidae*, but they were not able to differentiate between *Cervidae* and *Bovidae* families. A phylogeny analysis was performed by Irwin et al.[19] and Honeycutt et al.[25] by using *cytochrome b* and *cytochrome c* oxidase subunit II (*COII*) genes in different species of mammals, including *Cervidae* and *Bovidae,* and different topologies were observed between *cytochrome b* and *COII* genes. According to Irwin et al.[19], on the basis of study of *cytochrome b* gene, *Cervidae* and *Bovidae* were not differentiated. The observations based upon the modern molecular genetics studies, recommend close relation of *Cervidae* with *Bovidae* then with *Moschidae*. But *Giraffidae* family appears more distant from *Cervidae* and closer to *Antilocapridae*.

Variations at genetic level is a bottom material for survival of animals. It act as the genetic source for prediction of future conservation of animals. Molecular markers plays initial guide for evaluation of the genetic variation. Therefore, the information on this level of genetic variation is prerequisite for designing effective strategy for wildlife conservation.

Overall, low level of generic diversity revealed with higher level of genetic differentiation among different populations. The genetic analysis showed the generic variation at species level. Combined effects of field and molecular techniques standardize the conditions for genomic amplification and analysis for status of population structure in *cervidae* species.

The mitochondrial genome sequence will directly facilitate conservation and genetic epidemiology research in *cervidae* family. Moreover, it will also assist in genome conservation of the endangered species of deer. The published mitochondrial genome sequenced data in NCBI database will serve as a source of valuable information to solve problem of specie identification and Blackbuck-related crimes in wildlife forensics.

Conflict of Interest

No conflict of interest has been found.

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Research Article

Effects of Light Color on Growth Performance, Histomorphometric Features of Small Intestine and Some Blood Parameters in Chukar Partridges *(Alectoris chukar)* [1]

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Abstract

The study was conducted in order to compare the effects of light-emitting diode (LED) light with a different color on performance parameters, histomorphometric features of the small intestine and some blood parameters in growing partridges. For this purpose, a total of 450 Chukar partridges *(Alectoris chukar)* were perdivided into three experimental groups including green light (GL), daylight (DL) and blue light (BL), and 250-lumen lighting. Each group contained 150 partridges with 5 repetitions per group. Experimental groups balanced according to initial live weight and raised under the same feeding and environmental conditions within the scope of the needs of partridges during 35 days. GL group showed higher live weight (P<0.05), live weight gain (P<0.01) and feed intake (P<0.05). Feed conversion and viability rates were similar among the groups (P>0.05). Higher villus height (VH), villus width, villus surface area were obtained in GL group, followed by DL and BL (P<0.001). The differences in crypt depth (CD) and VH/CD were not significant (P>0.05). Serum glucose (P<0.01) and lactate dehydrogenase levels (LDH) (P<0.05) were increased in GL groups. Serum high-density lipoprotein level (HDL) was found to be higher in DL group (P<0.01). Consequently, GL group had better results in growing partridges. GL could be utilized under intensive farming conditions for partridges.

Keywords: *Fowl, Illumination color, Biochemical parameters, , Growth performance, Histological examination*

Kınalı Kekliklerde *(Alectoris chukar)* Işık Renginin Büyüme Performansı, İnce Bağırsak Histomorfometrik Özellikleri ve Bazı Kan Parametreleri Üzerine Etkileri

Öz

Bu çalışma, farklı renkte ışık yayan diyot lambaların (LED) kekliklerde büyüme performansı, ince bağırsağın histomorfometrik özellikleri ve bazı kan parametreleri üzerine etkilerini karşılaştırmak amacıyla yapılmıştır. Bu amaç için, toplam 450 Kınalı keklik *(Alectoris chukar)* yeşil ışık (YI), gün ışığı (GI) ve mavi ışık (MI) grupları olmak üzere 3 gruba ayrılmış ve 250 lümen aydınlatma uygulanmıştır. Her grup, grup başına 5 tekrar içeren 150 keklikten oluşmuştur. Deney grupları, kekliklerin başlangıç canlı ağırlıklarına göre belirlenmiş olup, büyüme dönemleri göz önünde tutularak, 35 günlük deneme süresince her gruba eşit beslenme ve çevresel koşullar sağlanmıştır. YI grubu canlı ağırlık (P<0.05), canlı ağırlık artışı (P<0.01) ve yem tüketimi (P<0.05) açısından yüksek değerler göstermiştir. Yem dönüşümü ve yaşama gücü oranları gruplar arasında benzer tespit edilmiştir (P>0.05). YI grubunda villus yüksekliği (VY), villus genişliği, villus yüzey alanı daha yüksek olup, bu grubu sırasıyla GI ve MI (P<0.001) grupları takip etmiştir. Kript derinliği (KD) ve VY/KD'deki farklılıklar önemli bulunmamıştır (P>0.05).YI gruplarında serum glukoz (P<0.01) ve laktat dehidrojenaz seviyeleri (LDH) (P<0.05) artmıştır. Serum yüksek yoğunluklu lipoprotein düzeyi (HDL) GI grubunda yüksek tespitedilmiştir (P<0.01). Sonuç olarak, YI grubundan kekliklerin büyüme döneminde daha iyi sonuçlar elde edilmiştir. Entansif koşullarda keklik yetiştiriciliği yaparken YI'dan faydalanılabilir.

Anahtar sözcükler: *Av kuşu, Aydınlatma rengi, Biyokimyasal parametreler, Büyüme performansı, Histolojik inceleme*

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INTRODUCTION

In poultry farming, the light is determined to have crucial functions to carry out rhythmic and synchronized functions including important metabolic events of the body, to control body temperature, to control activities such as feed consumption, growth, maturity, and reproduction, as well as releasing, stimulating, and controlling hormones [1,2]. While conventional incandescent and fluorescent lamps have been used as the source of light until the recent past, there have been technological advancements in illumination in the last few years and these lamps have started to be substituted with light emitting diodes (LED). The most important advantages of LEDs are that they have high energy saving (consumption of energy of 80% lesser compared to incandescent lamp, 50% lesser compared to fluorescent lamp), are long-lasting, have high reliability, low costs of maintenance and the wavelength to ensure sufficient light stimulation for poultry species [3-5].

Color of light is the associations caused by beams at different wavelengths in the brain. Like mammals, poultry can see the part of light whose the wavelength varies between 380 nm and 780 nm and which is described as color [6]. The eye has different perception sensitiveness for lights with diverse wavelengths. Eye of poultry has a structure different from mammals. The light is detected more densely by retinal and extra-retinal photoreceptors [7]. Extra-retinal pineal gland and hypothalamus play a role in certain homeostatic, physiological, and reproductive events; whereas, retina directs growth and behaviors [1].

Perception levels of the light with different wavelengths vary across the poultry species [8]. In this sense, it is important to identify the wavelength that is appropriate to the goal of farming for species. Previous studies revealed that generally light with long wavelength or higher density contains higher energy and can reach to hypothalamus because of higher capability to penetrate in skull, thereby better results have been obtained in development of sexual organs, reaching to sexual maturity, activation of sexual hormones, egg production, and metabolic activities such as longer laying periods [1,7,9,10]. It was determined that the light with shorter wavelength or lower density could be easily detected by retinal receptor and retinal stimulation was sufficient in terms of growth and fattening performance [6,11,12]. On the other hand, poultry performance is also significantly affected by behaviors of poultry. Density, wavelength, and source of the light are known to be the important environmental factors that have an effect on behaviors and physiology of poultry animals [13,14]. It was found for laying hens that red light decreased aggressiveness [4], green light relieved and rested broilers [15], white light elevated plasma corticosterone levels in songbirds [16], also color of the light are effective on blood biochemistry such as glucose, triglyceride, cholesterol, uric acid [12,17] and immunological parameters [11,18].

Because it is thought that a different species such as partridge may react differently to the light spectrum. The study was aimed to determine the effect of green light, daylight and blue light on the growth performance, some blood parameters, and the intensity of histomorphometric characteristics of the small intestine during the growth period (1-35 days) of the Chukar partridges *(Alectoris chukar)*.

MATERIAL and METHODS

Ethical Approval

The study was conducted in Chukar partridge production station affiliated with Turkish Republic Ministry of Forestry and Water Affairs. Approval of the Ministry and Fırat University Animal Experimentation Local Ethics Committee was obtained for the study (FUHADYEK, Approval number: 2016/127).

Experimental Design

Daily chicks from incubator which was available in the station were weighed randomly and distributed into experimental groups to equalize initial live weights. Experimental groups were formed as green light (GL), daylight (DL), and blue light (BL) led groups (Single-chipped, outdoor, 60 leds/M, DC 12 V, 4.4 W/M). Every experimental group was planned with 5 repetitions. The study was conducted by using 30 partridges in each repetition, 150 partridge in each group, and 450 Chukar partridges *(Alectoris chukar)* in total. The ingredients and composition of the diet are shown in *Table 1*. Feed and water were supplied *ad libitum* during the study (1-35 days). Conditions of poultry house were regulated without causing the differences between experimental groups within the scope of the needs of partridges. Partridge chicks were raised in the special wire cages with 5 tiers in the size of 95 x 50 x 22.5 cm (length x width x height). Each section of the cage was illuminated using 250-lumen lighting. The 24 h light was applied during first three days, later it was gradually lowered to 16 h light/8 h dark program. During the trial, live weights were measured weekly. On the day of weighing, the feed was removed from in front of partridges at 12 a.m. and all partridges in the study were recorded weighing in groups with 5 birds at 8 a.m. The 5 birds in each group were weighed at once. Feeds were supplied by weighing every day and remaining feeds were recorded weekly and feed conversion was calculated. Dead animals were recorded daily. Viability rate of experimental groups was calculated *(Fig. 1)*. At the end of 35 days, the animals were slaughtered by randomly choosing 2 partridges from each repetition, 10 from each group, and 30 partridges in total. Bloods of slaughtered partridges were collected in serum tubes with gel. Serum were removed by centrifuging blood samples at 1792 g for 10 min. Small intestine samples (duodenum) were taken following the slaughter and kept under appropriate conditions until the analyses were performed.

Table 1. *Ingredients and composition of diet*

Feed Ingredients	**(%)**
Maize	40.50
Wheat	9.51
Soybean meal, 48%	30.20
Maizebran	9.95
Vegetableoil	4.70
DL-Methionine	0.34
Dicalciumphosphate	2.90
Calciumcarbonate	1.00
L-Lysinehydrochloride	0.25
Salt	0.40
Vitamin*-mineral** mixture	0.25
Composition	**(%)**
Drymatter	90.2
Crude protein	20.4
Crudecellulose	3.17
Crudefat	6.72
Crudeash	7.28
Calcium	1.18
Availablephosphorus	0.64
Sodium	0.19
Methionine + cystine	1.00
Lysine	1.32
Threonine	0.72
Metabolicenergy, kJ	12552.00

** Vitamin mix (per 1 kg): Vit. A 5.000 IU, Vit. D_3 500 IU, Vit. E 10 mg, Vit. K_3 2 mg, Vit. B_2 4 mg, Vit. B_{12} 10 mg; ** Mineral mix (per 1kg): Manganese 120 mg, ferrous 40 mg, zinc 16 mg, copper 16 mg, cobalt 200 mg, iodine 1.25 mg, selenium 0.30 mg*

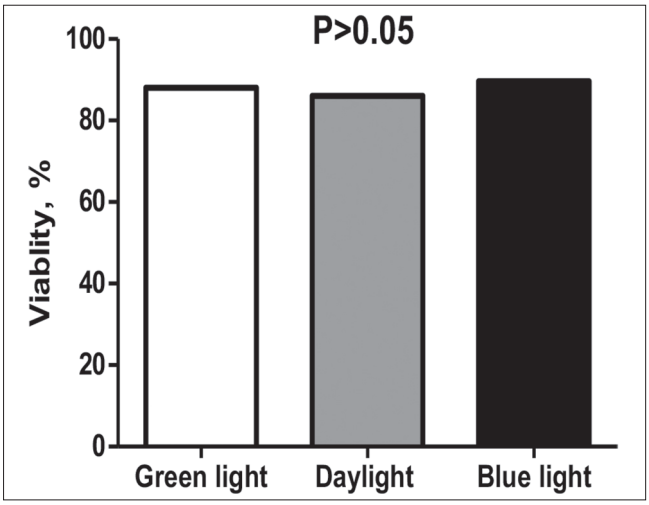

Fig 1. Effect of light color on viability of Chukar partridges

Morphometric Analyses of Small Intestine Segment

Tissue samples were taken from the duodenum and fixed in 10% neutral-buffered formalin for 24 h. They were then

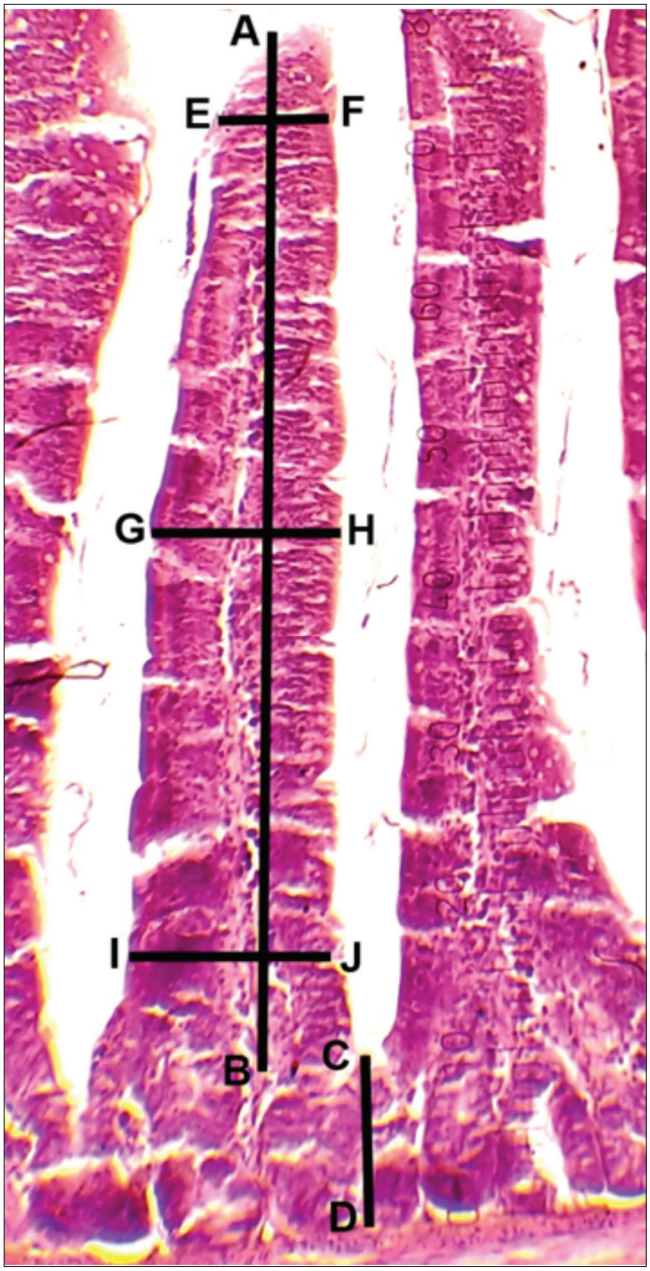

Fig 2. Section of the duodenum hematoxylin-eosinstained, showing the measured parameters. Villus height [AB], crypt depth [CD], villus edge widths [EF], villus middle width [GH] and villus bottom widths [IJ] (x200)

dehydrated through graded ethanol and embedded in paraffin. Five micrometers thick sections were obtained by a microtome and placed on glass slides. Cross sections were processed according to conventional hematoxylin and eosin staining method [19]. Sections were examined by a light microscope (Olympus CX31, Olympus USA) equipped with a digital imaging system (Olympus DP20, Olympus USA). Villus surface area was calculated according to Solis de los Santos et al.[20]. In all histomorphometric parameters, villus heights (VH), villus bottom widths, villus edge widths and crypt depths (CD) related to villi were measured from 7 consecutive villus-crypt complexes. Villus heights (AB) was measured from joining point of villi

Table 2.*Treatment groups means and standard errors as well as P-values of performance traits of Chukar partridges in different ages*

Days	Green Light (GL)	Day Light (DL)	Blue Light (BL)	Significance (P)
Live weights (LW), g/day/bird				
1	14.92±0.08	14.92±0.03	14.92±0.05	NS
7	28.62±0.22	28.87±0.34	28.76±0.25	NS
14	60.02±0.54	60.08±0.68	59.74±0.41	NS
21	95.58±0.87^{a}	92.44±0.59^{b}	90.33±1.22^{b}	**
28	138.36±1.32^{a}	135.48±0.75^{ab}	134.44±1.06^{b}	*
35	178.54±1.71^{a}	175.68±2.19^{ab}	171.67±1.26^{b}	*
Live weight gains (LWG), g/day/bird				
1-7	1.95±0.06	1.99±0.04	1.97±0.06	NS
8-14	4.48±0.08	4.45±0.03	4.42±0.04	NS
15-21	5.08±0.19	4.62±0.16	4.37±0.24	NS
22-28	6.11±0.24	6.14±0.04	6.30±0.33	NS
29-35	5.74±0.12	5.74±0.12	5.31±0.12	NS
1-35	4.67±0.03^{a}	4.59±0.03^{ab}	4.47±0.03^{b}	**
Feed intake (FI), g/day/bird				
1-7	4.41±0.09	4.69±0.14	4.29±0.11	NS
8-14	16.11±1.34	15.39±1.71	15.06±0.71	NS
15-21	24.22±0.10^{a}	19.44±0.20^{b}	21.57±1.51^{ab}	**
22-28	22.26±1.93	19.44±1.46	17.84±0.27	NS
29-35	22.56±2.29	20.48±2.19	17.27±0.18	NS
1-35	17.91±0.99^{a}	15.96±0.46^{ab}	15.20±0.30^{b}	*
Feed conversion ratio, g FI/g LWG				
1-7	2.27±0.09	2.35±0.12	2.17±0.11	NS
8-14	3.59±0.26	3.45±0.41	3.40±0.15	NS
15-21	4.76±0.21	4.20±0.18	4.93±0.23	NS
22-28	3.63±0.41	3.16±0.21	2.85±0.05	NS
29-35	3.93±0.45	3.56±0.30	3.25±0.08	NS
1-35	3.83±0.21	3.47±0.07	3.40±0.06	NS

NS: *Non-significant, * P<0.05, ** P<0.01, ^{a,b} The differences among the mean values with different superscripts (a, b: P<0.05) in a row within the different illumination groups are significant according to the ANOVA and post-hoc Tukey HSD tests*

and crypts to top points of the villus. CD was measured from the middle point of two neighboring villi to base of crypts. While villus edge widths (EF) was measured from just below the top point of the villus, villus bottom widths (IJ) was measured from just above the joining point of villi and crypts. Also, the middle widths (GH) was measured from the middle of the villus heights *(Fig. 2)*. By using the measured values, ratios of villus height/crypt depth (VH/CD) were also calculated.

Blood Parameters

Serum glucose, triglyceride,high density lipoprotein (HDL), creatine, uric acid, urea, lactate dehydrogenase (LDH), and blood urea nitrogen (BUN) values were measured via autoanalyzer (Mind-Way B5-2000 M), calcium level via atomic absorption spectrophotometer (Perkin Elmer AAnalyst 800 Atomic Absorption Spectrometer-Flame), Vitamin D level via high performance liquid chromatography (HPLC) (Shimadzu) (25-0H Vitamin D_2/D_3, ImmuChrom GmbH-IC3401-160114).

Statistical Analyses

After tests of Shapiro-Wilk for normality, Levene's test for homogeneity of variance was used. All data including performance parameters, blood parameters and histo-morphometric features of small intestine were subjected to analysis of variance. Significant differences were compared with Tukey HSD test. Viability rate of groups were compared with Chi-square test. All analyses were performed using SPSS ver. 21 for Windows [21]. Differences were important at P≤0.05. Data were presented as means ± SEM.

RESULTS

The effect of light colors on performance parameters in Chukar partridge is shown in *Table 2*. The live weight of GL

Table 3. *Treatment groups means and standard errors as well as P-values of histo-morphometric features of small intestine of Chukar partridges*

Parameters (µm)	Green Light (GL)	Day Light (DL)	Blue Light (BL)	Significance (P)
Villus height (VH)	112.91±2.93^{a}	109.10±3.67^{a}	94.10±2.80^{b}	***
Crypt depth (CD)	11.50±0.47	10.64±0.60	10.29±0.53	NS
Villus edge width	13.02±0.46^{a}	12.02±0.46^{ab}	9.68±0.33^{b}	***
Villus middle width	16.37±0.54^{a}	14.93±0.51^{ab}	11.22±0.42^{b}	***
Villus bottom width	19.77±0.70^{a}	17.50±0.67^{b}	13.77±0.63^{c}	***
VH/CD	9.81±0.65	10.25±0.65	9.14±0.55	NS
Villus surface area (VSA)	5813.60±230.79^{a}	4989.54±178.61^{b}	3431.81±145.25^{c}	***

VSA: *(2x3.14) x (Mean of Villus width/2) x (Villus height),* ***NS:*** *Non-significant, *** P<0.001, ^{a,b,c} The differences among the mean values with different superscripts (a, b, c: P<0.05) in a row within the different illumination groups are significant according to the ANOVA and post-hoc Tukey HSD tests*

Table 4. *Treatment groups means and standard errors as well as P-values of blood parameters of Chukar partridges*

Parameters	Green Light (GL)	Day Light (DL)	Blue Light (BL)	Significance (P)
Vit D, mg/dL	11.92±1.58	14.24±1.56	10.70±0.59	NS
Calcium, mg/dL	6.81±0.29	6.55±0.30	6.46±0.39	NS
Glucose, mg/dL	350.26±6.42^{a}	321.00±9.96^{ab}	304.96±5.34^{b}	**
Triglyceride, mg/dL	68.94±7.64	58.84±4.03	55.74±7.37	NS
HDL, mmol/dL	108.28±7.48^{b}	141.60±4.11^{a}	117.38±5.45^{b}	**
Uricacid, µmol/dL	1.91±0.42	2.12±0.50	3.54±0.57	NS
Urea, mg/dL	6.75±0.94	4.79±0.53	5.33±0.51	NS
LDH, µkat/L	23.35±1.67^{a}	18.30±1.42^{ab}	18.20±0.80^{b}	*
BUN, mg/dL	3.20±0.37	2.20±0.35	2.60±0.24	NS

HDL: *High density lipoprotein,* ***LDH:*** *Lactatedehydrogenase,* ***BUN:*** *Blood urea nitrogen,* ***NS:*** *Non-significant, * P<0.05, ** P<0.01, ^{a,b} The differences among the mean values with different superscripts (a, b: P<0.05) in a row within the different illumination groups are significant according to the ANOVA andpost-hocTukey HSD tests*

group was found to be higher in days of 21 (P<0.01), 28 and 35 (P<0.05), followed by DL and BL. Live weight gain was also different among the groups in the period of 1-35 days. GL group showed better performance in live weight gain than DL and BL (P<0.01). Feed intake in days 15-21 (P<0.01) and 1-35 (P<0.05) were significantly higher in GL group. However, feed conversion *(Table 2)* and viability rates *(Fig. 1)* were similar among the groups (P>0.05).

When the effect of light colors on histo-morphometric features of the small intestine is examined in *Table 3*, villus height,villus width in the edge, middle and bottom of the duodenum, and villus surface area were found to be superior in GL group compare with DL and BL, respectively (P<0.01). The crypt depth of duodenum and the ratio of villus height to crypt depth were similar among the groups (P>0.05).

Table 4 shows the blood parameters of partridges subjected to different light color. Serum glucose (P<0.01) and LDH (P<0.05) levels showed statistically higher value in GL group, followed by DL and BL. However, serum HDL was found higher in DL group than another experimental group. The differences in levels of Vit D, calcium, triglyceride, uric acid, urea, and BUN weren't significant among the experimental groups (P>0.05).

DISCUSSION

The studies on the sensitivity of poultry to light spectrum have been continuing intensively [10,22-24]. The superiority of LEDs to other sources of light increases the use of this light source in poultry houses day by day [5]. Because perception levels of LED lamps at various wavelengths are different, a lot of researchs in broiler [1,9,25] and less in other poultry species [10,17,23] were determined to have different effects on performance in recent years, however, no information could be reached in partridge species. Investigatings in the present study showed that green LEDs increase feed intake of partridges significantly *(Table 2)*. In parallel with feed intake, live weight gains of partridges and live weights on the day of 35 were significantly high in this group. There were also no significant differences among the groups in terms of feed conversion and viability rates. On the other hand, better results were obtained from daylight illumination compared to blue light and the lowest values were obtained in the blue light group. When the other studies on growth and fattening performance were reviewed, green light was emphasized to lead to an increase in skeletal muscle cells of broilers, expression of growth hormone receptor genes, the number and uniformity of myofibrils in early period [12,25]. Sarica [26] stated

that green illumination had a delaying effect on the ovary and testicle development of quails, therefore, increased the growth performance compared to red and white light because it retarded sexual maturation. In different studies revealed that melatonin, which has important effects on regulation of body rhythm, cell regeneration, and immunity, was reported to be synthesized mostly under green light followed by red, white, and blue light [27]. Associated with increased levels of meletonin, the fact that the present study provided higher feed consumption and better live weight gain for partridge chicks grown under green light may be associated with feeling better and coping with environmental factors better. Similarly, Pan et al.[8] stated that while the light with a short wavelength (blue and green) induced the growth of broilers in early periods, the light with a long wavelength (yellow) induced the growth in the late period. It was found that every 100 nm (455 to 620 nm) increase of light's wavelength in early period decreased live weight by 15.4 g, every 100 nm of increase in the late period elevated live weight by about 16.4 g. Hassan et al.[17] also reported that the highest body weight and weight gain of duck were obtained in green light treatment compared with the white, yellow and blue lights during first 21 days. During d 22-42, weight gain increased in both the green and blue treatments in a like manner. Poultry behaviors are another factor influencing poultry performance. Studies on broilers revealed that red and white light increased activity in chicks, white light increased aggressiveness compared to red therefore negatively influenced growth, and broilers felt more peaceful at a wavelength shorter than 580 nm (blue, green, yellow) [15].

In addition to above-mentioned results, challenging effect of green light on growth was considered to be arising from the changes in bowel structure. When the results in *Table 3* were examined, there was a substantial increase in villus height and villus width of bowel in the green light group and villus width was higher than other groups all along the whole villus. These developments in intestinal villi increased the surface area of villi. These results were thought to influence digestion and absorption positively [20,28]. Similarly, compared to white and blue light, green light increased intestinal villus length and the ratio of villus length to crypt depth in early periods in broilers (days of 1-21) and decreased crypt depth and blue light had similar results in the late period (until 49) [11]. In addition, green light in early period and blue light in late period reinforced intestinal immunologic barrier by influencing the number of intestinal intraepithelial lymphocytes, goblet cells, and IgA+ cells [11], and also caused birds to become more resistant to environmental stress by improving general immunity [18].

When the findings of blood parameters were examined *(Table 4)*, it was observed that all parameters studied were within the normal ranges reported for partridges [29]. However, blood glucose and LDH levels were significantly higher in the green light group compare with blue light. Blood HDL level was higher in daylight group, conversely. Light applications were stated to have an important effect on the release of hormones and on glucose level [30,31]. Elevated LDH might have related that it induces the energy transformation process which is required to reversibly convert pyruvate, in parallel to increasing glucose concentration, into lactic acid and for cellular usage of glucose [32]. In other studies, their results were similar to blue light group of the present study, reported that there were significant relationships between blood pressure, blood lipids and blood glucose; exposure to blue light causes a decrease in blood pressure and positively effects blood glucose level and blood lipids [33]. Hassan et al.[17] also stated lower cholesterol level in blue light than green in duck. On the contrary, Yang et al.[12] determined that in broilers green light alone significantly increased blood high-density lipoprotein cholesterol (HDL-CH) level; whereas, blue light alone low-density lipoprotein cholesterol (LDL-CH) level, a combination of both colors increased both performance parameters and blood glucose levels.

In conclusion, the green light was identified to stimulate the growth of partridges during the growth period. It also improved the intestinal structure in the direction of increasing absorption and changed hormonal mechanism positively. Accordingly, the use of green light during growth period in the enterprise performing intensive partridge farming is considered will be improve performance.

Conflict of Interest

We declare that there is noconflict of interest with any financial organization regarding the material discussed in the manuscript.

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Research Article

Immunohistochemical Localization of Catalase in Geese *(Anser anser)* Kidney [1]

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Abstract

The aim of this study is to observe the histologic structure of kidney tissue of geese and to determine the immunohistochemical localization of catalase in it. Six female geese were used in our study. After fixation in Bouin's solution and routine histological process, the kidney tissues taken from geese were embedded in paraffin blocks. To detect catalase immunoreactivity in the tissues, the avidin-biotin-peroxidase complex (ABC) technique was used. Crossmann's modified triple stain for histological examination was applied to the samples. It was seen that the geese kidney included numerous lobules and each lobule consists of cortical and medullary tissues. The strong immunoreactivity of catalase was observed in the proximal convoluted tubules in the cortex. Immunohistochemical reaction was also observed in intermediate tubules and the thick segment of Henle's loops. Also it was determined that the immunoreactivity in the basal epithelial cells of the secondary branches of ureters and the intercalated cells in the distal convoluted tubules and collecting duct. There was no catalase immunoreactivity in the renal corpuscles. In conclusion, localization of catalase in the proximal convoluted tubules, in the intermediate tubules, in the thick segments of Henle's loops and in the intercalated cells is considered that these parts of the geese kidney are involved in the antioxidant defence. This study is thought to contribute to the planned future studies about antioxidant system in the kidney of poultry.

Keywords: *Catalase, Goose, Kidney, Immunohistochemistry*

Kaz *(Anser anser)* Böbreğinde Katalaz'ın İmmunohistokimyasal Lokalizasyonu

Öz

Bu çalışmada amaç, kazların böbrek dokusunun histolojik yapısı ve katalazın immunohistokimyasal lokalizasyonunun belirlenmesidir. Çalışmamızda 6 adet ergin dişi kazdan alınan böbrek doku örnekleri kullanıldı. Alınan doku örnekleri Bouin solüsyonunda tespit edildikten sonra rutin histolojik doku takibi yapılıp ardından parafinde bloklandı. Dokulardaki katalaz immunoreaktivitesini incelemek için Avidin-Biotin-Peroksidaz Kompleks (ABC) tekniği kullanıldı. Dokuların genel histolojik görünümlerini incelemek için de Crossmann'ın modifikasyonu olan üçlü boyama tekniği uygulandı. Yapılan histolojik incelemelerde kaz böbreğinin her birinin ayrı ayrı korteks ve meduldadan oluşan çok lopçuklu bir yapıya sahip olduğu görüldü. Dokulardaki katalaz immunoreaktivitesi en yoğun olarak kortekstekı tubulus proksimalis konvoluta kısımlarında belirlendi. Bununla birlikte immünohistokimyasal reaksiyon intermediyer tubullerde ve çıkan Henle kısımlarında da gözlendi. Ayrıca, üreterlerin kollarındaki bazal epitel hücrelerinde, tubulus distalis konvolutalarda ve toplayıcı borucuklarda bulunan interkalat hücrelerde de yer yer reaksiyon olduğu görüldü. Korpuskulum renislerde katalaz immunoreaktivitesine rastlanmadı. Sonuç olarak, antioksidan bir enzim olan katalazın tubulus proksimalis konvoluta, intermediyer tubul, çıkan Henle bölümü ve interkalat hücrelerde lokalize olması bu kısımların kaz böbreğindeki antioksidan savunma bölgeleri olduğunu düşündürmektedir. Bu araştırmanın kanatlı böbreğinde antioksidan savunma sistemi ile ilgili yapılması planlanan başka çalışmalara katkı sağlayacağı düşünülmektedir.

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INTRODUCTION

The histological structure of kidney tissue in poultry is observed as a multi-lobule structure with separate cortex and medulla. There is a thin connective tissue between the cortex and the medulla. Two different types of nephrons are present in the kidney of poultry. The first one is the nephron structure called mammalian or medullary type. In this type of nephron; renal corpuscle, proximal convoluted tubule, thin and thick segments of Henle's loop, distal convoluted tubule and collecting tubule are observed. In the mammalian type nephrons, the renal corpuscle is larger [1,2]. Another type of nephron in the poultry kidney is the reptilian type nephron which is also called as cortical type. The renal corpuscle is smaller in this nephron type. Also, there is no Henle's loop in the reptilian type nephron. There is a short part called intermediate tubule between proximal convoluted tubule and distal convoluted tubule [1-4]. In the cytoplasm of the epithelial cells located in the proximal convoluted tubule, there are plenty of mitochondria and a large number of peroxisomes [5]. Catalase is present in peroxisomes within the cell [6]. Intercalated cells are located among epithelial cells of both distal convoluted tubules and the collecting tubules [7]. There are secondary branches of the ureter in the poultry kidney [1,2].

During the normal functions of the cells, a number of molecules called reactive oxygen species are formed and they are very harmful to the cells [8]. The most important source of these molecules in the cell is the mitochondria. The harmful effects of these molecules are prevented by the antioxidant defence system [8-10]. Catalase is one of antioxidant enzymes in this system. Catalase shows antioxidant activity by detoxifying hydrogen peroxide (H_2O_2) [8,11,12]. The kidney has high oxidative metabolic activity so it has a high risk of oxidative tissue damage [13]. Degraded antioxidant defence system plays a critical role in the pathogenesis of chronic renal failure [12]. Lack or insufficiency of catalase and other antioxidant enzymes causes renal tissue damage and interstitial fibrosis, resulting in loss of renal function [12-14].

Poultry species are living organisms with high metabolic activity. However, it is stated that poultries are more resistant to oxidative damage compared to the mammals. This may be related to the production of less reactive oxygen species or a strong antioxidant defence system in the poultry [15].

The aims of this study were to investigate the histologic structure of the geese *(Anseranser)* kidney tissue and to determine the immunohistochemical localization of catalase in it.

MATERIAL and METHODS

This study was approved by the Local Ethics Commission of Experimental Animals of Kafkas University (KAÜ-HADYEK). In our study, kidney tissue samples taken from 6-12 month old, 6 female geese *(Anser anser)* butchered for consumption were used. Tissue samples taken for histological and immuno-histochemical examinations were fixed in Bouin's solution. Routine histological tissue process was performed, and the tissues were blocked in paraffin. Tissue sections were taken from paraffin blocks at a thickness of 5 µm. The sections were stained with Crossman's modified triple staining technique [16] to examine the general histological structure of the tissues after deparaffinization and rehydration procedures. Avidin-Biotin-Peroxidase complex (ABC) technique [17] was used to determine catalase immunoreactivity in tissues. Anti-polyvalent HRP kit (Thermo Scientific, TP-125-HL) was used for this purpose. Endogenous peroxidase activity was prevented by applying 3% H_2O_2 to the sections. Afterwards, the masked antigens were exposed by the application of microwave irradiated antigen retrieval (MW-AR). For this purpose, 800 watt heat was applied to the sections in citrate buffer solution (0.1 M, pH: 6.0) in microwaves for 10 min. At the end of this period, the sections were washed with PBS (Phosphate buffer solution, 0.1 M, pH 7.2). Samples had been incubated in the blocking buffer (Thermo Scientific, TA-125-UB) for 10 min, they were washed with PBS. After the sections were then incubated for 1 h at room temperature with anti-catalase primary antibody (abcam, ab1877, 1: 500 dilution). Biotinylated secondary antibody (Thermo Scientific, TP-125-BN) was applied for 30 min following incubation. Then, streptavidin peroxidase (Thermo Scientific, TS-125-HR) was applied for 30 min. In the final stage, DAB (Diaminobenzidine) (Thermo Scientific, TP-015-HD) was applied to make this reaction visible. Negative control was applied to determine the specificity of the reaction. Cross-sections were stained with hematoxylin to determine nuclei. Preparations were examined by light microscopy and photographed. Grading was performed to determine the intensity of the reaction according to regions (0: no reaction, 1: mild reaction, 2: medium reaction, 3: very intense reaction). For statistical analysis, slides were randomly selected from each subject and 120 tubulus proximalis, 120 intermadiate tubulus, 120 thick segment of Henle's loop and 120 intercalated cells were also randomly selected. Selected regions of each subject were graded in terms of the reaction intensity which ranged from 0 to 3. SPSS 16.0 for Windows was used to compare the intensity of the catalase immunoreactivity. To determine differences of the reaction intensity between different regions of geese kidney, Tamhane's T2 of Post-Hoc Multiple Comparison in One-Way ANOVA was used because of homogeneity variance result.

RESULTS

The histological examination revealed that the goose kidney had a multi-lobule structure, each consisting of cortex and medulla. Two types of nephron structure and their different tubular structures were seen in poultry kidney.

In the cortex; renal corpuscle, proximal convoluted tubule, distal convoluted tubule *(Fig. 1-A)*, sections of collecting tubule, perilobular collecting duct, intermediate tubules of reptilian type nephrons, blood vessels and connective tissue regions were identified. In the medulla; thin and thick segments of Henle's loop of mammalian type nephrons, medullary collecting duct, secondary branches of ureters, vessels and connective tissue were seen. More darkly stained intercalate cells were observed between epithelial cells in the distal convoluted tubule *(Fig. 1-B)* and collecting tubule in both cortex and medulla. In addition, partial lymphoid tissue foci were also found in the geese kidney.

In our immunohistochemical examinations to determine the localization of catalase in renal tissue *(Fig. 2-A)*, no evidence of catalase immunoreactivity was observed in the renal corpuscle. No reaction was observed in the negative controls which were performed to check whether the catalase immunoreactivity was specific or not *(Fig. 2-B)*. Cytoplasmic and granular strong catalase immunoreactivity was determined in the proximal convoluted tubules *(Fig. 3)*. Immunohistochemical reaction was also observed in intermediate tubules of reptilian type nephrons *(Fig. 4-A)*. However, the reaction observed in the intermediate tubules was found to be milder than that of proximal convoluted tubules. Generally, there was no

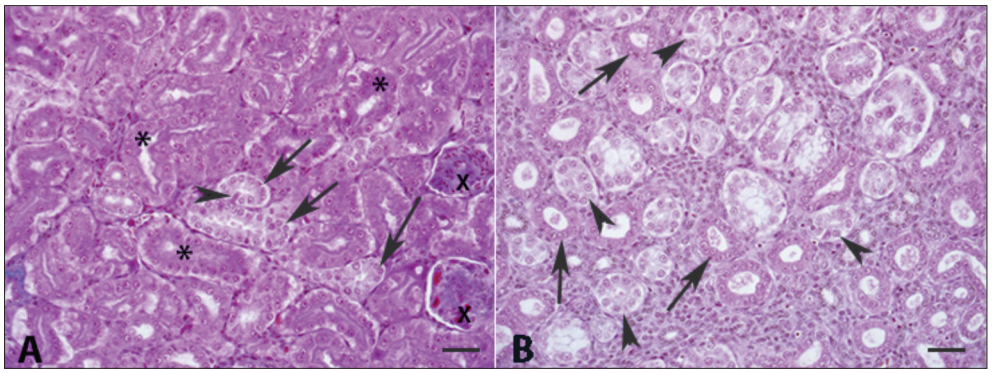
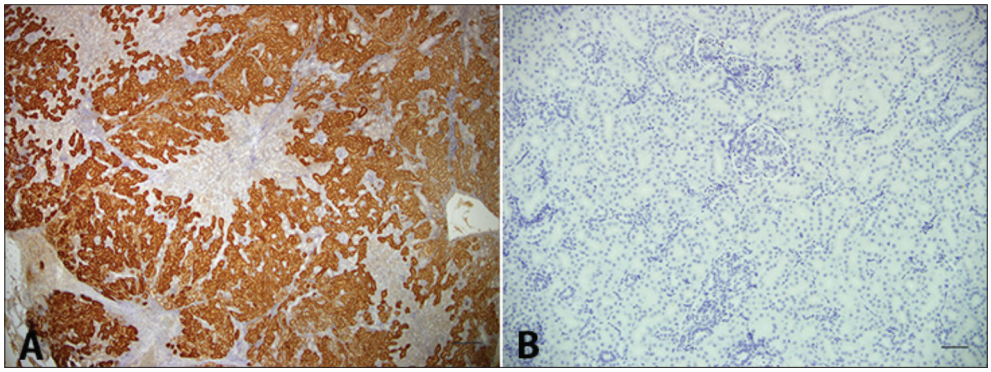


Fig 1. General view of the geese kidney. Crossman's modified triple staining. **A)** X: Renal corpuscle, * Proximal convoluted tubules, *Arrows:* Distal convoluted tubules, *arrow head:* intercalated cell. Bar: 50 µm; **B)** Medulla. *Arrows:* Thick segments of Henle's loops, *arrow heads:* medullary collecting ducts. Bar: 50 µm

Fig 2. A) General view of catalase immunoreactivity in the kidney. Bar: 500 µm; **B)** Negative control. Bar: 100 µm



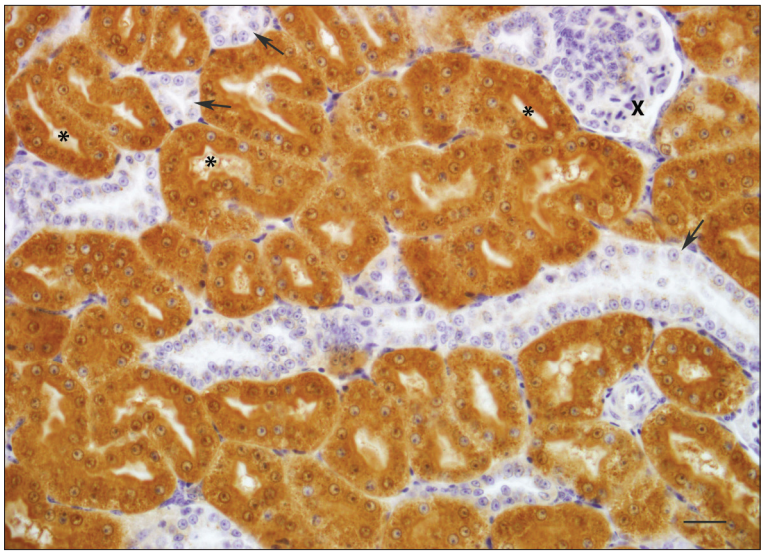


Fig 3. Catalase immunoreactivity in the renal cortex. **X:** Renal corpuscle, * Proximal convoluted tubules, *Arrows:* Distal convoluted tubules. Bar: 50 µm

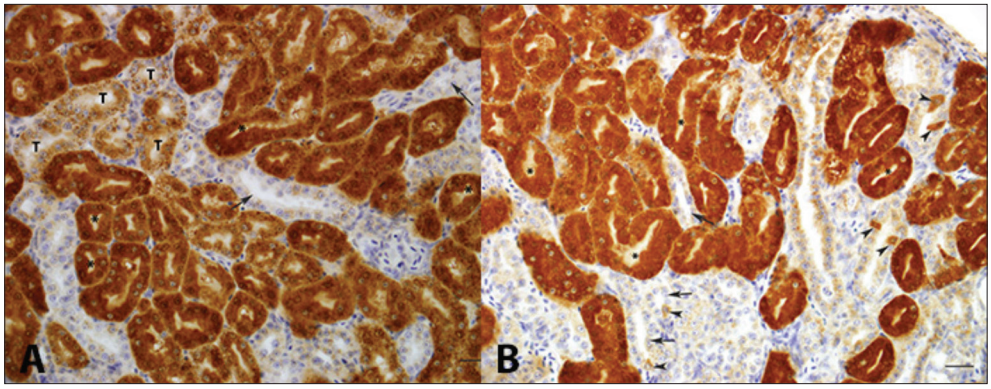


Fig 4. Catalase immunoreactivity. **A)** * Proximal convoluted tubules, *Arrows:* Distal convoluted tubules, T: Intermediate tubules. Bar: 50 µm. **B)** * Proximal convoluted tubules, *Arrows:* Distal convoluted tubules, Arrow heads: Intercalated cells. Bar: 50 µm

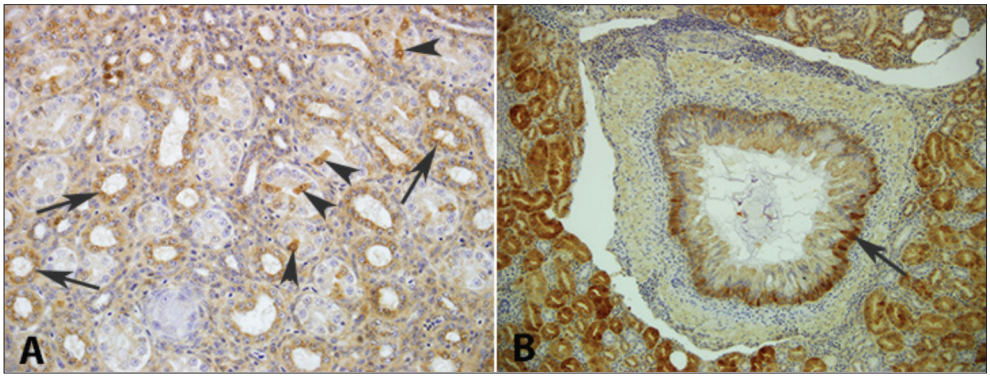


Fig 5. A) Catalase immunoreactivity in the medulla. *Arrows:* Thick segments of Henle's loops, *arrow heads:* the intercalated cells in the medullary collecting ducts. Bar: 50 µm; **B)** Catalase immunoreactivity in the secondary branch of the ureter. *Arrow:* Basal epithelial cells. Bar: 100 µm

Table 1.*The intensity of the catalase immunoreactivity according to the regions of geese kidney*

Parts of Kidney	N	Min	Max	Mean±SD	F
Proximal Convoluted Tubule	120	2	3	2.96±0.18[a]	434.15
Thick Segment of Henle's Loop	120	1	2	1.16±0.37[b]	
Intermediate Tubule	120	1	2	1.39±0.49[c]	
Intercalated Cells	120	1	3	1.46±0.57[c]	

Different superscripts mean statistically significant difference. ***SD:*** *Standart Deviation,* ***F:*** *F value*

immunoreactivity in the epithelial cells of distal convoluted tubules while a moderate reaction was observed in the intercalated cells of these tubules *(Fig. 4-B)*. Similar reactions were also observed in the intercalated cells in the collecting tubules. In the medulla, there was no reaction in the thin segment of Henle's loops of the mammalian type nephrons while mild-degree catalase immunoreactivity was observed in the thick segment of Henle's loops *(Fig. 5)*. Immunoreactivity was also observed in the intercalate cells in medullary collecting ducts *(Fig. 5-A)*.

It was found that the strongest catalase immunoreactivity was in the proximal convoluted tubules in terms of statistical analysis between different parts of the geese kidney. However, a weak immunoreactivity was found in the intermediate tubules, in the thick segments of Henle's loops and in the intercalated cells *(Table 1)*.

The catalase immunoreactivity was observed in the basal epithelial cells in the secondary branches of ureters *(Fig. 5-B)*. The catalase immunoreactivity was determined in macrophages in the connective tissue. No catalase immunoreactivity was observed in the vascular wall forming layers.

DISCUSSION

In this study, we evaluated immunohistochemical distribution of catalase enzyme and general histological structure in the geese kidney. The histological structure of the kidney shows that it is composed of nephrons and collecting tubules as parenchyma. However, kidney tissue in poultry is different from mammals because two different types of nephron are seen together [1,3]. In our study, it was seen that the histological findings of the geese kidney were parallel with the data of the mentioned literature.

Reactive oxygen species are continuously produced during the life of the cell as a result of mitochondrial oxidative metabolism. Cells are constantly fighting with these free radicals. In the antioxidant defence system, both antioxidant enzymes and natural antioxidant molecules play an

important role [18]. Catalase, which detoxifies hydrogen peroxide, is one of important antioxidant enzymes. When reactive oxygen species cannot be controlled due to deficiency of antioxidant enzymes, various damages are seen in tissues [11]. For the evaluation of these harmful effects of free radicals, not only the amount of the free radicals produced should be considered but also the effectiveness of these enzymatic and chemical antioxidant systems should be considered [18]. At this point; the low amount of free radicals produced [15] or the transcription and translation of an active gene related to oxidative stress resistance [19] or the production and activity of antioxidant enzymes in poultry may be related to the higher resistance to oxidative damage in the poultry than that in mammals.

There are few studies about catalase and antioxidant system in the tissues of the poultry [20,21]. However, no such studies could be encountered in the poultry kidney. In studies on the mammalian kidney, it was reported that catalase plays a very effective role in the maintenance of the renal functions [12-14]. In a study on the immunohistochemical distribution of catalase in mouse kidney, cytoplasmic reaction that was granular in style was reported as like our findings [22]. In our study, it was seen that the catalase was localized more intensely in proximal convoluted tubule. This result is similar to the previous studies on mouse kidney [22,23]. The most intensive catalase immunoreactivity in these tubules may be related to both production of free radicals as a result of intense metabolic activity and presence of many peroxisomes in their cells. We think that presence of reaction in intermediate tubules of cortical type nephron may be due to the same reasons.

In our study, immunoreactivity was also found in intercalate cells which are located between epithelial cells of distal convoluted tubule and collecting tubules. Intercalate cells are highly active cells that regulate acid-base balance in the kidney and have abundant mitochondria [24]. The presence of catalase immunoreactivity in these cells, in which the reactive oxygen species are produced, is an indicator of cellular antioxidant defence. Previous studies in mammals reported no catalase immunoreactivity in the Henle's loops [22,25]. Contrary to these literatures, in our study it was determined that a small amount of catalase immunoreactivity was found in the thick segments of Henle's loops in geese kidney. This may be related to the differences between species. In addition, we did not encounter any studies on catalase immunoreactivity in basal epithelial cells in the secondary branches of the ureters.

In conclusion, we investigated the immunohistochemical localization of catalase which is an important antioxidant enzyme in the kidney tissue of geese. The results showed proximal convoluted tubules, thick segments of Henle's loops and intermediate tubules as antioxidant defence regions of the poultry kidney. Intercalated cells in distal convoluted tubule and collecting tubule have also a role as antioxidant defence in the poultry kidney. In conclusion, localization of catalase in the proximal convoluted tubules, in the intermediate tubules, in the thick segments of Henle's loops and in the intercalated cells is considered that these parts of the geese kidney are antioxidant defence parts. This study will contribute to the planned future studies about antioxidant system in the kidney of poultry.

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Research Article

Assessment of the Effect of Beef and Mutton Meat Prices on Chicken Meat Prices in Turkey Using Different Regression Models and the Decision Tree Algorithm

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Abstract

Determination of the extent to which increases in the price of red meat is reflected in the price of chicken meat will shed light to the sector in many areas, including production planning. The purpose of this study is to present how the increases in the price of red meat are reflected in the consumer prices of chicken meat and to determine the estimation rate of the consumer prices of chicken meat by analysing the monthly average consumer prices of chicken meat, beef and mutton (TRY/kg) in Turkey between January 2005 and September 2018 using different regression models and the decision tree algorithm. The results of the study indicate that in predicting the prices of chicken meat using the prices of beef, the cubic regression equation gives accurate predictions at a rate of 91.4%, and in predicting the prices of chicken meat using the prices of mutton, the cubic regression equation gives accurate predictions at a rate of 89.9%. It was estimated that 1 unit increase in the prices of beef would result in an increase by 1.35 unit in the prices of chicken meat, and a 1 unit increase in the prices of mutton would result in an increase by 0.39 unit in the prices of chicken meat.

Keywords: *Red meat, Chicken meat, Price, Regression, Turkey*

Türkiye'de Dana ve Koyun Eti Fiyatlarının Tavuk Eti Fiyatlarına Etki Düzeylerinin Farklı Regresyon Modelleri ve Karar Ağacı Algoritması İle Değerlendirilmesi

Öz

Türkiye'de kırmızı ette yaşanan fiyat artışlarının tavuk eti fiyatlarına yansımasının ne düzeyde olduğunun belirlenmesi, sektörün üretim planlaması başta olmak üzere pek çok hususta sektöre ışık tutacaktır. Bu çalışmanın amacı, 2005/01 Ocak ile 2018/09 Eylül döneminde aylara göre Türkiye geneli tavuk, dana ve koyun eti tüketici ortalama fiyatlarının (TL/kg) farklı regresyon modelleri ve karar ağacı algoritması ile analiz edilerek, kırmızı ette yaşanan fiyat artışlarının ne ölçüde tavuk eti tüketici fiyatlarına yansıdığını ortaya koymak ve tavuk eti tüketici fiyatlarının tahmin oranlarının belirlenmesidir. Çalışmanın sonuçlarına göre dana eti fiyatlarını kullanarak tavuk eti fiyatlarının tahmin edilmesinde kübik regresyon denklemi ile %91.4 açıklama oranında başarılı bir tahmin yapılırken, koyun eti fiyatlarında kübik regresyon denklemi ile %89.9 açıklama oranında başarılı bir tahmin yapılabileceği belirlenmiştir. Dana eti fiyatlarında meydana gelecek 1 birimlik değişimin kübik regresyona göre tavuk eti fiyatlarında 1.35 birimlik bir artışa, koyun eti fiyatlarında meydana gelecek 1 birimlik değişimin ise tavuk eti fiyatlarında 0.39 birimlik bir artışa yol açacağı tahmin edilmiştir.

Anahtar sözcükler: *Kırmızı et, Tavuk eti, Fiyat, Regresyon, Türkiye*

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INTRODUCTION

Foods of animal origin are important for sustainability of individual and social development. Easy access to and availability of such healthy foods that are one of the essential needs of humans is one of the common characteristics of developed countries. However, animal products are not supplied to all segments of the society at affordable prices and in sufficient amounts in Turkey.

In addition to the problems in the supply of red meat in Turkey, the amount of consumption of animal products is restricted by price level and consumers' income level, two major factors underlying the preference for meat [1]. In Turkey between 2005 and 2018, per capita meat consumption increased by 3.06% for sheep, 211.29% for beef and 50.92% for chicken meat [2]. The sudden increase in the consumption of beef can be associated with imports after 2009. In the same period, meat prices increased. Chicken meat prices showed an increase of 214.24%, beef prices increased 273.80% and mutton prices increased by 349.59% [3]. As chicken meat is more affordable than red meat, consumers considerably shift their preferences to chicken meat when the price of red meat increases [4-7]. The major reason for this is that chicken meat and red meat are substitute goods [1]. Although costs are the primary factor affecting the formation of red meat and chicken meat prices, it can be said that there are also different variables that affect the prices [8].

Inputs such as feed raw materials, broiler breeder prices [9], packaging materials and energy costs are the factors that affect the price of chicken meat in Turkey [10]. On the other hand, consumer preferences [4] and socio-economic, psychological, seasonal factors also have an important role on the market price and supply-demand balance of chicken meat [11].

In the formation of red meat demand and price, production costs [12], consumer preferences [13], import decisions [14], government interventions in the red meat market, implemented policies and subsidies [15] are effective.

The studies delving into the interaction between red meat and chicken meat in Turkey focus on the effects of taxes and beef prices on consumption of chicken meat [16], the factors affecting the consumption of red meat and chicken meat by households [17] and the factors affecting the consumption of fresh red meat [18].

Studies on livestock breeding use different linear and non-linear regression models. Such models were used in estimating the prices of beef and feed [19] and the optimum growth curve for cattle [20] and sheep [21]. The decision tree method, which is an alternative to general linear models [22], is widely used in estimating the price of chicken meat [23], determining factors affecting the consumption of red meat [24], estimating the factors affecting the birth weight of dairy cows [25] and determining the factors affecting milk yield [26].

The purpose of this study is to present how the increases in the price of red meat are reflected in the consumer prices of chicken meat and to determine the estimation rate of the consumer prices of chicken meat by analyzing the monthly average consumer prices of chicken meat, beef and mutton in Turkey between January 2005 and September 2018 using different regression models and the decision tree algorithm.

MATERIAL and METHODS

Data Set

The material of the study consists of the monthly average consumer prices of chicken meat, beef and mutton (TRY/kg) in Turkey between January 2005 and September 2018. The prices of chicken meat, beef and mutton used in the study were obtained from the monthly consumer prices data set of the Turkish Statistical Institute (TURKSTAT) [3]. The beginning of the data in the TurkStat system was 2005 and this year was taken as a starting point.

Analysis Method

First, the chicken meat prices, the dependent variable in the analysis, were subjected to a goodness of fit test. Then, all input variables were estimated independently one by one using appropriate regression models. SPPS 25 and EasyFit 5.4 Professional [27] were employed to evaluate the data.

Regression Curve Estimation Models were used in the first stage of the analysis. Estimation models for one or more curves can be obtained by regression analysis. The relationship between variables can have a linear or another type of distribution. In such cases, it is better to use different models.

Among the models given in *Table 1*, the model with the

Table 1. *Regression models for estimating the consumer price of chicken meat*

Regression Models	Equation
Linear	$Y = b_0 + (b_1 * t)$
Logarithmic	$Y = b_0 + (b_1 * \ln(t))$
Inverse	$Y = b_0 + (b_1 / t)$
Quadratic	$Y = b_0 + (b_1 * t) + (b_2 * t^2)$
Cubic	$Y = b_0 + (b_1 * t) + (b_2 * t2) + (b_3 * t^3)$
Power	$Y = b_0 * (t^{**} b_1)$ or $\ln(Y) = \ln(b_0) + (b_1 * \ln(t))$
Compound	$Y = b_0 * (b_1^{**}t)$ or $\ln(Y) = \ln(b_0) + (\ln(b_1) * t)$
S-curve	$Y = e^{**}(b_0 + (b_1/t))$ or $\ln(Y) = b_0 + (b_1/t)$
Growth	$Y = e^{**}(b_0 + (b_1 * t))$ or $\ln(Y) = b_0 + (b_1 * t)$
Exponential	$Y = b_0 * (e^{**}(b_1 * t))$ or $\ln(Y) = \ln(b_0) + (b_1 * t)$

Y = dependent variable, b_0 = regression equation's constant term, b_1= regression coefficient, t = value of independent variable

highest coefficient of determination according to the result of the regression analysis was selected and evaluated.

In the analysis section of the study, the relationship between the dependent variables and the independent variable is presented according to the result of the curve estimations.

Then, in an attempt to conduct comparative analyses of major decision tree algorithms, C4.5(J48) [28], Decision Stump [29], Hoeffding Tree [30], LMT [31,32], Random Forest [33], RandomTree [34] and RepTree [35] algorithms on WEKA (Waikato Environment for Knowledge Analysis) were used. The original data set was randomly divided into ten equal parts using the 10-fold cross validation method. Subsequently, one of the parts was kept as validation data to test the model, and the remaining nine parts were used as training data. The cross validation process was carried out 10 times, using each of the 10 parts as validation data for once [36].

In obtaining the comparative results from the decision tree algorithms, no method was employed to select any of the attributes in the data set, and all attributes were taken into account in developing the classification model. The data set does not contain any missing values. For this reason, it was not subjected to pre-processing. The price of chicken meat was assessed using the CRT (Classification And Regression Tree) algorithm.

RESULTS

In order to identify the relationship between the consumer prices of chicken meat and the consumer prices of beef and mutton, analyses were conducted using 10 different regression models. The results are given in *Table 2* and *Table 3* below. The explanatory model of the cubic regression model for beef and mutton was found to be the highest according to the R^2 values of the regression models developed to estimate the consumer prices of chicken meat.

In estimating the consumer prices of chicken meat using the consumer prices of beef, the cubic regression equation (1) gives accurate estimations with a percentage of variance explained of 91.4%. It was estimated that a 1 unit increase in the consumer prices of beef would result in a 1.35 unit increase in the consumer prices of chicken meat according to the cubic regression model.

Table 2. *Models for estimating the consumer prices of chicken meat by different regression equations for the consumer prices of beef*

Equation		Model Estimates					Coefficients Estimates			
		R^2	F	sd1	sd2	P	Constant	b1	b2	b3
CONSUMER PRICES OF BEEF	Linear	0.862	1014.590	1	163	0.001	1.838	0.171		
	Logarithmic	0.885	1256.042	1	163	0.001	-7.041	4.202		
	Inverse	0.858	988.745	1	163	0.001	10.282	-87.320		
	Quadratic	0.877	578.430	2	162	0.001	0.207	0.313	-0.003	
	Cubic	0.914	573.363	3	161	0.001	-7.830	1.404	-0.047	0.001
	Compound	0.791	615.495	1	163	0.001	2.781	1.030		
	Power	0.862	1014.210	1	163	0.001	0.565	0.744		
	S	0.884	1244.513	1	163	0.001	2.516	-15.894		
	Growth	0.791	615.495	1	163	0.001	1.023	0.029		
	Exponential	0.791	615.495	1	163	0.001	2.781	0.029		

Table 3. *Models for estimating the consumer prices of chicken meat by different regression equations for the consumer prices of mutton*

Equation		Model Estimates					Coefficients Estimates			
		R^2	F	sd1	sd2	P	Constant	b1	b2	b3
CONSUMER PRICES OF MUTTON	Linear	0.886	1265.337	1	163	0.001	2.071	0.169		
	Logarithmic	0.891	1325.864	1	163	0.001	-5.623	3.819		
	Inverse	0.835	823.157	1	163	0.001	9.741	-70.229		
	Quadratic	0.897	708.898	2	162	0.001	1.163	0.251	-0.002	
	Cubic	0.899	478.607	3	161	0.001	0.048	0.406	-0.008	7.155E-05
	Compound	0.808	684.750	1	163	0.001	2.901	1.029		
	Power	0.873	1115.996	1	163	0.001	0.721	0.678		
	S	0.868	1074.325	1	163	0.001	2.421	-12.847		
	Growth	0.808	684.750	1	163	0.001	1.065	0.029		
	Exponential	0.808	684.750	1	163	0.001	2.901	0.029		

$$\text{Price of Chicken Meat} = -7.83 + 1.40 * x_i - 0.047\, x_i^2 + 0.00055\, x_i^3 \quad \text{(for price of beef)} \quad (1)$$

In estimating the consumer prices of chicken meat using the consumer prices of mutton, the cubic regression equation (2) gives accurate estimations with a percentage of variance explained of 89.9%. It was estimated that a 1 unit increase in the consumer prices of mutton would result in a 0.39 unit increase in the consumer prices of chicken meat according to the cubic regression model.

$$\text{Price of Chicken Meat} = 0.48 + 0.41 * x_i - 0.0076\, x_i^2 + 0.0000072\, x_i^3 \quad \text{(for price of mutton)} \quad (2)$$

As is seen in *Table 1* and *Table 2*, there is a statistically significant relationship between the consumer prices of beef and mutton and the consumer prices of chicken meat. However, the relationship of chicken meat prices with the beef prices is more significant, albeit slightly.

The results of the analyses conducted to relationship between the consumer prices of beef and the consumer prices of chicken meat (TRY/kg) and relationship between the consumer prices of mutton and the consumer prices of chicken meat (TRY/kg) are given in *Fig. 1* and *Fig. 2* below.

The results of the analyses conducted to identify the relationship between the consumer prices of chicken meat and the consumer prices of beef using CRT (Classification And Regression Tree) algorithm are given in *Fig. 3* below.

According to the CRT (Classification And Regression Tree) algorithm for the consumer price of chicken meat, the consumer price of beef affects the price of chicken meat significantly in the first step, and can be divided into two categories as ≤23.234 TRY and >23.235 TRY. It is 57.6% more effective to estimate the consumer price of chicken meat when the consumer price of beef is less than or equal to 23.234 TRY and 42.4% more effective to estimate the consumer price of chicken meat when the consumer price of beef is above 23.235 TRY.

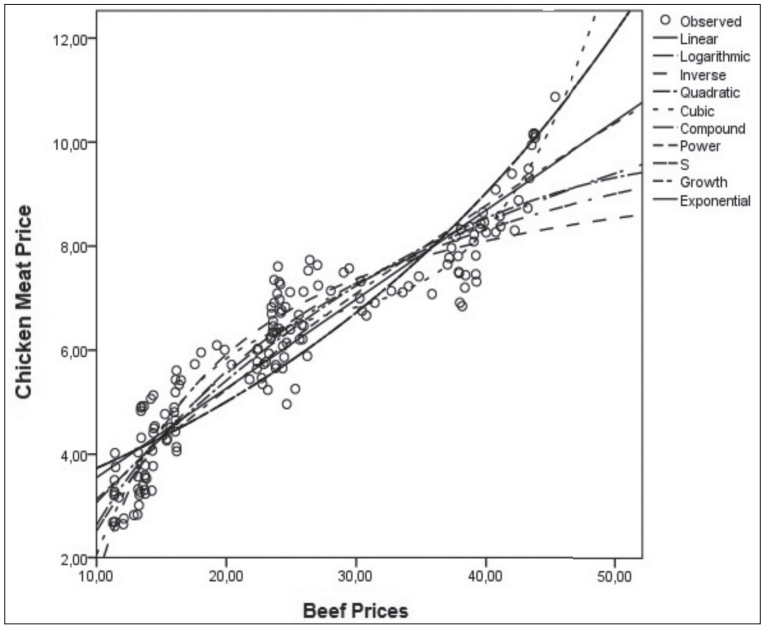

Fig 1. Relationship between the consumer prices of beef and the consumer prices of chicken meat (TRY/kg)

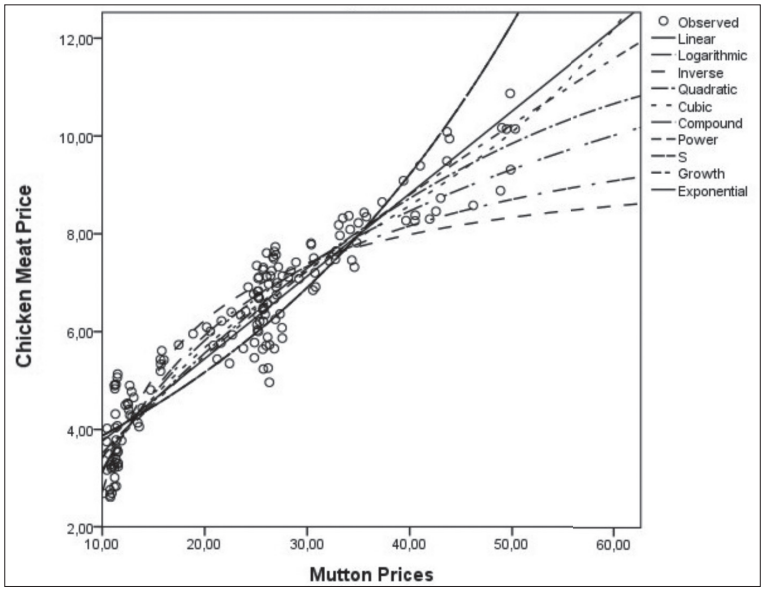

Fig 2. Relationship between the consumer prices of mutton and the consumer prices of chicken meat (TRY/kg)

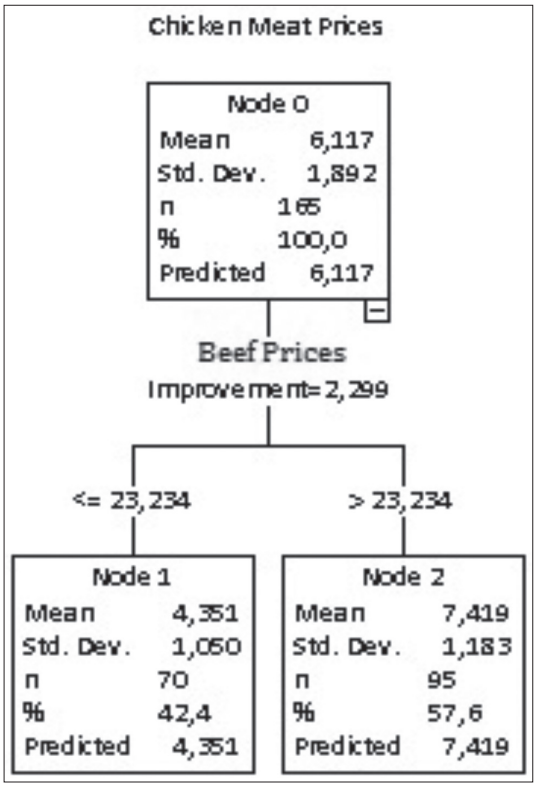

Fig 3. CRT algorithm for consumer price of chicken meat (TRY/kg)

DISCUSSION

This study is to present how the increases in the price of red meat are reflected in the consumer prices of chicken meat and to determine the estimation rate of the consumer prices of chicken meat. Formation of the prices of chicken and red meat is affected by many factors. They include production costs, feed amount and prices, natural and seasonal conditions, prices of substitute goods, consumer income and preferences, and purchasing power. These factors are dominant in the formation of retail prices of chicken meats [8,37]. Review of the main factors affecting the price of chicken meat is important to understand the issue. The levels of the effect of the cost items constituting the production cost and of external factors on the formation of prices differ.

In Turkey, production costs are the most important factors affecting the market price of chicken meat [9]. A study reports that a 10 percent increase in the price of soybean used in the poultry sector results in a 3.84 percent increase in the price of chicken meat in Turkey, and that the price of chicken meat and the prices of electricity and soybean are in an equilibrium relationship [7]. A study concerning the prices of chicken meat reported that a 50 percent decrease in the price of corn in China resulted in a 32 percent decrease in the price of chicken, whereas a 50 percent decrease in the price of soy flour resulted in a 16 percent decrease in the price of chicken [38]. It is understood that chicken meat prices decreased by 68% and feed prices decreased by 24% between the years 1990 and 2004. in the same study, it was shown that the feed was due to import origin [9].

A correlation analysis was performed with the aim of determining the level of relation between real whole chicken prices and feed prices in 1994-2006 period and the correlation coefficient was computed as ($r = + 0.731$ and $P<0.05$). This result demonstrates that, in the pricing of chicken meat, other factors are effective besides the feed input cost [8].

According to the regression model (r^2:0.93) established for the demand for chicken meat between 1983 and 1998, 1% increase in the price of chicken meat reduced the demand of 0.030%, 1% increase in beef meat increased the demand of 0.001% and 1% increase in the price of mutton meat increased the demand of 0.000002% [39].

Apart from the production costs, another factor that affects the prices of chicken meat is the presence of substitute goods. Substitute goods may affect the consumer demand for each other according to their quality and price. A study reports that the consumer prices of mutton affect the consumption of chicken meat [39]. It is reported that the price of chicken meat and people's level of income increase the consumption of beef [1]. Another study focusing on the increases in the prices red meat and chicken meat reported that the need for animal protein was met by consuming cheaper products, and that the increase in the consumption of chicken meat stemmed from the increases in the price of red meat [18].

In a study delving into the effects of taxes and the price of beef on the consumption of chicken meat in Turkey with a partial equilibrium approach, the tax elasticity was calculated to be -0.23, the price elasticity of demand for chicken meat 0.37, the income elasticity of demand for chicken meat 0.95 and chicken meat/beef elasticity -0.81 [16].

In another study aimed at identifying the factors that affect the consumption of red meat and chicken meat in 2.690 households living in provincial centers in Turkey using an ideal demand analysis model, the expenditure elasticities of chicken meat and beef were calculated to be 0.9394 and 0.8691, respectively [17].

The products with the highest income elasticity of demand were found to be goat meat (0.53), mutton (0.48) and beef (0.32), whereas the income elasticities of demand for chicken and fish were calculated to be 0.08 and 0.11, respectively. Additionally, the price elasticities of demand for chicken and fish were found to be -0.32 and -0.20, respectively [40].

A 1% increase in the beef price would decrease beef consumption by approximately 7.6% in the long run. A 1% change in the price of chicken meat will result in a long run change of approximately 4.2% in beef consumption [1]. In same study, In the long run, beef consumption, chicken meat prices, and income level contribute to beef prices by 18.95%, 13%, and 0.19%, respectively. The results also indicate that a 30.5% portion of chicken meat prices is explained by its own innovations whereas the contributions of beef consumption, beef prices, and the per capita income level are 4.17%, 64.44%, and 0.87%, respectively.

It was estimated that a 1 unit increase in the consumer prices of beef would result in a 1.35 unit increase in the consumer prices of chicken meat according to the cubic regression model. It was estimated that a 1 unit increase in the consumer prices of mutton would result in a 0.39 unit increase in the consumer prices of chicken meat according to the cubic regression model.

The increase in beef prices has lead Turkish consumers to shift from beef to chicken meat [1]. According to one study, it was stated that the reason for the preference of chicken meat was its suitable price compared to other meat [41]. The results of the study indicate that in estimating the consumer prices of chicken meat using the consumer prices of beef and mutton, the cubic regression equation has a high explained variance, suggesting that it is a good model to make a successful estimation. It can be said that the consumer prices of beef and mutton affect the consumer prices of chicken meat, and that the effect of the consumer price of beef on the consumer price of chicken meat is higher than that of the consumer price of mutton.

The main reason why the effect of the consumer price of beef is higher than that of the consumer price of mutton is that the output and the marketing opportunities of beef are higher. Furthermore, consumer habits and health reasons should be noted that the consumer is removed from the mutton. It can be said that the consumer prices of beef affect the consumer prices of chicken meat according to the decision tree algorithm.

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Research Article

The Correlation Between Anti-Müllerian Hormone Concentrations and Reproductive Parameters in Different Age Groups in Purebred Arabian Mares

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Abstract

The objectives of this study were to examine correlations of serum anti-Müllerian hormone (AMH) levels with age, ovarian function and fertility performance in purebred Arabian mares and to assess the potential use of AMH concentrations as a fertility indicator for the selection of breeder animals. For this purpose, thirty-six non-lactating purebred Arabian mares with no previous fertility problems constituted the material of the study. The animals were assigned to groups according to their age: Group I (aged 4-8 years), group II (9-18 years) and group III (19-25 years). Mean serum AMH concentrations were significantly higher (P<0.001) in group II (0.873±0.096 ng/mL) than in groups I (0.466±0.051 ng/mL) and III (0.347±0.068 ng/mL). Furthermore, serum AMH concentration was positively correlated with the daily increase in ovarian follicle diameter as well with mares with higher conception rates. It was also negatively correlated with anovulation and number of mating per conception. In summary, a strong relationship was found between peripheral AMH concentrations and fertility performance in purebred Arabian mares.

Keywords: *Mare, Anti-Müllerian hormone, Age, Reproductive performance*

Farklı Yaş Guruplarındaki Safkan Arap Kısraklarda Anti Müllerian Hormon Konsantrasyonları İle Reprodüktif Parametrelerin Korelasyonu

Öz

Bu çalışmanın amacı, safkan Arap kısraklarda serum anti-Müllerian hormon (AMH) seviyelerinin yaş, ovaryum fonksiyonları ve fertilite performansı ile korelasyonlarını incelemek ve AMH konsantrasyonlarının damızlık hayvan seçimi için fertilite göstergesi olarak potansiyel kullanımını değerlendirmektir. Bu amaçla çalışmanın materyalini herhangi bir fertilite problemi bulunmayan ve laktasyonda olmayan 36 Safkan Arap kısrak oluşturdu. Hayvanlar yaşlarına göre 3 gruba ayrıldı: Grup I (4-8 yaş), grup II (9-18 yaş) ve grup III (19- 25 yaş). Ortalama serum AMH konsantrasyonları, grup II'de (0.873±0.096 ng/mL) grup I (0.466±0.051 ng/mL) ve III'e (0.347±0.068 ng/mL) göre anlamlı derecede yüksek bulundu (P<0.001). Kısraklarda serum AMH konsantrasyonunun, günlük ovaryum folikül çap artışıyla ve gebelik oranları ile pozitif korelasyon gösterirken anovulasyon ve gebelik başına düşen aşım sayısı ile negatif korelasyon gösterdiği görüldü. Özetle, safkan Arap kısraklarda periferik AMH konsantrasyonları ile fertilite performansı arasında güçlü bir ilişki tespit edildi.

Anahtar sözcükler: *Kısrak, Anti-Müllerian hormon, Yaş, Reprodüktif performans*

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INTRODUCTION

The anti-Müllerian hormone (AMH) is a homodimeric glycoprotein belonging to the transforming growth factor beta family and is secreted only from the gonads in both males and females [1,2]. In addition to causing regression of the Müllerian ducts during embryonic development, it is also involved in regulating development of primordial follicles [3-5]. AMH contributes to maintenance of oocyte reserves by reducing the sensitivity of developing follicles to follicle-stimulating hormone (FSH) and thereby limiting the number of actively developing follicles [3,6]. Measuring the AMH level has a particularly wide range of uses in women, including determination of the ovarian reserve, which is the number of functional follicles, monitoring the transition into the menopausal period, diagnosis of polycystic ovarian syndrome and granulosa cell tumours and determination of low ovarian response and prognosis in *in vitro* fertilization applications [2]. Furthermore, it has been reported that AMH can be also used for the diagnosis of ovarian remnant syndrome in bitches [7,8] and granulosa cell tumours in mares [3,9,10] as well as a fertility indicator in ewes [11], cows [12] and bitches [13].

In mares, AMH is secreted by the granulosa cells of preantral and small antral follicles [3,14] and its plasma concentration was found to be positively correlated with the number of follicles having a diameter of 6-20 mm [14]. AMH is secreted at a lower level by follicles with a diameter greater than 30 mm [3] and its plasma concentration increases with higher numbers of developing follicles [15]. Although serum AMH concentrations may show individual variations among mares, these concentrations reportedly do not vary with the different stages of the estrous cycle or pregnancy [3,9].

Anti-Müllerian hormone concentrations decrease with the ageing process in mice [16], bitches [13] and women [17]. In older mares in which the follicular stage is prolonged, the occurrence of ovulation-related problems is significantly increased [18]. It has been reported that the number of antral follicles were decreased in senile mares (>19 years old), which causes plasma AMH concentrations to be lower than those of middle-aged mares (aged 9-18 years) [14]. To date, although there are some studies that investigated the influence of AMH on fertility performance in mares, the correlations between reproductive parameters and AMH levels and age are not yet clearly understood [14,19,20].

The aim of this study was to examine the correlation of serum AMH concentrations with age and certain reproductive parameters during the spring transition and in particular, to investigate the feasibility of using AMH values as a fertility indicator for breeder animal selection in purebred Arabian mares, which have a relatively short breeding period and are of very high economic value in the northern hemisphere.

MATERIAL and METHODS

Ethics

In this study, the Ethics Committee report (Approval number: 2016/20) was received before application in accordance with the directions of the Dollvet Inc. Experimental Animals Local Ethics Board.

Animals

The subjects of this study were 36 non-lactating Purebred Arabian mares, which were assessed as not conceiving in the previous breeding season. These mares were referred to the Animal Hospital of Harran University, Faculty of Veterinary Medicine between February 10-June 30, 2017 and were confirmed as having no apparent fertility problems upon gynecological clinical examination (ultrasonographic examination of uterus and ovaries and inspection of cervix, vagina and vulva). The animals were assigned to three groups according to their age: Group I (young animals aged 4-8 years, n=12), group II (middle-aged animals aged 9-18 years, n=12) and group III (senile animals aged 19-25 years, n=12).

AMH Assay

Between the 10th and 15th of February, blood samples were taken from the *V. jugularis* into dry tubes and were allowed to clot. Once coagulated, the samples were centrifuged at 3000 rpm for 15 min. The sera were stored at -20°C until analyzed. Serum AMH levels were determined by the ELISA (DRG Instruments ELISA Mat 2000) using a commercial kit (Beckman Coulter, AMH Gen II, USA). All serum assays were performed in duplicate. The lower and upper limits of detection were 0.375 ng/mL and 150 ng/mL, respectively. The respective intra- and inter-assay coefficients of variations were <8% and <10%, respectively. The standard curve range was 0.07-22.5 ng/mL, and the limit of detection value of the ELISA kit was <0.1 ng/mL.

Ultrasonographic Examinations

During the breeding season (February 15-June 30), repeated daily ultrasonographic examinations (SIUI, CTS-800, linear probe, 5 MHZ, Guangdong, China) were made until ovulation occurred. These examinations provided information on the stages of the sexual cycle, daily development of ovarian follicles, single or multiple ovulation status and the presence of anovulatory follicles. Mares with a follicle diameter greater than 35 mm and showing a positive response to stallion teasing were mated every other day until ovulation with stallions known to have normal fertility parameters by andrological examination. In mares that were detected to have ovulated, the first pregnancy examinations were performed on the 15th day post-ovulation. Mares that were found to have conceived were further examined on days 30 and 45. The mares that displayed a healthy gestation at the third examination were considered to be definitively

Table 1. *Mean AMH concentrations (ng/mL) and reproductive outcomes of the mares*

Group	Age (year) (X±Sx)	AMH (ng/mL) (X±Sx)	DIFD (%) (X±Sx)	FDO (mm) (X±Sx)	NIMC (number) (X±Sx)	CR (%)
I	6.08±1.12	0.466±0.05[a]	2.52±0.14[a]	42.72±0.80	1.62±0.26	10/12 (83.3)[ab]
II	12±2.32	0.873±0.096[b]	2.89±0.29[a]	43.69±0.93	1.20±0.20	11/12 (91.67)[b]
III	20.84±2.78	0.347±0.068[a]	2.11±0.06[b]	44.12±0.65	2.00±0.29	5/12 (41.67)[a]
P value		0.001	0.002	0.779	0.168	0.013

AMH: *Anti-Müllerian hormone,* ***DIFD:*** *Mean daily increase in follicle diameter,* ***FDO:*** *Follicle diameter at the time of ovulation,* ***NIMC:*** *Number of mating per conception,* ***CR:*** *Conception rate, [a,b] Means with different superscripts in the same column show statistically significant differences*

pregnant. Throughout this period, mares that suffered embryonic and early fetal death were also recorded.

Statistical Analyses

Statistical analysis of the data was performed with the Statistical Package for the Social Sciences (SPSS for Windows; version 22.0, USA) program. The normal distribution of the variables and analytical methods were examined using visual (histogram and probability plots) and Kolmogorov-Smirnov/Shapiro-Wilk tests. Descriptive analyses were given as means±standard error for normally dispersed and normally non-dispersed variables. The homogeneity of variances was determined by the Levene test. The data were analyzed by a one-way ANOVA test because age, AMH and follicle diameter at the time of ovulation were normally distributed. These parameters were compared between the groups using the Kruskal-Wallis test because it was determined that the mean daily increase in follicle diameter and number of mating per conception did not show a normal distribution. Two-handed comparisons were made using the Mann-Whitney U test and assessed using the Bonferroni correction. P values <0.05 were considered as statistically significant results. Comparisons were done with the post-hoc Tukey test, when the difference between the groups was significant. Correlation coefficients and statistical significance were calculated by the Spearman test for correlations between AMH and some reproductive parameters that were not normally distributed or between AMH and ordinal variables. The normal distributions with AMH (age and follicle diameter at the time of ovulation) were calculated by Pearson's test. P value of <0.05 was used to establish statistical significance.

RESULTS

Serum AMH concentrations, mean daily increase in follicle diameter, follicle diameter at the time of ovulation, number of mating per conception and conception rate in the different study groups are shown in *Table 1*. The mean serum AMH concentrations were 0.466±0.051 ng/mL in the young mares (group I), 0.873±0.096 ng/mL in the middle-aged mares (group II) and 0.347±0.068 ng/mL in the senile mares (group III). The mean serum AMH concentration calculated for all the mares used in the study was 0.515±0.324 ng/mL. The differences in mean serum AMH concentrations between groups I and II (P<0.01) and between groups II and III (P<0.001) were statistically significant while the difference between groups I and III was insignificant (P>0.05). With respect to the daily follicle diameter increase among the groups, the difference between groups I and II was not significant, while the differences between groups I and III, and between groups II and III, were statistically significant. Anovulation and embryonic mortality rates for groups I, II and III were 1/12, 0/12, 2/12 and 2/12, 0/12, 2/12, respectively and there was no statistical significance among the groups.

Table 2. *Correlation of Anti-Müllerian Hormone values with age of the mares and reproductive outcomes*

Reproductive Outcomes	Anti-Müllerian Hormone	
	r	P
Age of mares	-0.243	0.153
Daily increase in follicle diameter	+0.463	0.013
Ovulated follicular diameter	-0.104	0.564
Multiple ovulation	-0.047	0.787
Anovulation	-0.440	0.007
Conception	+0.608	0.000
Number of mating per conceptions	-0.562	0.001
Embryonic death	-0.072	0.675

In the present study, it was ascertained that in purebred Arabian mares, serum AMH concentrations were significantly and positively (+) correlated with daily increase in follicle diameter (r=+0.463) and conception rate, and were negatively (-) correlated with the anovulation rate (r=-0.440), number of mating per conception (r=-0.562) and follicle diameter at the time of ovulation (r=-0.104) *(Table 2)*.

DISCUSSION

Almeida et al.[9] reported the mean serum AMH concentrations in cyclic, pregnant, and ovariectomized mares were 0.96±0.08 ng/mL, 0.72±0.05 ng/mL and 0.06±0.003 ng/mL, respectively. These authors reported that there were no differences between pregnant and cyclic mares or between the different stages of the estrous cycle for AMH concentration. Vernunft et al.[15] reported that plasma AMH concentrations ranged between 0.6±0.04 and 1.2±0.05

ng/mL, depending on the number of developing follicles in the ovaries. In the present study, the mean serum AMH concentrations measured in purebred Arabian mares were 0.466±0.051 ng/mL in young animals aged 4-8 years, 0.873±0.096 ng/mL in middle-aged animals aged 9-18 years, and 0.347±0.068 ng/mL in senile animals aged 19-26 years. The AMH concentrations measured in the different age groups in this study showed that the AMH levels of group II, composed of middle-aged mares, not only fell within the AMH range previously reported by Vernunft et al.[15], but also displayed similarity to the concentrations measured by Almedia et al.[9] in cyclic mares. The levels of AMH measured by both these groups being similar to the concentrations detected in middle-aged mares in the present study was attributed to these authors having used cyclic animals with no reproductive health problems. Furthermore, in this study the lowest number of mating per conception and the highest conception rate were also detected in group II which displayed AMH concentrations similar to those reported in previous studies. In addition, in the present study, the lowest conception rate and the highest number of mating per conception were detected in the senile mares in group III which had the lowest AMH concentrations. Claes et al.[14] reported the mean number of antral follicles as 13.6 in senile mares, 25.5 in young mares and 27.8 in middle-aged mares. In parallel with these numbers, these authors reported the mean plasma AMH concentrations as 0.21 ng/mL in senile mares, 0.47 ng/mL in middle-aged mares and 0.29 ng/mL in young mares. In the present study, similar to the results reported by Claes et al.[14], serum AMH levels were highest in the middle-aged mares and lowest in the senile mares. Furthermore, when assessed together with the results reported by Claes et al.[14], the lowest conception rate and the highest number of mating per conception in the senile mares, together with their reduced serum AMH levels, were considered to be also related to the reduced number of antral follicles found in these animals.

In the present study, the maximum follicle diameter at the time of ovulation was largest in the senile mares (group III) and was smallest in the young mares (group I). These data are in agreement with those reported by Ginther et al.[21]. Likewise, consistent with the results of Ginther et al.[21], in the present study, the daily increase in follicle diameter was found to be lowest in the senile mares and highest in the middle-aged mares. These findings support the hypothesis of Ball et al.[3], who proposed that AMH plays an important role in follicular recruitment and selection of the dominant follicle.

In this study, it was ascertained that the serum AMH concentrations measured during the spring transition period in mares were positively correlated with the daily increase in follicle diameter and conception rate in the breeding season and were negatively correlated with the anovulation rate and number of mating per conception. The lowest serum AMH concentrations were measured in the senile mares (group III). Furthermore, in that group the lowest daily increase in follicle diameter and conception rates were positively correlated with AMH concentrations; as well as with the highest anovulation rate and number of mating per conception, which were negatively correlated with AMH concentrations.

It was reported that, in parallel with reduced gonadal functions and ovarian reserves in older women, AMH concentrations were also decreased [17]. In females, the major source of AMH is the granulosa cells of early antral follicles [2,3]. Korkmaz et al.[13] indicated that the number of granulosa cells of preantral follicles shows a striking decrease with advanced age in bitches. Granulosa cells, which physically support oocyte development and provide the necessary micro-environment for this process, are capable of active differentiation [5]. In the present study, a decrease was observed in the AMH concentrations of purebred Arabian Mares with advanced age, which was associated with the increased number of mating per conception and anovulation rate and decreased conception rate, all of which may be attributed to possibly decreased numbers of preantral granulosa cells occurring with ageing. The lowest number of antral follicles detected in senile mares by Claes et al.[14] supports this suggestion. In a previous study [14], it was reported that there is a relationship between peripheral AMH concentrations and age which directly affects the fertility outcomes of mares. In contrast to the report by Ball et al.[3] and Hanlon et al.[20] declared that there was no relationship between AMH and fertility in mares. In contrast, this relationship was seen clearly in our study with purebred Arabian Mares: when they were allocated to young, middle and older ages, peripheral AMH concentrations were correlated with fertility outcomes.

In conclusion, serum AMH concentrations were highest in middle-aged mares (aged 9-18 years) and lowest in senile mares (aged 19-26 years). According to our results, AMH is positively correlated with the daily increase in follicle diameter, follicle diameter at the time of ovulation and conception rates in mares. It was also negatively correlated with the number of mating per conception. Therefore, AMH is a reliable parameter for selection of Purebred Arabian Mares undergoing breeding and routine reproductive examinations as an indicator of their fertility.

Declaration of Conflicting Interests

The authors(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Research Article

The Effect of Oregano Oil *(Origanum vulgare)* on the Fattening Performance and Blood Oxidant-Antioxidant Balance in Post-weaned Tuj Lambs

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Abstract

The purpose of this study is to investigate the effect of oregano oil (Origanum vulgare) at on fattening performance and blood oxidant-antioxidant balance such as glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GPx) catalase (CAT) enzyme activities, malondialdehyde, ceruloplasmin, nitric oxide, albumin, total protein and globulin in post-weaned Tuj breed lambs. The lambs were average 155 days old, and were divided into 3 groups regardless of gender, and in total 18 lambs were used. In the study the control group (C) was fed with basal ration; while the experimental groups were orally fed with oregano oil supplementation as a commercial product in daily doses of 1 mL/lamb/day (T1) and 2 mL/lamb/day (T2), respectively, in addition to the basal ration, via sterile injections. The experiment was maintained for a total of 52 days of which 10 days were adaptation period. In the study, it has been seen in post-weaned Tuj breed lambs that using different doses of oregano oil had no effect on live weight gain, daily live weight gain, daily feed consumption, and feed conversion ratio (P>0.05). In the study, it was determined that the addition of oregano oil to the ration made no significant difference on fattening performance and MDA, GSH, CAT, ceruloplasmin, nitric oxide, albumin, total protein and globulin values of blood oxidant-antioxidant parameters. SOD and GPx values were significantly affected by the addition of oregano oil to the lamb rations at the point of examining the effect on blood oxidant-antioxidant balance (P<0.05). As a result, it has been determined that oregano oil did not affect the performance parameters, it significantly affected the oxidant-antioxidant balance in lambs.

Keywords: *Oxidant-antioxidant balance, Tuj, Lamb, Performance, Oregano oil*

Sütten Kesilmiş Tuj Irkı Kuzularda Kekik Yağı *(Origanum vulgare)* Kullanımın Besi Performansı ve Kan Oksidan-Antioksidan Denge Üzerine Etkisi

Öz

Bu çalışmanın amacı sütten kesilmiş Tuj ırkı kuzularda kekik yağının performans ve glutatyon (GSH), süperoksit dismutaz (SOD) oranları, glutatyon peroksidaz (GPx) katalaz (CAT) enzim aktiviteleri, seruloplazmin, nitrik oksit, albumin, total protein ve globulin kan oksidan-antioksidan denge üzerine etkisinin araştırılmasıdır. Kuzular ortalama 155 günlük yaşta olup cinsiyet gözetilmeksizin 3 gruba ayrılmış ve toplamda 18 kuzu kullanılmıştır. Çalışmada kontrol grubu (K) bazal rasyonla beslenirken deneme gruplarına bazal rasyona ek olarak sırasıyla 1 mL/kuzu/gün (T1) ve 2 mL/kuzu/gün (T2) dozlarında ticari ürün olan kekik yağı ilavesi steril şırıngalar ile hayvanlara günlük olarak oral şekilde verilmiştir. Deneme, 10 gün adaptasyon dönemi olmak üzere toplam 52 gün sürdürülmüştür. Çalışmada kesilmiş Tuj ırkı kuzularda kekik yağının farklı dozlarda kullanımının canlı ağırlık artışı, günlük canlı ağırlık artışı, günlük yem tüketimi ve yemden yararlanma oranını etkilemediği görülmüştür (P>0.05). Çalışmada rasyona kekik yağı katılmasının, besi performansı ve kan oksidan antioksidan parametrelerinden MDA, NO, GSH, CAT, seruloplazmin, albümin, total protein ve globulin değerlerinin üzerinde önemli bir farklılık oluşturmadığı saptanmıştır. Kan oksidan antioksidan denge üzerine etkinin irdelenmesi noktasında SOD ve GPx değerleri kuzu rasyonlarında kekik yağı ilavesinden anlamlı düzeyde etkilenmiştir (P<0.05). Sonuç olarak kekik yağının kuzularda performans parametrelerini etkilemediği, kan oksidan-antioksidan denge üzerine olumlu etkilerinin olduğu tespit edilmiştir.

Anahtar sözcükler: *Oksidan-antioksidan denge, Tuj, Kuzu, Performans, Kekik yağı*

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INTRODUCTION

Because the antibiotics and the antimicrobial agents, used in animal feeding for many years as growth stimulants, have given rise to the development of resistance in pathogenic bacteria in humans, and due to the residues they can generate in animal products, the demands for such products is decreasing day by day [1,2]. In recent years, with the prominence of consumer demand in terms of product characteristics, the desire to obtain more natural and more suitable products for human health has made the field of animal feeding with new searches a current issue, and many of the researchers have focused their attention on natural and aromatic plants, and essential oils obtained from them [3,4]. Oregano is a common term for the plant family *Lamiaceae*, which has more than 60 species, and known by their general aroma and taste [5]. Among these species *Thymus, Origanum, Satureja, Thymbra* and *Coridothymus* are of great importance both prevalently and economically [6]. Turkey is the country with the world's most important exporter thyme. A large part of the thyme exported from Turkey is *Origanum* species [7]. Both oregano itself and its extracts (oregano essential oil) contain substances that induce such as antiseptic, antioxidant, antimicrobial and aroma-regulating effects. It has been reported that oregano contains *phenols, thymo, carvacrol, monoterpene hydrocarbons, p-cymene and γ-terpinene* [8]. Oregano is rich in *carvacrol*, and at a lesser extent in *phenolic monoterpenoids* (particularly thymol) [9].

The short-lived reactive atoms and molecules with un-conjugated electrons in their outer orbitals are known as free radicals and the enzymatic and non-enzymatic structures trying to prevent radicals and their reactions are defined as antioxidants [10]. In normal functioning cells, there is a balance between the free radicals produced as a by-product of cell function and the antioxidants which inactivate them, and is described as an oxidant-antioxidant balance. Oxidative stress occurs when there is a free radical production in the cells that overcomes the antioxidant defense system; leads to protein and DNA oxidation, and lipid peroxidation in cells; and consequently causes cell destruction and tissue damage [10]. Therefore, studies on the potential antioxidant effects of plant extracts have accelerated especially in recent years. Farag et al.[11] examined the relationship between the chemical composition and antioxidant properties of essential oils, and stated that due to the presence of phenolic OH groups acting as a hydrogen donor to the peroxide radicals released during the first step of the lipid oxidation, thymol owns high antioxidant activity by reducing the formation of hydroxy peroxide.

In recent years, several studies focused on the aromatic plants and their extracts. Oregano oil has antioxidant properties. Therefore, oregano oil has been used in many studies because of its antioxidant properties. Turkey is very important in the world in the export of oregano. Oregano oil used in our study were obtained from the Mediterranean region of Turkey. There are many studies in the literature on the use of oregano in lambs. However, no study was seen in the literature about the use of oregano oil in Tuj breed lambs. Therefore, the aim of this study is to investigate the effect of oregano oil on fattening performance and blood oxidant-antioxidant balance such as glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GPx) catalase (CAT) enzyme activities, malondialdehyde (MDA), ceruloplasmin, nitric oxide (NO), albumin, total protein and globulin in post-weaned Tuj breed lambs.

MATERIAL and METHODS

Animals, Experimental Design and Feed

This study was carried out with the permission of the Kafkas University Animal Experiments Local Ethics Committee (Decision No: KAU-HAYDEK/2018-086) report. In the study, Tuj breed lambs, being raised in Kars, Ardahan and Igdir provinces, which is a local sheep breed in Turkey were used as an animal material. The average age of the lambs was 155 days. Lambs were divided into 3 groups, regardless of gender, with a mean live weight of 34.75±0.22 kg, and a total of 18 lambs were used. The Experiment was conducted in Kafkas University Faculty of Veterinary Medicine, Prof. Dr. Ali Riza AKSOY Training, Research and Implementation Farm. The experiment was maintained for a total of 52 days of which 10 days were adaptation period. During the study, the animals were housed in individual boxes (180 cm × 150 cm × 120 cm; height, length, width, respectively) equipped with feeders dispensing pasture grass and concentrate feed separately. During the study, the animals were fed with pasture grass and lamb grower feed.

In the study; dry matter, energy and other nutrient requirements of animals were calculated according to NRC [12] standards. The daily amount of concentrate feed provided to the lambs was 600 g per animal. Pasture grass and water was supplied *ad libitum* during the trial. Animals were fed mainly with roughage. The content of the concentrated feed is given in *Table 1*. In adaptation period that lasted 10 days lambs got accustomed to the experimental diet. All lambs were treated against internal and external parasites. The study was conducted in Kars. Kars is a place where important pastures are at for Turkey. Tuj lambs used in the study are a region-specific race. Tuj lambs are fed with pasture-based feed in the region. For this reason, in our study, while feeding tuj lambs with the pasture *ad libitum,* 600 g of concentrated feed were given daily. In this wise, the effect of using oregano oil based on pasture feeding method on performance and blood oxidant and antioxidant balance was investigated.

In the study, control group (C) was fed with basal ration. In addition to basal ration, the experimental groups were fed with a supplementation of oregano oil *(Origanum vulgare,* BOTALIFE®), a commercial product at doses of 1 mL/lamb/

Table 1. *Ingredient composition of concentrate feed*

Ingredients	Amount, %
Wheat	3.75
Barley	17.5
Corn	12.5
Soya bean meal	2.5
DDGS	2.5
Sunflower seed meal (28-30 CP)	2.5
Sunflower seed meal (36 CP)	2.5
Safflower meal	6.25
Cotton seed meal (28-30 CP)	4.6
Wheat bran	20
Corn bran	6.3
Leaf cotton (25 CP)	6.25
Molasses	9
Marble powder	3.25
Salt	0.5
Vitamin mineral premix	0.1

[1] CP: Crude Protei
[2] DDGS: Dried Distillers Grains with Solubles
[3] The vitamin & mineral premix provided the following (per kg): 4.000.000 IU Vit. A, 800.000 IU Vit. D_3, 5.000 IU Vit. E, 400 mg Vit. B_2, 2 mg Vit. B_{12}, 5.000 mg Vit. PP, 1.000 mg D-pantothenic acid, 20.000 mg choline, 50 mg Co, 5.400 mg Fe, 185 mg I, 6.900 mg Mn, 800 mg Cu, 6.400 mg Zn, 14 mg Se

day (T1) and 2 mL/lamb/day (T2), respectively. Oregano oil was given orally by sterile syringes to the experimental groups daily. Oregano oil used in the study, *Origanum vulgare* belong to the species of plants are grown in Turkey. Oregano oil used in the study contains 4.15% thymol and 75.3% carvacrol. The essential oil acid profile of the commercial oregano oil is presented in *Table 2*.

Feed Analysis

Nutrient analysis of the feed was determined according to the method reported in AOAC [13]; while NDF (Neutral Detergent Fiber) and ADF (Acid Detergent Fiber) analyses for pasture grass were determined according to the method reported by Goering and Van Soest [14].

Determination of Performance Parameters

Animals were weighed before morning feeding in the beginning and on the 0th, 10th, 31st and 52nd days of the trial. At the end of the trial, daily feed consumption and feed conversion ratios of each group were calculated. Feed convertion ratio was calculated as the proportion of daily feed consumption to daily weight gain (kg/kg).

Biochemical Analyses

On the 10th and 52nd days during the experimental period, after separating a fair amount of the blood samples, taken from the *V. jugularis* of the animals with anticoagulant (EDTA) tubes, as whole blood, plasma of the remaining

Tablo 2. *The essential oil acid profie of the commercial oregano oil (%)*

Ingredient	%	Ingredient	%
α-*pinene*	0.29	spathulenol	0.37
α-*thujene*	0.30	b-caryophyllene oxide	1.01
α-terpinene	0.66	carvone	0.25
b-myrcene	0.79	α -*terpineol*	0.32
α-*fellanderen*	0.30	borneol	0.99
g-terpinen	2.39	b-bisabolen	2.08
p-simen	2.89	timol	4.15
linalyl acetate	0.35	carvacrol	75.30
linalool	6.36		

blood was obtained. Samples taken were centrifuged at 3000 rpm for 15 min, and stored at -20°C until the analyses were carried out.

Superoxide dismutase, GPx and CAT antioxidant enzyme activities in plasma were determined by ELISA device (Epoch, Biotek, USA) using commercial kits (Cayman Chemical Co., USA). Whole blood reduced glutathione (GSH) analysis was determined colorimetrically (Epoch, Biotek, USA) according to the method issued by Beutler et al.[15]; while MDA in plasma by Yoshoiko et al.[16], NO by Miranda et al.[17], ceruloplasmin by Colombo and Ricterich [18], and albumin and total protein levels by commercial test kit (Biolabo, France). The globulin was determined by subtraction of the albumin from the total protein according to Doumas et al.[19].

Statistical Analysis

For the significance of the differences between the statistical calculations belonging to the groups and the mean values of the groups, one-way analysis of variance (ANOVA) method was used, and for the significance control of the difference between the groups, the appropriate next stage test (Tukey) was applied. For this purpose, SPSS packaged software was used [20].

RESULTS

The amounts of nutrients and metabolizable energy values of concentrated feed and pasture grass are shown in *Table 3*. The performance parameters of the study are given in *Table 4*. In the study, statistically significant difference between the groups regarding average live weights, live weight gains, daily live weight gains, daily feed consumption and feed conversion ratio was not found (P>0.05). It has been observed that for the oregano oil use in post-weaned Tuj lambs, the difference between the groups, in terms of the performance parameters, was not statistically significant (P>0.05). It has been observed that the addition of oregano oil to lamb rations does not affect the performance parameters, but improves it.

Blood parameters of the study are given in *Table 5*. The differences between the groups on MDA, NO, GSH, CAT,

Table 3. *Nutrient and energy* levels of the feeds (%)*

Feeds	DM	CP	EE	CF	CA	Calcium	Phosphorus	ADF	NDF	ME
Concentrate	87.36	16.25	2.36	11.03	7.99	0.64	0.42	-	-	2531
Pasture grass	92.1	9.13	-	-	7.6	0.65	0.16	38.70	62.60	1767

***DM:** Dry matter, **CP:** Crude protein, **EE:** Ether extract, **CF:** Crude fiber, **CA:** Crude ash; **ADF:** Acid detergent fiber, **NDF:** Neutral detergent fiber, **ME*:** Metabolic Energy (kcal/kg)*

Table 4. *The effect of oregano oil on the live weight, live weight gain, daily live weight gain, daily feed consumptions, feed consumptions and feed conversion ratio of lambs*

Fattening Performance Parameters	Control		T1 1 mL/day/lamb		T2 1 mL/day/lamb		Significance
	X	Sx	X	Sx	X	Sx	P
Live Weight, kg Days							
0	34.58	0.23	34.83	0.42	34.83	0.52	0.886
10	35.81	0.25	36.13	0.34	36.06	0.47	0.814
31	38.18	0.2	38.7	0.23	38.93	0.34	0.160
52	41.05	0.07	41.4	0.33	41.65	0.44	0.441
Live Weight Gain, kg, Days							
0-10	1.23	0.12	1.3	0.11	1.23	0.12	0.889
10-31	2.36	0.08	2.56	0.19	2.86	0.24	0.195
31-52	2.86	0.23	2.7	0.18	2.71	0.18	0.818
0-52	6.46	0.25	6.56	0.37	6.81	0.48	0.805
Daily Live Weight Gain, g Days							
0-10	123.33	12.01	130	11.25	123.33	12.01	0.889
10-31	112.7	3.82	122.22	9.25	136.51	11.64	0.195
31-52	136.5	11.24	128.57	8.60	129.36	8.98	0.818
0-52	124.39	4.98	126.28	7.24	131.09	9.29	0.805
Daily Feed Consumption (dry matter), g Days							
0-52	1511.77	43.56	1448.30	101.34	1441.01	97.95	0.814
Feed Convertion Ratio Days							
0-52	12.32	0.84	11.91	1.56	11.46	1.37	0.898

***K:** Control, **T1:** 1mL/day/lamb oregano oil, **T2:** 2 mL/day/lamb oregano oil. Statistically not significant (P>0.05). All values are given as mean ± standard error of mean (SEM), (n=6)*

ceruloplasmin, albumin, total protein and globulin values of oregano oil use in weaned Tuj lambs were not statistically significant (P>0.05). According to the results, in terms of SOD and GPx, the use of oregano oil in post-weaned Tuj breed lambs was found to be statistically significant (P<0.05).

DISCUSSION

The use of aromatic plants and extracts in ruminant breeding is increasing each passing day. It has been reported that essential oils stimulate the digestive system of animals and increase the efficiency of digestive secretions [21]. Due to these features, essential oils have a positive effect on performance parameters. In the study conducted, it was observed that the use of oregano oil in different doses in post-weaned Tuj lambs did not affect live weight gain, daily live weight gain, daily feed consumption, and feed conversion rate. There are many current studies using thyme and other plant extracts that support the results of our study [22-26]. In another study, it has been reported that the use of *cinnamic aldehyde* or *carvacrol* (200 mg/kg diet) does not affect dry matter consumption, live weight gain, and feed conversion ratio parameters [27]. In contrast to the results obtained, it was reported in a different study that the use of oregano oil in lamb feeding did not affect the feed conversion rate, and that the daily feed consumption and daily live weight gain were higher [28]. There are also studies reporting that the use of plant extracts in lambs has positive effects on dry matter consumption, live weight gain and feed conversion parameters [29-31].

The observed differences between the results are considered to be affiliated with the factors such as: plant species from which the oregano oil is obtained, oregano oil dose, the *carvacrol* and *thymol* ratio in oregano oil, the composition of the ration, the roughage-weighted feeding method, race

Table 5. *The effect of oregano oil on MDA, NO, GSH, CAT, SOD, GPx, ceruloplasmin, albumin, total protein and globulin*

Oxidant-Antioxidant Parameters	Days	Control		T1		T2		Significance
		X	Sx	X	Sx	X	Sx	P
MDA (µmol/L)	10	2.70	0.04	2.75	0.03	2.77	0.04	0.479
	52	2.69	0.04	2.73	0.02	2.76	0.03	0.407
NO (µmol/L)	10	12.24	0.94	12.32	1.30	12.75	1.14	0.948
	52	12.48	1.10	12.93	1.01	12.97	0.97	0.934
GSH (mg/dL)	10	38.21	1.93	38.16	2.14	38.24	1.95	1.000
	52	37.84	1.67	37.51	1.89	39.80	2.89	0.736
SOD (U/mL)	10	1.06^{c}	0.07	1.72^{b}	0.07	2.40^{a}	0.08	**0.000***
	52	1.02^{c}	0.07	1.70^{b}	0.07	2.27^{a}	0.02	**0.000***
CAT (nmol/min/mL)	10	28.07	1.39	32.23	1.55	32.42	2.05	0.156
	52	27.80	0.89	30.36	1.18	31.81	1.35	0.077
GPx (nmol/min/mL)	10	297.31^{b}	8.47	311.08^{ab}	4.22	32.54^{a}	6.00	**0.046***
	52	294.81^{b}	6.04	320.97^{ab}	7.10	321.72^{a}	7.41	**0.022***
Ceruloplasmin (mg/dL)	10	21.75	1.46	22.12	1.54	19.48	1.40	0.413
	52	19.57	0.9	18	1.02	18.14	1.08	0.488
Albumin (g/dL)	10	2.9	0.06	2.91	0.11	2.94	0.08	0.944
	52	2.87	0.07	2.95	0.08	2.87	0.06	0.709
Total protein (g/dL)	10	7.15	0.05	7.06	0.08	7.19	0.05	0.404
	52	7.18	0.10	7.16	0.13	7.20	0.06	0.952
Globulin (g/dL)	10	4.25	0.1	4.15	0.16	4.24	0.07	0.819
	52	4.31	0.16	4.21	0.14	4.33	0.07	0.789

K: *Control,* ***T1:*** *1 mL/day/lamb oregano oil,* ***T2:*** *2 mL/day/lamb oregano oil. Statistically not significant (P>0.05). All values are given as mean ± standard error of mean (SEM), (n=6);* ^{a,b,c} *The differences between the mean values with a different letter in the same row were statistically significant (P<0.05) * P<0.001*

of the animal, oregano oil's administration route to the animal.

Oxidation events occur constantly in the metabolism of a living being, and reactive oxygen substances, taken from outside, accelerate these oxidation events. Antioxidants are substances that prevent autoxidation/peroxidation progress by reacting with the radicals very quickly [32]. Endogenic antioxidants are non-enzymatic antioxidants composed of glutathione, albumin and ceruloplasmin, and enzymatic antioxidants that form the SOD, CAT, GPx and glutathione reductase enzymatic defense line [33,34]. The SOD enzyme is the first line of the antioxidant defense system. It plays a critical role in eliminating superoxide radicals [35]. Glutathione peroxidase is found in the cytoplasm of cells, and protects cells against oxidative damage caused by H_2O_2 [36]. In the present study, it was observed that the use of oregano oil in lambs did not affect MDA, NO, GSH, CAT, ceruloplasmin, albumin, total protein, and globulin values, but significantly affected SOD and GPx values. Compared to the control group, SOD and GPx values increased in direct proportion to the increasing doses of oregano oil. The research results are consistent with current studies using thyme and other plant extracts. In the study in which 11 different plant extracts, including thyme, were used for lamb feeding, it was reported that the blood antioxidant activity was not affected [37]. Gumus et al.[24] reported in their study that the use of oregano oil in the lambs not effected liver SOD values but GSH and CAT values, and also affected GSH, SOD, and CAT enzyme activities in *M. longissimus dorsi* tissue muscle significantly. Furthermore, in some studies using lamb extract from lambs, glutathione peroxidase activity (P<.05) has been positively affected and antioxidant parameters have been improved [26,31,38].

In conclusion, it was determined that the addition of oregano oil into the ration did not have a significant effect on fattening performance and MDA, NO, GSH, CAT, ceruloplasmin, albumin, total protein, and globulin values of blood oxidant-antioxidant parameters. SOD and GPx values were significantly affected by the addition of oregano oil in lamb rations at the point of examining the effect on blood oxidant-antioxidant balance. Oregano oil had no effect on performance parameters in lambs, but significantly affects SOD and GPx values in blood. Therefore, it is determined that blood oxidant-antioxidant balance parameter results; have the potential to protect the cells against oxidative damage caused by free radicals, are able to decrease the peroxidation by strengthening the antioxidant structure in blood, and can be effective in protecting the oxidative stress which decreases the efficiency and resistance of the animals.

In the light of the data obtained from this study, it was concluded that the use of oregano oil, which is among the natural herbal products as an alternative to antibiotics, in Tuj lambs will make an important contribution to the breeders and the researchers, who will work later in this field, since it clarifies the changes occurred in the performance and blood parameters.

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Research Article

Expression of Follicle-stimulating Hormone, Luteotropic Hormone, Estrogen and Progesterone Receptors in Ovary, Oviduct and Endometrium After Estrus Induction in Ewe

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Abstract

Induction of estrus is an effective management tool for increasing the pregnancy rate in ewes, and there is a long estrus interval after lambing in spring in Small-tail Han ewe. In this study, norgestrel releasing intra-vaginal devices (PRID) and PRID + pregnant mare serum gonadotrophin (PMSG) were used for estrus induction in Small-tail Han ewe, and expression of follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), estrogen receptor (ER) and progesterone receptor (PGR) mRNA in the ovarial stroma, oviduct ampulla and endometrium was analyzed by quantitative real-time PCR. The results showed that the estrus-induction with PRID and PRID + PMSG almost had the same effects on increasing the percentage of animals in estrus and conception rate of the ewes, and the estrus-induction had no significant effect on the expression of LHR, ER and PGR mRNA in the oviduct ampulla, ovarial stroma and endometrium, and FSHR in the ovarial stroma and endometrium. Furthermore, the estrus-induction could significantly decrease the expression of FSHR mRNA in the ovine oviduct ampulla ($P<0.05$). Therefore, it was suggested that down-regulation of FSHR in the oviduct ampulla after estrus induction may be helpful for fertilization and early embryo development.

Keywords: *Ewe, Endometrium, Estrus induction, Ovarial stroma, Oviduct ampulla, Receptor*

Koyunlarda Östrus İndüksiyonu Sonrası Ovaryum, Oviduct ve Endometriyumda Folikül Uyarıcı Hormon, Luteotropik Hormon, Östrojen ve Progesteron Reseptör Ekspresyonu

Öz

Östrusun indüksiyonu, koyunlarda gebelik oranını artırmak için etkili bir yönetim aracıdır ve kısa kuyruklu Han koyununda ilkbaharda kuzulamanın ardından uzun bir östrus aralığı söz konusudur. Bu çalışmada, kısa kuyruklu Han koyunlarında östrus uyarımı için intravaginal salınan norgestrel (PRID) ve PRID + gebe kısrak serum gonadotropini (PMSG) kullanıldı. Ovidukt ampullası, ovaryum stroması ve endometriyumdaki folikül uyarıcı hormon reseptörü (FSHR), luteinleştirici hormon reseptörü (LHR), östrojen reseptörü (ER) ve progesteron reseptörü (PGR) mRNA ekspresyonu düzeyleri kantitatif real-time PCR ile analiz edildi. Sonuçlar, koyunlarda PRID ve PRID+PMSG ile östrus indüksiyonunun, östrustaki hayvan yüzdesi ve konsepsiyon oranını arttırmada neredeyse aynı etkilere sahip olduğunu ve östrus indüksiyonunun ovidukt ampullası, ovaryum stroması ve endometriyumdaki LHR, ER ve PGR mRNA ekspresyonu ile ovaryum stroması ve endometriyumdaki FSHR üzerinde anlamlı bir etkisi olmadığını gösterdi. Bunun yanısıra, östrus indüksiyonunun, koyunlarda ovidukt ampullasında FSHR mRNA'nın ekspresyonunu önemli ölçüde azaltabileceği belirlendi ($P<0.05$). Bu nedenle, östrus indüksiyonundan sonra ovidukt ampullasında FSHR'nin down-regülasyonunun, fertilizasyon ve erken embriyo gelişimi için faydalı olabileceği düşünüldü.

Anahtar sözcükler: *Koyun, Endometriyum, Östrus indüksiyonu, Ovaryum stroması, Ovidukt ampullası, Reseptör*

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INTRODUCTION

There are long-day and short-day breeders in seasonally reproductive animals, and ewe is a short-day breeder. Small-tail Han sheep is known as a year-round breeder in Northern China. However, there is a long estrus interval after lambing in spring in Small-tail Han sheep. The induction of estrus is an effective management tool for increasing the pregnancy rate in ewes [1,2], which can control the reproductive process and improve efficiency of extensive production in sheep. Induction of estrus with an intravaginal progesterone-releasing device (IPRD) and GnRH can enhance pregnancy rates in adult ewes, and ewe lambs out of the breeding season [3]. Exogenous gonadotrophins, such as pregnant mare serum gonadotrophin (PMSG), are used to stimulate follicular growth, which increase the estrus response and ovulation rate [4,5], and also lead to a tighter synchrony of ovulation in out-of-season estrus induction and cyclic ewes [6,7].

Pulsatile gonadotropin-releasing hormone (GnRH) is secreted into the hypophyseal portal blood system from hypothalamic neurons. GnRH activates its receptors on the anterior pituitary, which leads to the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [8,9]. FSH and LH exert divergent effects and regulate essential reproductive processes, such as gametogenesis, steroidogenesis, and ovulation [10], which are through binding to the FSH receptor (FSHR) and LH receptor (LHR) on the granulosa cell surface in the gonads, uterus, breasts, and other tissues, respectively [11,12]. Steroidogenesis includes synthesis of the estrogen, progesterone, and androgens. The expression of estrus behavior is related with the peripheral blood estradiol concentrations during estrus in cows, and progesterone also exerts a priming role for the full display of estrus behavior [13]. In general, progesterone blocks the sexual behavior induced by estradiol in female sheep [14]. Estrogen and progesterone regulate the uterine endocrine and paracrine signals through their receptors during the estrus cycle and pregnancy in sheep [15,16]. There was no study on PMSG-estrus induction during estrus interval after lambing in spring in Small-tail Han sheep. Therefore, the present study was aimed at the effects of estrus induction on reproductive efficiency, and expression of FSHR, LHR, estrogen receptor (ER) and progesterone receptor (PGR) in ovarial stroma, oviduct ampulla and endometrium after estrus induction in Small-tail Han sheep.

MATERIAL and METHODS

Animals and Experimental Design

The experiment was carried out under natural conditions during February (a long estrus interval after lambing for Small-tail Han ewes) in Boading, Hebei province, China, and all procedures were approved by the Hebei University of Engineering Animal Care and Use Committee. A total of 70 healthy ewes after weaning, ranging in age from 1.5 to 2 years, were used in this study. During the trial, ewes were group-housed in straw-bedded pens with hay fed ad libitum and supplemented daily according to NRC (2007) based on the nutritional requirements. Norgestrel releasing intravaginal devices (PRID) and PMSG were from Sansheng Biological Technology Co., Ltd., Ningbo, China. PRID was inserted into vagina of the ewe for 14 days. In group 2 (n = 27), 250 IU of PMSG was administered 1 day before sponge withdrawal, but no PMSG was administered in group 1 (n = 27). In the control group (n = 16), ewes were not treated with PRID and PMSG. All of the ewes were monitored daily for estrus using vasectomized rams after the PRID withdrawal, and mated twice with intact rams at a 12-h interval after the detection of sexual receptivity. The pregnancy of ewe was evaluated by transrectal ultrasonography on day 40 post coition. Percentage of animals in estrus is the number of ewes showing estrus/number of total treated ewes in each group x 100, and conception rate is the number of pregnant ewes/number of mated ewes in each group x 100. Ovarial stroma, oviduct ampulla and endometrium were sampled from the ewes of showing estrus and the ewes without estrus (n = 6 for each group) after the PRID withdrawal for 72 h at a local slaughterhouse. The samples were frozen in liquid nitrogen for quantitative real-time PCR (qRT-PCR) assay.

RNA Extraction and qRT-PCR Assay

The samples of ovarial stroma, oviduct ampulla and endometrium were crushed into fine powders in liquid nitrogen, and the powders were digested in TRIzol (Tiangen Biotech Co., Ltd., Beijing, China), and the total RNA was extracted according to the manufacturer's instructions. The cDNA was synthesized with an All-In-One RT MasterMix (with AccuRT Genomic DNA Removal Kit; abm Biotech Co., Ltd., Jiangsu, China), and an EvaGreen qPCR MasterMix-No Dye (abm Biotech) was used for qRT-PCR. The primer sequences of FSHR, LHR, ER, PGR and GAPDH were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. *(Table 1)*. The $2^{-\Delta\Delta Ct}$ analysis method was used to calculate the relative expression values for the qRT-PCR assay, with GAPDH as the endogenous control [17].

Statistical Analysis

The data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System Package version 9.1 for Windows (SAS Institute, Cary, NC, USA). Experimental sample groups consisted of six biological replicates for the qRT-PCR assay. Groups were considered significantly different at $P<0.05$.

RESULTS

It is showed in *Table 2* that there was no ewe in estrus in control group, and the majority of ewes came into estrus between 24 and 48 h after PRID removal. It was obvious that estrus induction with PRID or PRID + PMSG

Table 1. *Primers used for qRT-PCR*

Gene	Primer	Sequence	Size (bp)	Accession Numbers
FSHR	Forward	TGTCTTGGAAGCGATAGAGGC	108	NM_001009289.1
	Reverse	GGGAAGGTTCTGGAAGGCAT		
LHR	Forward	CAGCAAGGAGACCAAATAATGAAAC	187	NM_001278566.1
	Reverse	TGAGGGTGTAGACAGAGAGTT		
ER	Forward	GATGGAGTGGCTGGAGTGAG	236	XM_015097472.1
	Reverse	GCCTTTCATTTCTTTTCTTACCTGG		
PGR	Forward	GCTTGAATACATTTATCCAGTCC	114	XM_015100878.1
	Reverse	GAAGAGATTTCACCATCCCT		
GAPDH	Forward	CACCCTCAAGATTGTCAGC	107	NM_001190390.1
	Reverse	CAGTGGTCATAAGTCCCTCC		

Table 2. *Effects of oestrus induction on pregnancy rate of Small-tail Han ewes*

Group	Number of Ewes	Ewes in Estrus (%)				Conception Rate (%)
		0-24 h	24-48 h	48-72 h	0-72 h	
Control	16	0	0	0	0	0
PRID	27	3.70% (1/27)	48.15% (13/27)	33.33% (9/27)	85.19% (23/27)	86.96% (20/23)
PRID +PMSG	27	18.52% (5/27)	44.44% (12/27)	18.52% (5/27)	81.48% (22/27)	90.91% (20/22)

PRID, norgestrel releasing intra-vaginal device; PMSG, pregnant mare serum gonadotrophin

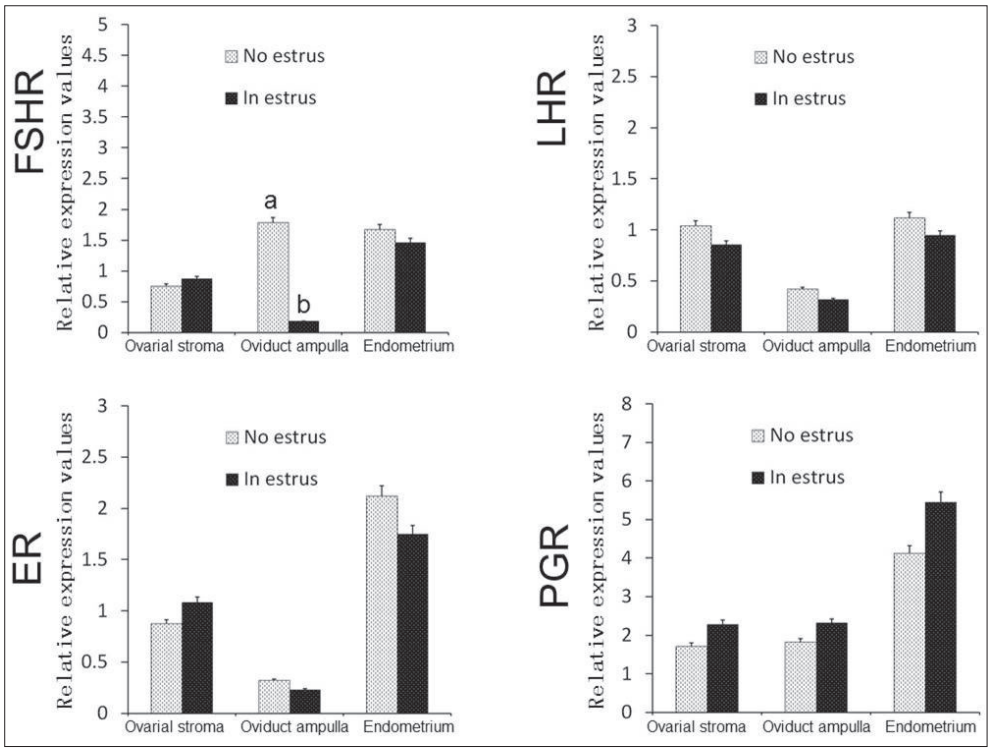

Fig 1. Relative expression values of FSHR, LHR, ER and PGR mRNA in the ovine oviduct ampulla, ovarial stroma and endometrium from the ewes in estrus and ewes without estrus. Note: Significant differences ($P<0.05$) are indicated by different letters within different column

could increase the percentage of animals in estrus and conception rate of the ewes during February. However, there was no significant difference between the group 1 and group 2 in the percentage of animals in estrus (0-72 h after PRID removal) and conception rates.

The qRT-PCR assay revealed *(Fig. 1)* that the relative level of FSHR mRNA was lower in the oviduct ampulla from the ewes in estrus than that from the ewes without estrus ($P<0.05$), but induced estrus had no significant effects on expression of FSHR mRNA in the ovarial stroma and endometrium ($P>0.05$). The relative level of LHR mRNA was low in the oviduct ampulla compared with that in the ovarial stroma and endometrium, and the relative level of ER mRNA was high in the endometrium, and low in the ovarial stroma compared with that in the oviduct ampulla. The relative level of PGR mRNA was high in the endometrium compared with that in the oviduct ampulla and ovarial stroma, but induced estrus had no significant affects on the expression of LHR, ER and PGR mRNA in the ovarial stroma, oviduct ampulla and endometrium ($P>0.05$; *Fig. 1)*.

DISCUSSION

Our results showed that there was a long estrus interval after lambing in spring in Small-tail Han, and estrus induction with PRID was as effective as with PRID + PMSG in increasing the percentage of ewes in estrus and conception rate. It has been reported that intravaginal sponge or controlled internal drug release dispenser (CIDR) can induce majority of ewes come into estrus between 24 and 48 h after sponge or CIDR removal in mature Merino ewes during December in the Great Southern region of Western Australia [18]. The treatment of either CIDR or intravaginal sponge in combination with PMSG can significantly enhance the estrus response, pregnancy and lambing fecundity rates in Awassi ewes during the non-breeding season [19]. There is a uniform effect on the reproductive performance for the different doses of PMSG (300 IU, 400 IU, and 500 IU) following a 12-day treatment with intravaginal sponges in Awassi ewes during the transition period [5]. Therefore, it is suggested that estrus induction with PRID only can increase the percentage of ewes in estrus and conception rate in spring in Small-tail Han ewes.

As a central hormone in mammalian reproduction, FSH is produced and secreted by the pituitary gland, and essential for gonadal development and maturation, and gamete production [20]. Through binding to FSHR, FSH activates the extracellular domain of the FSHR, and induces the maturation of ovarian follicles [21]. The level of FSHR increases until the middle of estrus, and then drops sharply when the LH leads to ovulation [22]. The level of FSHR mRNA is not changed at days 13, 15, and 17 in the same follicles, but decreases in the preovulatory follicles in the pig ovary by day 19 of the estrus cycle [23]. The expression of FSHR protein decreases from early luteal phase to mid and late luteal phases in ovine deep endometrial glands and stroma during the estrus cycle [24]. FSHR expression was decreased in the oviduct induced by insemination or after superovulation [25]. Our results showed that estrus-induction could decrease the expression of FSHR mRNA in the oviduct ampulla, which indicated that down-regulation of FSHR in the oviduct ampulla after estrus induction may be helpful for fertilization and early embryo development.

LHR is essential for LH to regulate follicular maturation and ovulation, and luteal function [26,27]. Estradiol combined with progesterone enhances expression of LHR protein in the porcine oviduct, which is essential for LH-induced relaxation of the porcine oviduct [28]. LHR mRNA is expressed in the immature rat uterus, and declined to an extremely low level after human chorionic gonadotropin (hCG) treatment, which suggests that LH acts on the uterus to block contraction at ovulation [29]. The expression levels of LHR mRNA are relatively lower in uterine tissues and oviduct at the estrus stage in healthy non-pregnant adult Hu sheep [30]. LHR expression is weakly positive or declines in the theca cells of small follicles or large mature follicles on day 0 of the estrus cycle in the porcine ovary [31]. In this study, estrus-induction had no significant effect on expression of LHR mRNA in the oviduct ampulla, ovarial stroma and endometrium, which indicated that LH may have no obvious effect on regulating the physiological status of the oviduct ampulla, ovarial stroma and endometrium of the ewes in estrus.

As a primary female sex hormone, estrogen exerts its actions through ER, and estrogen-ER complex binds to specific DNA sequences, and controls gene expression to activate the transcription of the target genes [32]. ER protein and mRNA are expressed in ovaries of the ewes, and declines from day 2 of the estrus cycle during CL development [33]. The expression of ER mRNA in theca interna tissue and granulosa cells increase continuously during follicle growth in the bovine ovary during estrus cycle [34]. The level of endometrial ER mRNA is highest on day 1, declines between days 1 and 6, and increases between days 11 and 15 of the estrus cycle in ewes [35]. ER mRNA and protein are expressed in the epithelium and smooth muscular layer of the oviduct, but no significant changes are observed during estrus cycle [36]. Our results showed that estrus-induction had no significant effect on expression of ER mRNA in the oviduct ampulla, ovarial stroma and endometrium. Therefore, estrogen may be not obviously implicated in regulating the functions of the oviduct ampulla, ovarial stroma and endometrium of the ewes in estrus.

The biological actions of progesterone are mostly through binding to PGR [37]. PGR is expressed in the ampulla and isthmus of the oviduct, but PGR is not correlated with progesterone concentrations in heifers [38]. There is a reduction of PGR protein expression in most of oviductal and uterine cells after synchronization of estrus with

progestagen in sheep [39]. There is a significant increase of PGR content in the pituitary gland after treatment with GnRH, but PGR content is not altered obviously in the uterus of anestrus ewes [40]. The endometrial and myometrial PGR mRNA levels are highest on day 1 of the estrus cycle, and then decline thereafter in cyclic ewes [35]. The expression of PGR mRNA in follicles and theca interna tissue increases continuously with the development of the follicles in the bovine ovary during estrus cycle [34]. In this study, estrus-induction had no significant effect on expression of PGR mRNA in the oviduct ampulla, ovarial stroma and endometrium, which suggested that progesterone may have no obvious effects on the oviduct ampulla, ovarial stroma and endometrium of the ewes in estrus.

In conclusion, estrus-induction with PRID can increase the percentage of animals in estrus and conception rate of Small-tail Han ewes in spring. The estrus-induction had no significant effects on expression of the mRNA of LHR, ER and PGR in the ovine oviduct ampulla, ovarial stroma and endometrium, FSHR mRNA in the ovarial stroma and endometrium. However, estrus-induction could decrease the expression level of FSHR mRNA in the oviduct ampulla from the ewes in estrus, suggesting that down-regulation of FSHR in the oviduct ampulla after estrus induction may be helpful for fertilization and early embryo development.

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Research Article

Development of a Monoclonal Antibody to Detect αs1-casein in the Milk of Healthy and Mastitis-Affected Goats

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Abstract

This study aimed to evaluate the expression level of caseins during mastitis of goats. Whole goat caseins were used as primary antigens for mouse immunization. A monoclonal antibody (mAb) named 5B with high specificity to goat αs1-casein was developed. Further results showed that mAb 5B can successfully be applied to western blot and ELISA. In addition, immunofluorescence analysis using this mAb showed increased milk αs1-casein level in mastitis-affected goats. In conclusion, this study has established an effective tool to evaluate the expression level of αs1-casein during mastitis development.

Keywords: *αs1-casein, Monoclonal antibody, ELISA, Mastitis, Immunofluorescence, Goat*

Sağlıklı ve Mastitisli Keçi Sütünde αs1-kazeini Belirleyen Monoklonal Antikor Geliştirilmesi

Öz

Bu çalışma keçilerde mastitis sırasında kazein ekspresyon seviyesini değerlendirmeyi amaçlamıştır. Farelerde immünizasyon için birincil antijen olarak keçi kazeinleri kullanıldı. Keçi αs1-kazeine yüksek spesifite gösteren ve 5B olarak adlandırılan bir monoklonal antikor (mAb) geliştirildi. Sonuçlar mAb 5B'nin western blot ve ELISA'ya başarıyla uygulanabileceğini gösterdi. Ek olarak, geliştirilen mAb kullanılarak yapılan immünofloresan analizi, mastitisten etkilenen keçilerde süt αs1-kazein seviyesinin arttığını gösterdi. Sonuç olarak, bu çalışma ile mastitis gelişimi sırasında αs1-kazeinin ekspresyon seviyesini değerlendirmek için etkili bir faktör belirlenmiştir.

Anahtar sözcükler: *αs1-kazein, Monoklonal antikor, ELISA, Mastitis, İmmünofloresans, Keçi*

INTRODUCTION

Goat milk and its productions begin gaining attention because of their easy digestibility and lower allergenic properties compared to the cow milk [1]. An important role for goat milk is to provide the necessary nutrients for infants affected by cow milk allergy [2]. In addition, the nutritional value of goat milk has been widely accepted considering the total protein, fat, vitamin and mineral contents [3,4]. Caseins in milk account for more than 80% of total proteins and consist of αs1-, αs2-, β-, and κ-caseins and αs1-casein is one of the most important highly-phosphorylated proteins among caseins [3]. Goat milk contains less αs1-casein (5%-20%) compared to (30%-35%) cow milk, making goat milk more similar to human milk and less likely to cause allergy [3]. In addition, αs1-casein influences the coagulation properties of milk [3]. With the development of hybridoma technology, monoclonal antibodies (mAbs) against caseins were used to determine the content of β-casein in bovine milk [5]. However, up to the present, mAbs against goat caseins have not yet been developed.

In this paper, we used purified goat caseins for mouse immunization. Two strains of hybridoma cells targeting goat caseins (named 5B and 7H) were screened by indirect

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ELISA. To explore the effect of mastitis on αs1-casein, we analyzed the level of αs1-casein of goat milk samples collected from healthy goats, goats with sub-clinical mastitis and clinical mastitis by indirect competitive ELISA. Immunofluorescence of goat mammary epithelial cells was carried out to further examine αs1-casein expression profile in lipopolysaccharide (LPS)-induced mastitis *in vitro*.

MATERIAL and METHODS

Ethical Approval

Animal-related experiments in the present study were approved by the Research Ethics Committee of Northwest A&F University according to the guidelines of the Ministry of Health in China for the care and use of laboratory animals.

Antigen (Whole Goat Caseins) Preparation

Goat caseins were extracted using isoelectric precipitation. Briefly, 10 g of whole goat milk powder (purchased from Kabtrita, Holland) was dissolved in 10 mL of 2M Na_2HPO_4•$12H_2O$ buffer. The pH was adjusted to 4.2 using 1M CH_3COOH solution (preheated to 40°C). After complete precipitation at 37°C, the solution was centrifuged at 4000g for 10 min, and the precipitate was washed with 20 mL of 95% (v/v) ethanol 3 times to remove lipid. Next, 1:1 (v/v) ethanol-ether mixture was added to wash the precipitate 3 times. Final precipitation was washed twice with ether and was placed in a fume hood until all ether volatilized. Casein powder was preserved at -80°C and its purity and molecular weight were analyzed by SDS-PAGE [6].

Mouse Immunization, Cell Fusion, Hybridoma Cell Screening and Ascites Prepareation

Mouse immunization, cell fusion, hybridoma cell screening and ascites preparation were conducted as previously reported by our group [6].

Competitive Indirect ELISA

Competitive indirect ELISA was conducted as previously reported [6]. Specifically, the dilution of goat milk was 15 fold with PBST and the dilution of mAb 5B was 7 fold with PBST.

Milk Sample Collection and Processing

For clinical mastitis samples, milk with the detection of flakes and clots with gland swelling or systemic illness such as fever, depression, weakness and dehydration were selected. For sub-clinical mastitis diagnosis, the California Mastitis Test was used as previously reported [7]. All the milk was stored at 4°C after collection and was used for further assays within 1 h.

Isolation and Culture of Goat Mammary Epithelial Cells (MECs)

Goat MEC isolation was performed as previously reported [6]. After isolation, the cells were cultured in DMEM/F12 medium (Thermo Fisher Scientific, New York, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 1% GlutaMAX Supplement (Thermo Fisher Scientific) and 100 units mL^{-1} of penicillin-streptomycin (Sigma Aldrich, Missouri, USA). The culture medium was refreshed every 24 h. The cells were cultured at 37°C in 5% CO_2 and were passaged when they were 80% confluent.

LPS Stimulation and Immunofluorescence Staining

The *in vitro* mastitis model was established by adding LPS (Sigma Aldrich) to the culture medium at a final concentration of 10 μg mL^{-1}. Meanwhile, the control group was treated with culture medium of the same volume. All cells were treated for 12 h before immunofluorescence analysis. Procedures of immunofluorescence were the same as previously reported [8].

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Differences were assessed using t-tests or one-way ANOVA with Dunnett test. $P<0.05$ was considered significant.

RESULTS

Isoelectric point precipitation method was used for the purification of the goat casein and was identified by SDS-PAGE. After precipitation, caseins were purified and contained αs1-casein because lane 3 and lane 4 has an identical band at 23.9 kDa *(Fig. 1)*. Consistent with a previous study, purified αs1-casein showed a higher molecular weight when analyzed by SDS-PAGE than expected [9].

The titers of sera collected from the immunized mice 7-10 days after the forth immunization were determined by indirect ELISA. All immunized mice produced anti-goat

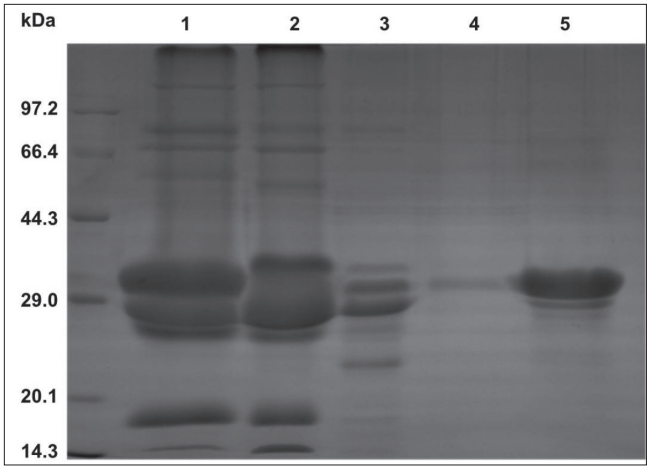

Fig 1. SDS-PAGE analysis of caseins. Lane 1, bovine milk powder; lane 2, goat milk powder; lane 3, total goat caseins; lane 4, goat αs1-casein; lane 5, bovine α-casein

casein antibodies. Several mice were selected for further experiment and were injected with 100 μg of immunogen intravenously 3 days before cell fusion. Then, hybridoma secreting antibodies specific to goat caseins but unrecognizable to bovine milk powder were selected by indirect ELISA, and were subcloned by limiting dilution method. Two stable hybridoma cell lines were established and named as 5B and 7H. These two hybridoma cell lines showed strong proliferation ability and high specificity to goat caseins and was chosen for further studies.

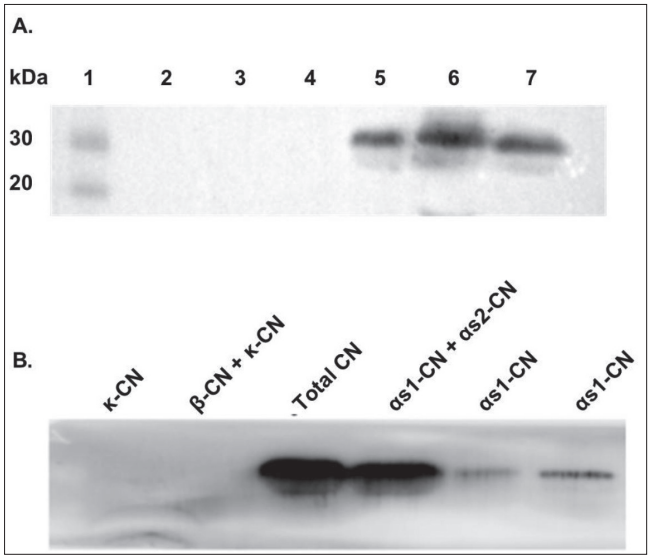

Fig 2. mAb specificity tests by western blot analysis. **(A)** Goat casein was loaded as a positive control, the content of goat casein and bovine casein was 1.7 μg, milk powder was at the concentration of 0.1 g/mL, the milk was 20-fold diluted. Lane 1, marker; lane 2, bovine α-caseins; lane 3, cow milk; lane 4, cow milk powder; lane 5, total goat casein; lane 6, goat milk; lane 7, goat milk powder. **(B)** Isolation of goat caseins and recognition of 5B to αs1-casein as determined by western blot. Goat caseins are purified by cation-exchange chromatography. Each lane is loaded with the correspondingly labeled proteins

Cross-reactivity of mAbs 5B and 7H was analyzed by indirect ELISA using goat casein, bovine milk, goat milk, goat milk powder and bovine milk powder as antigens. The results showed that these two mAbs both specifically recognized goat caseins and had no cross-reactivity with bovine caseins *(Table 1)*, which was supported by western blot analysis using mAb 5B *(Fig. 2A)*. In addition, the specificity of mAb 5B was further evaluated using different subtypes of goat caseins. The result showed that this mAb only reacted with goat αs1-casein, while no other reactions existed between mAb 5B and purified β- and κ-caseins *(Fig. 2B)*.

In order to evaluate αs1-casein content in milk of goat with mastitis, OD_{450} values of different goat milk samples were compared by indirect competitive ELISA. As illustrated in *Fig. 3A*, milk from goats suffering from clinical mastitis had the lowest OD_{450} value, representing the highest levels of αs1-casein in these samples. In comparison, milk from goats with sub-clinical mastitis and healthy controls had relatively lower levels of αs1-casein compared to the goats with clinical mastitis ($P<0.05$). We further built an *in vitro* mastitis model by challenging goat MECs with LPS. Interestingly, LPS treatment resulted in significantly decreased cytoplasmic αs1-casein levels in MECs *(Fig. 3B-C)*.

Table 1. *Testing specificity of monoclonal antibodies 5b and 7b against goat casein by ELISA*

Antibody	Protein					
	Goat Casein	Goat Milk	Goat Milk Powder	Bovine Casein	Bovine Milk	Bovine Milk Powder
MAb 5B	1.762	1.531	1.824	0.080	0.075	0.082
MAb 7H	1.634	1.520	1.627	0.092	0.065	0.087

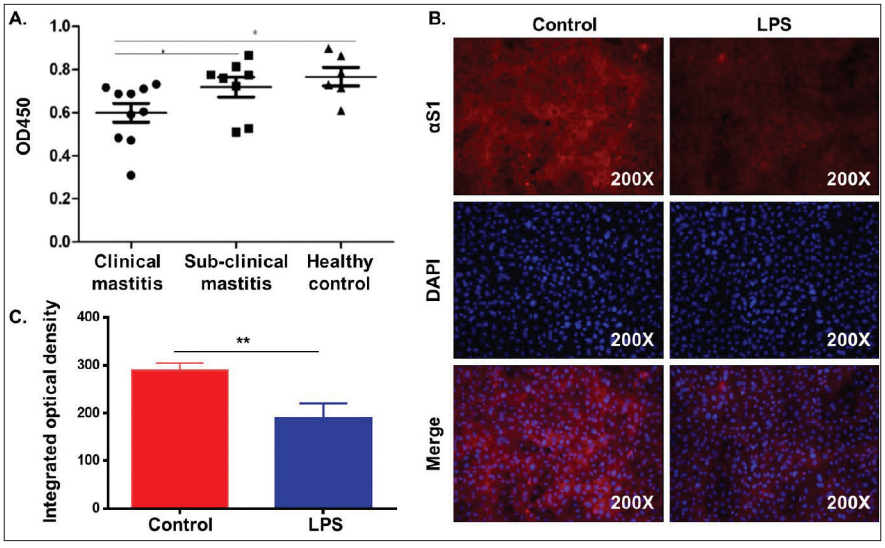

Fig 3. Analysis of αs1-casein level in goat milk samples and goat MECs. (A) Indirect competitive ELISA analysis of αs1-casein content in milk samples from goats with clinical mastitis (n=10), sub-clinical mastitis (n=8) or healthy goats (n=6). (B) Immunofluorescence analysis of αs1-casein expression levels in goat MECs with (LPS, n=4) or without LPS (Control, n=4) treatment. (C) Integrated optical density analysis of the immunofluorescence results in (B)

DISCUSSION

Casein expression level is an important index when evaluating the lactation function of the mammary gland. While mAbs against β-casein [10] and κ-casein [11] have been developed, no studies have reported the successful development of mAbs against αs1-casein, the major part (38%) of total caseins in milk [12]. In addition, considering that αs1-casein is the most important protein causing milk allergy [13], mAbs with high specificity to goat αs1-casein are therefore of critical importance for people to avoid milk allergy.

Mammary epithelial cells constitute an important part of the mammary gland and are the structural basis for the lactation of the mammary gland. The secretion of caseins is affected by the condition of MECs. By using mAbs against αs1-casein, we can measure its expression level in MECs under different conditions. Intriguingly, our data suggest that the concentration of αs1-casein was significantly increased in milk samples of clinical mastitis-suffered goats, indicating that the milk of these goats may be problematic and chances of allergy are higher. However, we found the expression of αs1-casein in the cytoplasm of MECs was decreased after stimulation of LPS *in vitro*, consistent with a previous study [14]. The severely milk yield loss in goats with clinical mastitis may be the main reason for the higher concentration of αs1-casein in milk of clinical mastitis-affected goats. However, we could not rule out the possibility that whether an enhanced αs1-casein secretion ability accounted for less αs1-caseins retention. Thus, future studies are needed to further explore the underlying mechanisms.

In conclusion, a mAb named 5B that specifically reacted with goat αs1-casein was developed in the present study. This mAb can successfully be applied to western blot, ELISA and immunofluorescence and served as a convenient tool to assess the dynamics of αs1-casein during mastitis development. More future studies investigating the biological function of αs1-casein and its relationship with certain diseases are encouraged. Specially, studies concerning the involvement of αs1-casein in milk allergy will lay important foundation for elucidating the underlying mechanisms and for effective therapy development.

Conflict of Interest

The authors declare that no conflict of interest exists.

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Research Article

Morphometric Study on the Digital Bones in the Domestic Cattle [1]

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Abstract

In this study, the phalanges of the forelimb and hindlimb of 18 adult Holstein breed cattle were used. Morphometric measurements were taken from 144 digital bones. In contrast to classical references, it was concluded that the greatest lengths (GLpe) were longer in the hindlimb than the forelimb for the phalanx proximalis and phalanx media. In the phalanx proximalis and phalanx media, the SD*100/GLpe index value was high in the forelimb and low in the hindlimb. It was observed that the differences between the Bp (Breadth of the proximal end) values of phalanx proximalis and Bd (Breadth of the distal end) values in phalanx media were significant for the inner bones of the forelimb and their hindlimb counterparts, while the other values were statistically not significant. The presence of an asymmetry between the osteometric measurements of the internal and external bones of the digits could only be observed between the GL values of the phalanx media of forelimb ($P<0.05$). We concluded that the asymmetry seen in the forelimb in Holstein breed cattle maybe a result of being kept on concrete ground as dairy cows.

Keywords: *Digital bones, Cattle, Morphometry*

Evcil Sığırlarda Parmak Kemikleri Üzerine Morfometrik Çalışma

Öz

Bu çalışmada, 18 adet erişkin Holstein ırkı sığıra ait ön ve arka ayak parmak kemikleri (phalanx proximalis, media ve distalis) kullanıldı. Toplam 144 parmak kemiğinin her birinden morfometrik ölçümler alındı. En büyük uzunluk (GLpe) ölçümlerinin hem Ph1, hem de Ph2'lerde, klasik kaynakların aksine arka ayaklarda daha fazla olduğu sonucuna varıldı. Ph1 ve Ph2'lerde SD*100/GLpe indeksinin ön ayaklarda daha yüksek, arka ayaklarda ise düşük olduğu gözlendi. Ph1'de Bp, Ph2'de de Bd değerlerinin ön ayakların internal kemikleriyle arka karşılıkları arasındaki farklarının istatistiksel açıdan anlamlı olması dışında, diğer farklılıkların istatistiki açıdan anlam taşımadığı gözlendi. Parmakların internal ve external kemiklerinin osteometrik ölçümleri arasında bir asimetrinin varlığı sadece ön Ph2'nin GL değerleri arasında gözlenebildi ($P<0.05$). Ön ayak kemikleri arasındaki bu asimetrinin, Holstein ırkı sığırların süt amaçlı yetiştiriciliği ve bunların nispeten sert zeminde tutulmuş olmalarının etkin olabileceği sonucuna varıldı.

Anahtar sözcükler: *Morfometri, Sığır, Parmak kemikleri*

INTRODUCTION

Ruminants have 4 digital elements and have two digits on each foot [1,2]. The abaxial pairs of the digit-forming elements have been reduced and functionally joined as a single bone as evidence of their common origin [1]. Each metapodium forms the manus with the medial and lateral digital bones found in pairs. Although the phalanx bones in hindlimb are generally similar to the phalanx bones in forelimb, phalanx proximalis and phalanx media are reported to be shorter in the hindlimb than forelimb [3,4]. This comparison in ruminants was not made only for the forelimb and hindlimb, but also for measurements in the inner and outer digits [5-7]. In some studies, the results of these comparisons show no statistical differences, although some present asymmetry [5-8]. In the comparison of phalanges forming the medial and lateral digits belonging to the same foot, it was reported that the average length of phalanx proximalis and phalanx media of the 4th digit was longer than those of the 3rd digit and the phalanx distalis of the 3rd digit had a larger mean value [2].

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The main differences observed in the forelimb and hindlimb were both length and width of phalanx proximalis and phalanx media, which is reported as being shorter in hindlimb in comparison to forelimb in classical anatomy resources [4], while Ocal et al.[8] reported that phalanx proximalis and phalanx media are shorter in the forelimb. Nevertheless, it was stated that the most significant difference was in the width.

It has been suggested that the double digits of the artiodactyls are not in equal length, indicating that they have different functions in posture and carrying weight [2]. Asymmetry in the lower extremities in cattle was observed especially in thoracic extremities [9]. It was reported that there were no significant differences between the corresponding measurements of the right and left extremities of the buffalos and the total length of the digital bones of the medial and lateral digits of the same extremities [5]. The movement of the animal on soft or hard ground has an effect on the emergence of asymmetric condition [2]. The anatomic position of the ruminant feet causes them to be exposed to significant physiological stresses that can result in pathological changes [10]. This may lead to the emergence of common aseptic inflammations expressed as laminitis [11]. Although the weight of the animal is carried by the central digits in Bovidae, it is stated that the fact that the digits are not of equal length has an effect on the localization and incidence of the pathologies observed in the digits [2,12]. It is stated that the digital bones are symmetrical in the oxen resulting in the equal weight loading to foot during standing and walking, which is the reason why the biomechanical lesions of the foot are less common than the cattle [5].

Approximately 90% of the clinical lameness cases in cattle are caused by digital lesions and 92% of these lesions affect the hindlimb [13-15]. It has been reported that approximately 2/3 of the digital lesions also affect the lateral hindlimb hooves [16,17]. The biomechanical properties of the feet of the ruminants cause a difference in the balance distribution of the weight between the rear hooves. Chronic overloading of the lateral hind hooves is considered a predisposing condition for cows. A similar situation exists in the forelimbs. While standing, the majority of the weight is carried by the medial hoof. The 3rd inner digit of the hoof has a greater length than the 4th digit [6]. It is therefore more affected by diseases such as sole ulceration.

In addition to the difference in the distribution of the load on the legs due to the differences in the length of the digits in ruminants, the way the front and rear legs are connected to the body is also thought to affect the biomechanical properties of the foot [2,6]. The fact that the forelimbs are connected to the muscles while the hindlimb connected in the joint style can affect the biomechanics of digital anatomy in these animals [3]. It is suggested that the effect of different hooves on the forelimb and hindlimb may be related to the anatomical features of these digits [16].

In this study, we aimed to reveal the morphometric differences between the digital bones of the forelimb and hindlimb and between the right and left digits in Holstein breed cattle. We believe that the obtained data will contribute to the evaluation and identification of artiodactyl digital bones in archaeozoological studies [18-21], easier evaluation of digital pathologies of the foot and better understanding of biomechanical properties by using morphometric data [22-24].

MATERIAL and METHODS

This study was accepted by the ethics committee of the Istanbul University (Decision number: 35980450-050.01.99).

In this study, digital bones of forelimb and hindlimb (phalanx proximalis, media and distalis) of 18 adult Holstein breed cattle were used obtained from the slaughterhouses of Istanbul region. For this purpose, each of the digital bones taken from the slaughterhouse was coded and recorded. Then these bones were boiled and subjected to maceration [25].

Morphometric measurements were obtained from each of 144 digital bones using digital calliper. The morphometric measurements are *(Fig. 1)* [26];

Phalanx Proximalis

1. Greatest length of the abaxial half (GLpe)
2. Breadth of the proximal end (Bp)
3. Smallest breadth of the diaphysis (SD)
4. Breadth of the distal end (Bd)

Phalanx Media

1. Greatest length of the abaxial half (GLpe)
2. Breadth of the proximal end (Bp)
3. Smallest breadth of the diaphysis (SD)
4. Breadth of the distal end (Bd)
5. Greatest length (in dorsal direction) (GL)

Phalanx Distalis

1. Greatest diagonal length of the sole (DLS)
2. Length of the dorsal surface (Ld)
3. Middle breadth of the sole (MBS)

All measurements were based on von den Driesch [26]. SPSS statistical package program (SPSS for Windows, version 21.0) was used for statistical analysis. Descriptive mean values and SD values were calculated. One-way analysis of variance (ANOVA) was used to evaluate all data. Tukey test was used to determine from which group the differences originated. Statistical differences between groups were presented as tables for each feature.

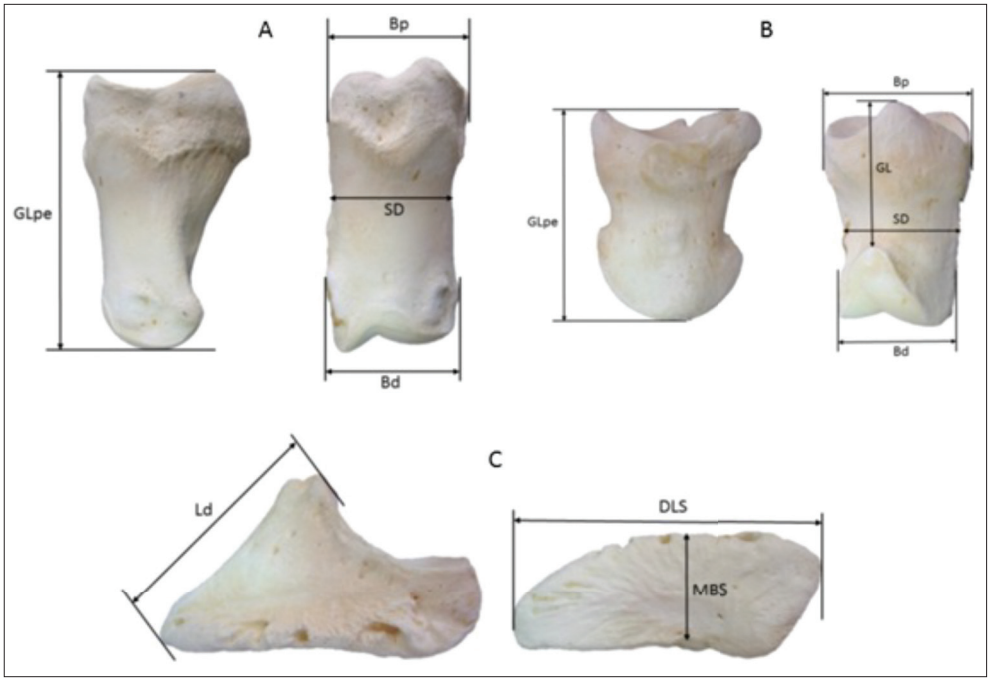

Fig 1. Osteometric measurements from digital bones. **A:** phalanx proximalis; **B:** phalanx media; **C:** phalanx distalis

RESULTS

The osteometric measurements of the phalanx proximalis of the forelimb and hindlimb are shown in *Table 1*.

There was no statistically significant difference in the GLpe measurements between phalanx proximalis of the forelimb and hindlimb, whereas the GLpe measurements showed a smaller value in the forelimb compared to the hindlimb. Statistically significant differences were found between the GLpe measurements of the phalanx proximalis of forelimb and hindlimb except the left forelimb ($P<0.05$). The difference between the internal phalanx proximalis of the left forelimb and the GLpe measurements of the external phalanx proximalis of the right hindlimb was not statistically significant.

Although Bp measurement results were relatively small in the hindlimb phalanx proximalis compared to the forelimbs, statistically significant differences were observed between the internal phalanx proximalis of the left forelimb and the Bp measurements of both the internal and external phalanx proximalis of the left hindlimb ($P<0.05$). In addition, the differences between both the phalanx proximalis of forelimb and hindlimb were not significant.

The SD value was relatively slightly higher in the phalanx proximalis of forelimb, but the differences between all phalanx proximalis of both the hindlimb and forelimb were not statistically significant. The same was true for Bd value.

Three different index values were calculated for phalanx proximalis in *Table 2*. These were Bp*100/GLpe, SD*100/GLpe and Bd*100/GLpe. While there were no statistically significant differences between the right and left digital bones, it was observed that the differences between all three index values of the phalanx proximalis of forelimb and hindlimb were significant ($P<0.05$). All three index values of the forelimb phalanx proximalis were higher than the hindlimb and the difference was statistically significant.

SD*100/GLpe index, which is considered as the fineness index, was higher than the hindlimb in the forelimb and the differences were statistically significant ($P<0.05$).

Osteometric measurements of phalanx media were given in *Table 1*. There were no statistically significant differences between the phalanx media of forelimb and hindlimb in both of the anterior and posterior areas. While GLpe measurements were relatively small in the phalanx media of forelimb compared to the hindlimb, there was a significant difference between the external phalanx media of the forelimb and the internal phalanx media of the hindlimb ($P<0.05$). Although the difference between the left forelimb external phalanx media and left hindlimb internal phalanx media was not statistically significant, the observed differences were remarkable.

Phalanx media's GL length in dorsal direction was different from that in GLpe. There were no significant differences in the GL measurement of the phalanx media between both the right/left and external/internal parts of the hindlimb, but some differences were found in the forelimb itself. In addition, the GL values of the forelimb were smaller than the hindlimb and the difference was statistically significant.

The GL value of the external phalanx media of the left and right forelimb was measured as the smallest value. No statistically significant difference was found between them.

Table 1. *Osteometric measurements of the phalanx proximalis and phalanx media of the forelimb and hindlimb*

M	Phalanges	N	Phalanx Proximalis				Phalanx Media			
			Mean (mm)	SD (mm)	Minimum (mm)	Maximum (mm)	Mean (mm)	SD (mm)	Minimum (mm)	Maximum (mm)
GLpe	External Ph of the left forelimb	18	72.37^{a}	2.548	68.28	78.52	48.85^{ac}	1.96	45.51	52.60
	Internal Ph of the left forelimb	18	72.84^{ab}	3.006	66.83	77.74	49.31^{ace}	2.29	45.08	53.65
	External Ph of the right forelimb	18	72.45^{a}	2.427	68.36	78.13	48.74^{ad}	1.89	45.47	52.85
	Internal Ph of the right forelimb	18	72.57^{a}	2.742	67.79	77.13	49.27^{ae}	2.37	45.27	53.24
	External Ph of the left hindlimb	18	75.97^{c}	3.018	71.40	82.05	50.53^{bcde}	2.15	47.45	54.37
	Internal Ph of the left hindlimb	18	76.05^{c}	2.819	71.88	82.27	51.05^{bcs}	2.40	47.76	55.48
	External Ph of the right hindlimb	18	75.44^{bc}	2.779	70.00	82.24	50.35^{bcde}	2.28	46.46	54.83
	Internal Ph of the right hindlimb	18	76.39^{c}	2.639	72.94	82.65	51.23^{be}	2.25	47.71	55.58
GL	External Ph of the left forelimb	18	-	-	-	-	25.90^{a}	1.51	23.59	29.29
	Internal Ph of the left forelimb	18	-	-	-	-	24.17^{bc}	1.28	21.32	26.19
	External Ph of the right forelimb	18	-	-	-	-	25.75^{ab}	1.39	23.02	27.49
	Internal Ph of the right forelimb	18	-	-	-	-	24.05^{c}	1.21	22.88	26.61
	External Ph of the left hindlimb	18	-	-	-	-	29.55^{d}	1.57	26.61	32.39
	Internal Ph of the left hindlimb	18	-	-	-	-	28.52^{d}	2.09	24.06	31.34
	External Ph of the right hindlimb	18	-	-	-	-	29.39^{d}	1.49	27.30	32.10
	Internal Ph of the right hindlimb	18	-	-	-	-	28.74^{d}	2.05	24.30	31.72
Bp	External Ph of the left forelimb	18	38.60^{cd}	1.425	36.01	41.09	37.25^{a}	2.08	32.71	40.78
	Internal Ph of the left forelimb	18	39.06^{ac}	1.714	36.08	42.06	37.24^{a}	1.96	33.19	40.59
	External Ph of the right forelimb	18	38.48^{cd}	1.396	35.73	41.15	37.38^{a}	1.85	32.70	40.08
	Internal Ph of the right forelimb	18	39.09^{ac}	1.559	35.83	41.56	37.07^{a}	1.93	32.87	40.20
	External Ph of the left hindlimb	18	37.41^{bd}	1.595	33.88	39.77	35.88^{a}	1.63	31.65	38.41
	Internal Ph of the left hindlimb	18	37.35^{bd}	1.685	33.82	40.28	36.58^{a}	1.79	33.16	39.85
	External Ph of the right hindlimb	18	37.51^{cd}	1.303	34.34	40.26	36.30^{a}	1.99	32.29	40.95
	Internal Ph of the right hindlimb	18	37.53^{cd}	1.875	33.35	40.33	36.16^{a}	2.03	33.13	39.40
SD	External Ph of the left forelimb	18	32.81^{a}	1.958	28.63	35.78	30.44^{a}	1.31	28.48	32.37
	Internal Ph of the left forelimb	18	33.13^{a}	1.545	30.42	35.71	30.34^{a}	1.52	27.86	32.85
	External Ph of the right forelimb	18	33.15^{a}	2.010	29.49	36.85	30.19^{a}	1.59	26.67	32.23
	Internal Ph of the right forelimb	18	33.31^{a}	1.759	30.28	35.67	30.43^{a}	1.56	27.50	32.91
	External Ph of the left hindlimb	18	31.76^{a}	1.698	27.80	34.19	29.11^{a}	1.73	25.08	31.95
	Internal Ph of the left hindlimb	18	32.09^{a}	1.771	29.13	35.14	29.64^{a}	1.61	26.63	32.45
	External Ph of the right hindlimb	18	31.81^{a}	1.751	27.75	35.02	29.09^{a}	1.89	24.83	32.66
	Internal Ph of the right hindlimb	18	32.14^{a}	1.676	28.62	35.12	29.73^{a}	1.82	26.19	32.69
Bd	External Ph of the left forelimb	18	35.95^{a}	2.573	30.08	40.43	31.37^{ac}	1.62	27.21	33.74
	Internal Ph of the left forelimb	18	35.74^{a}	1.983	32.09	39.66	32.58^{a}	1.68	28.20	34.26
	External Ph of the right forelimb	18	35.80^{a}	2.343	30.94	39.19	31.50^{ac}	1.77	27.48	34.49
	Internal Ph of the right forelimb	18	35.84^{a}	2.255	31.57	39.99	32.86^{a}	1.93	28.20	35.50
	External Ph of the left hindlimb	18	34.00^{a}	1.946	29.15	36.33	30.46^{bc}	2.39	24.70	36.49
	Internal Ph of the left hindlimb	18	34.96^{a}	1.866	31.48	37.60	29.90^{bc}	1.85	25.60	32.86
	External Ph of the right hindlimb	18	34.11^{a}	1.914	29.55	38.06	30.47^{bc}	2.16	24.91	33.80
	Internal Ph of the right hindlimb	18	34.82^{a}	2.200	30.76	39.16	29.86^{bc}	2.01	25.01	32.44

^{a,b,c,d,e} *Values within a column with different superscripts are significantly different (P<0.05).* ***M:*** *measurement;* ***GLpe:*** *greatest length of the abaxial half;* ***GL:*** *greatest length;* ***Bp:*** *breadth of the proximal end;* ***SD:*** *smallest breadth of the diaphysis;* ***Bd:*** *breadth of the distal end*

Table 2. *The indices of the phalanx proximalis and phalanx media of the forelimb and hindlimb*

Indices	Phalanges	N	Phalanx Proximalis				Phalanx Media			
			Mean (mm)	SD (mm)	Minimum (mm)	Maximum (mm)	Mean (mm)	SD (mm)	Minimum (mm)	Maximum (mm)
Bp*100/GLpe	External Ph of the left forelimb	18	53.38[a]	2.04	48.10	56.79	76.24[a]	2.70	69.52	80.45
	Internal Ph of the left forelimb	18	53.65[a]	2.01	50.35	56.60	75.59[ac]	3.74	70.12	82.17
	External Ph of the right forelimb	18	53.15[a]	1.99	49.01	56.50	76.71[a]	3.25	69.52	84.06
	Internal Ph of the right forelimb	18	53.90[a]	2.05	50.46	58.05	75.30[ac]	3.59	70.79	81.23
	External Ph of the left hindlimb	18	49.27[b]	1.97	45.43	51.90	71.07[b]	3.01	65.68	75.59
	Internal Ph of the left hindlimb	18	49.15[b]	2.46	45.47	54.04	71.70[b]	2.94	66.04	76.67
	External Ph of the right hindlimb	18	49.74[b]	1.44	47.11	52.74	72.18[bc]	4.15	66.08	83.10
	Internal Ph of the right hindlimb	18	49.15[b]	2.49	45.01	53.11	70.60[b]	3.10	65.31	76.32
SD*100/GLpe	External Ph of the left forelimb	18	45.35[a]	2.40	39.71	48.76	62.35[a]	2.20	58.91	66.61
	Internal Ph of the left forelimb	18	45.50[a]	1.52	43.25	48.85	61.57[a]	2.93	57.60	70.36
	External Ph of the right forelimb	18	45.76[a]	2.35	41.56	49.95	61.97[a]	3.15	56.70	68.55
	Internal Ph of the right forelimb	18	45.91[a]	1.92	43.25	50.19	61.78[a]	2.43	59.42	69.87
	External Ph of the left hindlimb	18	41.83[b]	2.23	37.84	45.49	57.64[b]	2.93	52.04	61.56
	Internal Ph of the left hindlimb	18	42.20[b]	2.00	39.36	46.03	58.11[b]	2.88	53.41	63.28
	External Ph of the right hindlimb	18	42.17[b]	2.14	38.19	45.87	57.82[b]	3.54	51.66	63.89
	Internal Ph of the right hindlimb	18	42.07[b]	1.78	38.62	44.91	58.05[b]	2.85	53.20	62.88
Bd*100/GLpe	External Ph of the left forelimb	18	49.66[a]	2.87	42.11	55.40	64.25[ac]	3.32	57.83	71.62
	Internal Ph of the left forelimb	18	49.06[a]	1.76	45.81	51.95	66.14[a]	3.29	59.63	73.16
	External Ph of the right forelimb	18	49.39[a]	2.35	43.61	53.12	64.66[a]	3.56	58.42	71.65
	Internal Ph of the right forelimb	18	49.38[a]	2.49	45.61	53.96	66.72[a]	3.23	60.76	71.85
	External Ph of the left hindlimb	18	44.78[b]	2.37	39.68	48.00	60.34[b]	4.76	51.26	70.25
	Internal Ph of the left hindlimb	18	45.98[b]	2.00	43.02	49.25	58.62[b]	3.53	52.53	66.56
	External Ph of the right hindlimb	18	45.23[b]	2.30	40.67	49.86	60.60[bc]	4.71	51.83	67.81
	Internal Ph of the right hindlimb	18	45.59[b]	2.64	41.51	52.71	58.31[b]	3.50	50.80	66.27

[a,b,c] Values within a column with different superscripts are significantly different (P<0.05).

There was a difference between the GL measurements of the internal phalanx media of the external phalanx media. The GL value of internal phalanx media was relatively large compared to the external ones. There was no statistically significant difference between the GL measurements of internal phalanx media of the right and left feet.

No statistical differences were found between the forelimb and hindlimb of the Bp and SD values. However, there were differences in the Bd value of phalanx media were only between internal and external phalanx media GL values. These differences were statistically significant. The GL value of the internal phalanx media of the forelimbs had the highest value.

Phalanx media index (Bp*100/GLpe; SD*100/GLpe and Bd*100/GLpe) values are given in *Table 2*. In general, there were significant differences between the index values of the phalanx media (except the right external phalanx media) of forelimb and hindlimb. The index values of phalanx media in the forelimb had a higher value. Differences between phalanx media index values of right and left feet were not statistically significant. There was no significant difference between Bp*100/GLpe value of the right rear external phalanx media and the index values of the front internal phalanx media. The same was also true for differences between the left front external and right rear external values of Bd*100/GLpe index value. For fineness index SD*100/GLpe, the forelimb had a higher value than the hindlimb and the differences were statistically significant (P<0.05).

The osteometric measurements of phalanx distalis and the index value of this bone are given in *Table 3*. The DLS value was higher in the forelimb than in the hindlimb. However, the highest value was in internal phalanx distalis. There was a statistically significant difference in DLS between the internal phalanx distalis in the forelimb and all phalanx distalis in the hindlimb (P<0.05). In the case of the external forelimb only significant difference was observed between the left phalanx distalis and right external hindlimb (P<0.05).

Table 3. *The measurements and indices of the phalanx distalis of the forelimb and hindlimb*

Measurements and Index	Phalanges	N	Mean (mm)	SD (mm)	Minimum (mm)	Maximum (mm)
DLS	External Ph3 of the left forelimb	18	72.76^{ac}	5.08	64.02	86.63
	Internal Ph3 of the left forelimb	18	75.80^{a}	6.51	64.03	93.81
	External Ph3 of the right forelimb	18	72.27^{acd}	4.98	64.12	85.06
	Internal Ph3 of the right forelimb	18	76.38^{a}	6.42	66.86	94.63
	External Ph3 of the left hindlimb	18	68.26^{bc}	3.99	61.85	79.52
	Internal Ph3 of the left hindlimb	18	68.59^{bc}	3.55	63.11	79.49
	External Ph3 of the right hindlimb	18	67.54^{bd}	3.98	61.67	79.69
	Internal Ph3 of the right hindlimb	18	68.33^{bc}	3.56	63.15	78.04
Ld	External Ph3 of the left forelimb	18	56.15^{ab}	3.61	49.86	64.35
	Internal Ph3 of the left forelimb	18	58.07^{b}	4.64	50.28	67.73
	External Ph3 of the right forelimb	18	55.06^{ab}	3.88	49.84	64.32
	Internal Ph3 of the right forelimb	18	58.59^{b}	4.66	51.66	68.70
	External Ph3 of the left hindlimb	18	56.11^{ab}	4.37	49.70	70.10
	Internal Ph3 of the left hindlimb	18	55.60^{ab}	2.80	50.72	61.05
	External Ph3 of the right hindlimb	18	54.13^{a}	3.06	50.06	60.83
	Internal Ph3 of the right hindlimb	18	55.46^{ab}	2.62	51.84	61.64
MBS	External Ph3 of the left forelimb	18	24.52^{ab}	1.12	22.17	25.95
	Internal Ph3 of the left forelimb	18	25.08^{a}	1.59	22.84	29.24
	External Ph3 of the right forelimb	18	25.02^{a}	1.41	21.77	27.14
	Internal Ph3 of the right forelimb	18	24.93^{a}	1.33	23.16	27.01
	External Ph3 of the left hindlimb	18	23.33^{b}	1.40	21.14	26.14
	Internal Ph3 of the left hindlimb	18	23.51^{b}	1.24	21.20	25.35
	External Ph3 of the right hindlimb	18	23.35^{b}	1.30	20.38	26.67
	Internal Ph3 of the right hindlimb	18	23.37^{b}	0.86	21.82	24.67
MBS*100/DLS	External Ph3 of the left forelimb	18	33.83^{a}	2.39	28.46	37.05
	Internal Ph3 of the left forelimb	18	33.27^{a}	3.08	26.44	40.73
	External Ph3 of the right forelimb	18	34.77^{a}	3.00	28.29	39.10
	Internal Ph3 of the right forelimb	18	32.81^{a}	2.71	26.60	38.48
	External Ph3 of the left hindlimb	18	34.29^{a}	2.80	29.90	39.98
	Internal Ph3 of the left hindlimb	18	34.32^{a}	1.96	30.79	36.92
	External Ph3 of the right hindlimb	18	34.69^{a}	2.88	29.29	41.67
	Internal Ph3 of the right hindlimb	18	34.28^{a}	1.96	30.61	37.24

^{a,b,c,d} *Values within a column with different superscripts are significantly different (P<0.05).* ***DLS:*** *greatest diagonal length of the sole;* ***Ld:*** *length of the dorsal surface;* ***MBS:*** *middle breadth of the sole*

Although the Ld value was relatively similar between the phalanx distalis of forelimb and hindlimb, the differences between the values of the internal phalanx distalis in the right external phalanx distalis of forelimb and hindlimb were significant (P<0.05). There were not any other statistically significant differences.

The MBS value was higher in the forelimb than in the hindlimb. The difference between them was statistically significant (P<0.05). There was no significant difference between the phalanx distalis value of the left external digit of forelimb and the others.

The MBS*100/DLS value, calculated using the length (DLS) and width (MBS) values of phalanx distalis, was slightly higher in the hindlimb but the differences were not statistically significant.

DISCUSSION

It is suggested that in ruminants which have an even number of digits in each foot, the pairs of digits are not of the equal length due to the asymmetry in these pair of digits, which indicates that they have different function in relation to stature and load bearing [2,9]. It was reported that

the differences between phalanx proximalis and phalanx media bones of cattle were more prominent than their width, but this was not associated with length and width as an index value [2,4,6,15]. The knowledge in classical anatomy books indicates that Ph1 and Ph2 is shorter in the hindlimb than in the forelimb and this information is not supported by morphometric data. In this study, it was concluded that the largest lengths of these bones (GLpe) were contrary to this discourse in both Ph1 and Ph2, and were longer in hindlimbs [4]. Similar situation was also supported by Ocal et al.[8]. While the difference in maximum length between the forelimb and hindlimb was significant for Ph1, there was a relationship between the externals of forelimb and the internals of hindlimb in Ph2. Among each limb values the GLpe values of these bones were the smallest.

It was observed that SD values were almost equal in the phalanx proximalis and phalanx media of forelimb and hindlimb and there was no significant difference between them *(Table 1)* [8]. Considering this, it is concluded that the value of SD*100/GLpe, which is called the fineness index of these bones, is more related to the length of the bones. This index value is higher in the forelimbs, rather than the SD width value of this value (GLpe) is significantly longer in the hindlimb. Bp in phalanx proximalis, Bd values in phalanx media did not show any statistically significant difference except that the differences between the inner bones of the forelimb and their hindlimb equivalents.

The importance of the difference of osteometric measurements is generally discussed in some studies to reveal the presence of asymmetry [6,7]. In the comparison of the external and internal phalanxes, it is usually observed that the mean length of the external (phalanx) proximalis and phalanx media of the 4th digit is greater than that of the third digit (internal) corresponding to this measurement, whereas the third (internal) phalanx distalis has a significantly greater average value [2]. Regarding the digits, the presence of an asymmetry between the osteometric measurements of the internal and external bones forming digits, could only be observed between the GL values of the phalanx media of forelimb.

In the majority of all other osteometric measurements, there were some significant differences between the corresponding bones of forelimb and hindlimb ($P<0.05$). The underlying reason for the asymmetry of the ruminant lower extremities especially the forelimb extremities observed in cattle despite the lack of a significant difference between the total lengths of the corresponding bones of the medial and lateral digits, is unknown [5,9]. However, considering the fact that the hardness of flooring where the animals live and walk on, the difference between the flooring conditions where cattle and dairy cows are kept may be the underlying reason of this asymmetry [2]. Therefore, we concluded that the asymmetry in the forelimb maybe a result of these cows of Holstein breed being raised as dairy cows and kept on concrete ground.

In the phalanx media of forelimb, especially the asymmetric condition in the GL measurements and the obvious difference in Bd measurements between the internals of forelimb and the phalanx media of hindlimb support the view that the body weight of the Bovidae is loaded on the central digits [2,12]. This is especially evident when the morphometric data of phalanx distalis are evaluated. Approximately 2/3 of the digital lesions affect the lateral hooves. In our study, the morphometric data of phalanx distalis are thought to be partially visible in the importance control of the difference in the forelimb and hindlimbs [16,17]. Especially in the phalanx distalis of hindlimb, there were significant differences in DLS measurement of all phalanx distalis. The front internal phalanx distalis have the largest DLS value. The difference between these bones and lateral phalanx distalis of hindlimb data is statistically significant. The lowest Ld value was obtained in the lateral phalanx distalis of hindlimb.

The age and body weight data were lacking for some cattle. Therefore, this information was not presented. The length and diameters of bones may be used as descriptive properties of cattle.

As a result, different distribution of stress to feet in cattle is thought to be related to the anatomical position of the feet. Particular differences are observed in phalanx media and phalanx distalis between the forelimb and hindlimb opposites of internal bones. The significant increase in SD*100/GLpe value, expressed as the fineness index, in forelimb in comparison to hindlimb is thought to be more significantly affected by the width value. This is because index value increases in correlation with the SD value. The same applies to the proximal and distal widths of phalanx proximalis and phalanx media. Not only the large values of the front phalanx proximalis and phalanx media, but also larger DLS and MBS values in the front phalanx distalis compared to the hinds, probably results in a larger contact area for digits. We believe that it may contribute to the fact that digital lesions are observed more frequently in phalanx distalis of hindlimb clinically, which are thought to have a smaller contact area.

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Research Article

Protective Effect of Resveratrol on Kidney and Liver Histopathology Induced by NMDA Receptor Antagonist Mk-801 in Mice [1]

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Abstract

TMK-801 is an antagonist of N-methyl-D-aspartate (NMDA) receptors. Sub-chronic administration of 1 mg / kg dose of MK-801 resulted in schizophrenia-like symptoms and degeneration in the brain of mice. In this study, it was investigated whether this dose could cause histopathological changes in kidney and liver tissues and resveratrol had a protective role against these changes. For this aim, 24 male mice were obtained and divided equally into 4 groups. The animals in the control group were intraperitoneally (i.p.) given 10 mL/kg saline solution, the animals in the experiment groups were i.p. given 1 mg/kg MK-801 alone, 40 mg/kg resveratrol alone and MK-801 by 1 mg/kg for 14 days. The kidney and the liver of the sacrificed mice were collected and routine histology procedure was applied. Glomerular shrinkage was observed in the kidneys after the measurements. Besides this finding, histopathological changes were observed in both kidney and liver tissue and resveratrol inhibited most of the harmful effects. In conclusion, MK-801 caused histopathological changes in both kidney and liver tissues, and resveratrol had a significantly protective role on this injury.

Keywords: *Histopathology, Kidney, Liver, Mice, MK-801, Resveratrol*

Farelerde NMDA Reseptör Antagonisti Mk-801 İle Böbrek ve Karaciğerde Oluşan Histopatolojik Değişikliklere Karşı Resveratrolün Koruyucu Etkisi

Öz

MK-801 N-methyl-D-aspartate (NMDA) reseptör antagonisti bir kimyasal ajandır. Bu kimyasalın sub-kronik 1 mg/kg dozunda uygulanması farelerde şizofreni benzeri semptomlar görülmesine ve beyinde dejenerasyona yol açmaktadır. Resveratrolünse hem böbrek hem de karaciğer üzerinde koruyucu etkisi vardır. Sunulan bu çalışmada hem bu dozun karaciğer ve böbrek gibi organlara histopatalojik bir zararının olup olmadığı hem de resveratrolün koruyucu etkisinin olup olmadığı araştırılmıştır. Bu amaçla 24 erkek fare alınmış ve 4 eşit gruba bölünmüştür. Kontrol grubuna intraperitoneal (i.p.) olarak %0.9'luk (10 mL/kg) fizyolojik tuzlu su verilmiştir. Deney gruplarındaki hayvanlara 14 gün boyunca MK-801 tek başına 1 mg / kg, resveratrol tek başına 40 mg/kg ve MK-801 ile birlikte i.p. verildi. Farelerin sakrifikasyonundan sonra karaciğer ve böbrek dokuları çıkartılmış, histolojik işlemlerden sonra hematoksilen-eozinle boyanmıştır. Ölçümler sonucunda glomeruluslarda istatistiksel olarak küçülme ile birlikte karaciğer ve böbrek dokusunda histopatolojik değişikliklere rastlanmıştır. Resveratrolün bu zararlı değişikliklere karşı oldukça fazla koruyucu etkisinin olduğu gözlenmiştir. Sonuç olarak MK-801'in kronik dozunun karaciğer ve böbrek üzerine zararlı etkileri olduğu ve resveratrolün bu zararlı etkilere karşı önemli miktarda bir koruyucu özelliğinin olduğu gözlemlenmiştir.

Anahtar sözcükler: *Histopatoloji, Böbrek, Karaciğer, Fare, MK-801, Resveratrol*

INTRODUCTION

N-methyl-D-aspartate (NMDA) receptors are an important factor in the synapse mechanism of the glutamatergic system [1,2]. Hypofunction of these receptors may cause cognitive and motor problems [3-8]. The chemical agent MK-801 is a blocking and neurotoxic antagonist of this receptor [6-8]. Blocking of this receptor by the 1 mg/kg

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sub-chronic dose of MK-801 causes neurodegeneration, demyelination, and schizophrenia-like symptoms in mice [7,8]. In addition, transporters and receptors of the glutamatergic system are found in many internal organs such as the testis, intestine, heart, lung, liver and kidney [9]. In the kidney, the NMDA receptor is expressed in the cortex, medulla and particularly in the renal proximal tubule [10,11].

Recently, low dose of MK-801 has been applied to kidney diseases as a therapeutic agent [12-14]. Administration of MK-801 at a dose of 0.5 mg/kg for 28 days relieved renal ischemia and reperfusion-induced glomerular and tubular functional problems [12,13]. In addition, NMDA receptor antagonist MK-801 improved the effects of gentamicin-induced renal injury [14].

However, there are some controversial points on the administration of the MK-801. MK-801 constricts the vessels of the kidney, reduces the rate of single nephron glomerular filtration, and produces abundant podocyte cytoskeleton [15-18]. In addition, MK-801 increased the detrimental effects of dexamethasone on the kidney, and the individual administration of MK-801 caused morphological changes on the kidney [19].

There is also limited information about the effect of MK-801 on the liver. Previous studies have reported that acute administration of NMDA antagonists may prevent from acute liver failure (ALF) and damage [20,21].

Resveratrol, a protective antioxidant, is a natural phytoalexin and is found especially in peanuts, grapes and red wine [22]. It is an NMDA receptor expression regulator and can be used as anti-oxidant, anti-inflammatory, anti-cancer, anti-viral, anti-ageing, anti-diabetic, and cardio-protectant [23,24]. In addition, it is also quite effective on some renal diseases such as diabetic nephropathy, drug-induced renal injury, ischemia-reperfusion, sepsis-induced, and obstructed and aging kidney [24]. In addition, resveratrol has a curative effect on the liver diseases [25].

This study investigated the possible deleterious effect of sub-chronic administration of 1 mg/kg MK-801 on the kidney and liver of mice and the possible protective role of resveratrol.

MATERIAL and METHODS

Animals

This study was performed on 24 male Balb/c mice (obtained from the Kobay Firm) under the permission of the Ethical Committee of Experimental Animals, Afyon Kocatepe University, AKUHADYEK-132-16. All the mice were housed at the Experimental Animal Research Centre of Afyonkarahisar, in the 22±2°C room temperature and a 12/12 h light/dark cycle and fed as *ad libitum*.

Groups and Dosages

The 24 mice were divided equally into 4 groups. The animals were grouped as control (i.p. 10 mL/kg saline solution), MK-801 (1 mg/kg), resveratrol (40 mg/kg) and resveratrol + MK-801 (40 mg/kg + 1 mg/kg) (n = 6 in each group). The saline, MK-801 and resveratrol doses in this study were chosen from previous studies [8,26]. All injections were performed for 14 days. In the third group, resveratrol was injected in the morning and MK-801 was injected in the afternoon.

Histological Tissue Processing

After the injections, all mice were sacrificed by decapitation and the kidney and liver tissues were collected into 10% formalin solution. After one week tissues were embedded into the paraffin and then trimmed into 5 µm thickness, consecutively. All the slides were stained with hematoxylin-eosin.

Tissue Evaluation

The mean shrinkage of the nephron was evaluated on a computer attached Olympus microscope by M-Shot software. The liver and kidney semiquantitative scoring was done according to a previous research [27].

Statistical Analysis

The data about the nephron and its compartments were estimated as means and standard deviations, and analysed using one-way analysis of variance (ANOVA) followed by Duncan posthoc test on the SPSS 16.0 software computer programme. A difference in the mean values of $P<0.05$ was considered to be significant.

RESULTS

Effect of Resveratrol on the Kidney

In the group, to which MK-801 was administered, a significant shrinkage ($P<0.001$) of the glomerular tuft was observed compared to control group *(Table 1)*. Administration of resveratrol significantly protected the mean glomerulus diameter of MK-801 treated mice ($P<0.001$). Moreover, compared to all the other groups, resveratrol did not change mean nephron diameter and bowman-space ($P>0.05$; *Table 1)*.

Effect of Resveratrol on Histopathological Examination

The histopathological changes in the kidney and liver of the animals in the experiment groups are shown in *Fig. 1* and *Fig. 2*, respectively. In the control group *(Fig. 1a, Fig. 2a)* and in the group supplied with resveratrol *(Fig. 1b, Fig. 2b),* histopathological observations in the kidney, and liver tissues were normal. In the MK-801 group, degenerative and necrobiotic changes in tubular epithelial cells as well as expansion and vacuolar degeneration of

Bowman's capsule were observed *(Fig. 1c)*. The double-nuclei hepatocytes in the periportal area and multifocal coagulation necrosis; severe hyperemia in the vessels; kuppfer cell activation; increase in the number of the bile ducts and focal mononuclear cell infiltration were observed in the liver tissue of the mice *(Fig. 2c)*. In the MK-801 + resveratrol group, slight histopathological changes were observed respectively in kidney and liver tissues *(Fig. 1d, Fig. 2d)*. Moreover, the quantitative assessment of the histopathological changes in the kidney and liver of mice from different groups is summarised in *Table 2* like this; -: no lesion; +: mild; ++:moderate; +++: severe. Our results demonstrated that resveratrol reduced the degenerative effects of MK-801 and protected the kidney and liver tissues.

DISCUSSION

The animal schizophrenia-model caused by MK-801 is used to observe the effects of this illness on the brain. Sub-chronic dose of 1 mg/kg MK-801 for 14 days was reported to cause schizophrenia-like symptoms in mice [7,8]. However, there was insufficient information about the effects of this dose on the liver and kidney. Therefore, this study focused on the effect of this dose on both the kidney and liver tissues. The effect of this dose was evaluated by histopathological inspections on the liver and kidney.

According to the literature, the MK-801 has protective effects on renal and liver degeneration [12-14,20,21]. However, in these studies, the protective dose was relatively lower than the sub-chronic dose. In addition, some previous studies have shown that 1 mg/kg MK-801 has adverse effects on the internal organs [28]. Previous studies have reported that administration of MK-801 to the kidney results in constriction of the renal vessels and reduces glomerular filtration rate and production of podocytes [15-18]. In this study, similar to these reports, the application of MK-801 to the kidney showed some histological changes. MK-801 at a dose of 0.3 mg/kg for 8 days caused a slight narrowing of the urinary cavity in the nephrons and the use of MK-801 with dexamethasone increased the severity of hyperemia and dilatation of these cavities [19]. However, in this study, application of MK-801 caused an expansion in the nephro-glomerular space, contraction of glomerulus diameter,

Table 1. *Measurement of the nephron and its compartments*

Groups	The Mean Nephron Diameter (µm)	The Mean Glomerulus Diameter (µm)	The Bowman-space (µm)
Control	145±8	116±6^{a}	27±2
MK-801	135±5	98±8^{c}	39±12
RES+MK-801	134±15	104±8^{bc}	31±10
RES	140±5	111±4^{ab}	32±7
P value	0.175	<0.001	0.084

Values are the mean ± S.D, n=6; ^{a,b,c} The mean glomerulus diameter with different letters in the same column shows statistically significant differences (P<0.05)

Table 2. *The semiquantitative scoring of the liver and kidney*

Tissue	Histopathological Findings	Control	Resveratrol	MK-801	MK-801 + Resveratrol
Liver	Double-nuclei hepatocytes in the periportal area and multifocal coagulation necrosis	- (7/7)	-(7/7)	+ (5/7) ++ (1/7) +++ (1/7)	- (7/7)
	Kuppfer cell activation	- (7/7)	-(7/7)	- (4/7) + (1/7) ++ (2/7)	- (6/7) + (1/7)
	Hiperemia in the vessels	- (7/7)	-(7/7)	+ (5/7) ++ (2/7)	- (7/7)
	Increase in the number of the bile ducts and focal mononuclear cell infiltration	- (7/7)	-(7/7)	+ (4/7) ++ (3/7)	- (7/7)
Kidney	Vacuolar degeneration in the glomerulus	- (7/7)	-(7/7)	- (1/7) + (4/7) ++ (2/7)	- (7/7)
	Expansion in the bowman-space	- (7/7)	-(7/7)	+ (2/7) ++ (5/7)	- (7/7)
	Degenerative and necrobiotic changes in the tubular epithelial cells	- (7/7)	-(7/7)	+ (4/7) ++ (2/7) +++ (1/7)	- (6/7) + (1/7)

The findings were evaluated and scored as follows: -: no lesion; +: mild; ++: moderate; +++: severe

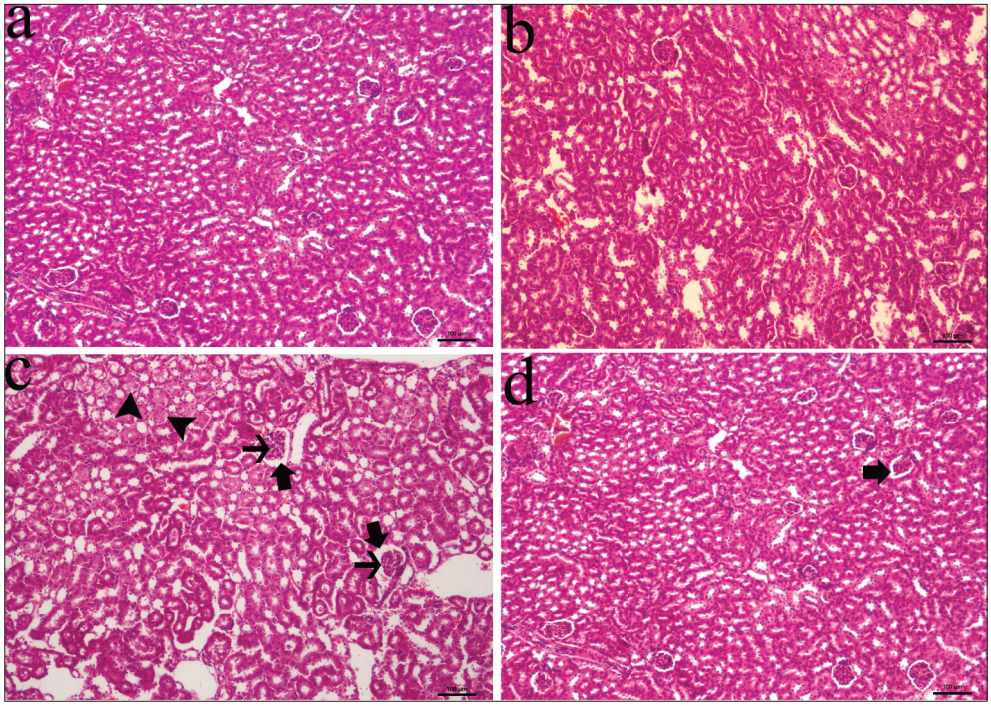

Fig 1. The effect of resveratrol on Mk-801 induced damage in kidney of mice. **a.** Control group, **b.** Resveratrol group, **c.** MK-801 group; *thick arrow:* Expansion in the bowman-space, *thin arrow:* Vacuolar degeneration in the glomerulus, *arrowhead:* Degenerative and necrobiotic changes in the tubular epithelial cells, **d.** MK-801 + Resveratrol group, *thick arrow:* Expansion in the nephroglomerular space

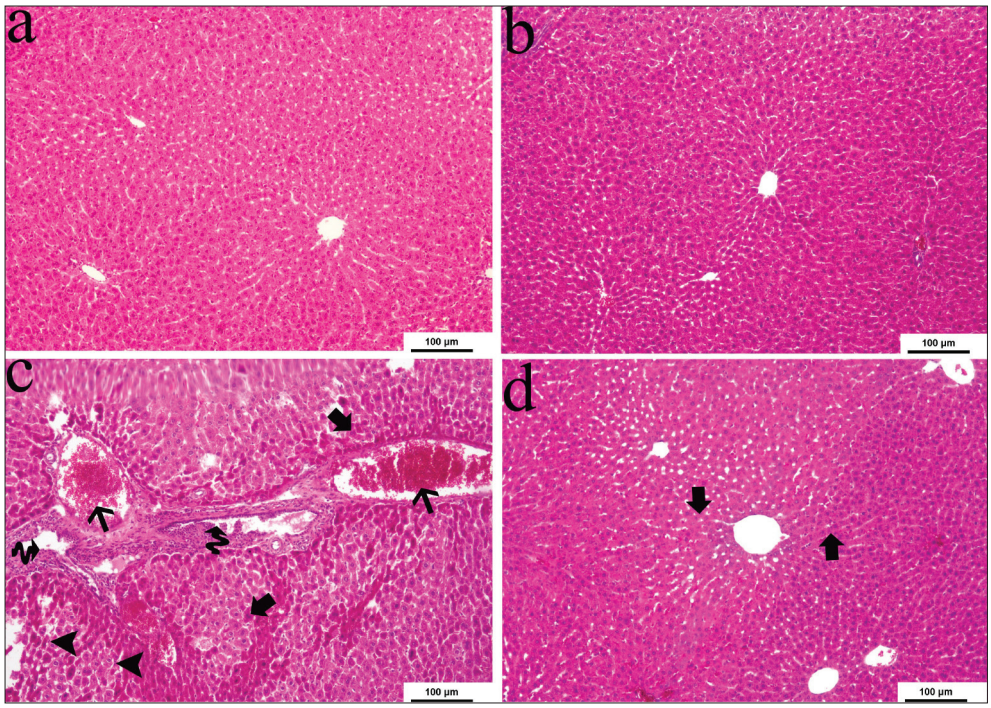

Fig 2. The effect of resveratrol on Mk-801 induced damage in liver of mice. **a.** Control group, **b.** Resveratrol group, **c.** MK-801 group, *thick arrow:* Double-nuclei hepatocytes in the periportal area and multifocal coagulation necrosis, *thin arrow:* Severe hyperemia in the vessels, *arrowhead:* Kuppfer cell activation, *curved arrow:* Increase in the number of the bile ducts and focal mononuclear cell infiltration, **d.** MK-801+Resveratrol group, Vacuolar degeneration areas around the central vein

vacuolar degeneration in glomerulus, and degenerative and necrobiotic changes in tubular epithelial cells. The administration of resveratrol has protected the many of these harmful effects. In one study, xenobiotics applied to the body caused histopathological deterioration in kidney and liver tissues and resveratrol protected against these disorders [29]. Beside the histopathological findings, a dose-dependent increase in cell death and apoptosis were also observed in the renal culture cells exposed to the MK-801. Thus, over excitation or blockade of the renal NMDA receptor leads to cell death [11].

There is insufficient information about the effect of subchronic administration of MK-801 on liver tissue. Recently, it has been reported that MK-801 has a positive effect on ammonia-induced activation of ALF [21]. ALF leads to activation of NMDA receptors in the brain, and neuronal damage ultimately results in death. Blocking NMDA receptors with MK-801 protects or delays death from ALF. But, in the clinics the routine or chronic administration of MK-801 has secondary effects and this is the biggest handicap for the treatment [21]. Additionally in the present study, double-nuclei hepatocytes in the periportal area,

multifocal coagulation necrosis, kuppfer cell activation, hyperemia in the vessels, increase in the number of the bile ducts and focal mononuclear cell infiltration in the liver and the protective effect of the resveratrol against these impairments were observed.

As a result, administration of resveratrol against MK-801 significantly protected kidney and liver tissues. Particularly, epithelial cells are NMDA receptor expression sites and resveratrol is thought to be important in protecting this site from necrosis.

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Research Article

Gossypin Protects Against Renal Ischemia- Reperfusion Injury in Rats

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Abstract

Renal injury occurring as a result of renal ischemia-reperfusion may lead to renal failure or even death. The aim of this study is to investigate possible protective effects of Gossypin on tissue damage occurred due to ischemia-reperfusion in rat kidney tissue. A total of 48 male Wistar albino rats were used in the study. These rats were randomly divided into 6 groups equally (n = 8). The created groups were control (C), sham (S), ischemia-reperfusion (I/R), I/R + DMSO, I/R + 400 µg/kg gossypin and I/R + 4 mg/kg gossypin. In the rats of sham group, the right nephrectomy was performed. In the rats of other groups rather than sham, the left renal artery was clamped after performing the right nephrectomy. Gossypin was administered intraperitoneally before the reperfusion. 24 h reperfusion was applied to the left renal after 1 h of ischemia. TNF-α, IL-1β, IL-6 and IL-10 levels were measured with spectrophotometric methods in the kidney tissues after the procedures were completed. Apoptosis and inflammatory pathways were evaluated histopathologically using Caspase 3 and NF-κB antibodies. There was a statistically significant decrease in IL-1β and IL-6 levels of the gossypin groups compared to the I/R group ($P<0.05$). As the level of TNF-α was decreased in the gossypin administered groups compared to the I/R group although not statistically significant, the level of IL-10 was increased. In the present study, we aimed to show that gossypin in renal I/R model is effective on inflammatory process and apoptosis and that it can be used in routine treatment to decrease the damage in all reasons that may cause I/R. In addition, this study can shed light on the studies to be done in this field in the future.

Keywords: *Renal ischemia reperfusion injury, Gossypin, Cytokines, Caspase-3*

Gossypin Sıçanlarda Böbrek İskemi- Reperfüzyon Hasarına Karşı Korur

Öz

Böbrek iskemi reperfüzyonu sonucu meydana gelen renal hasar, böbrek yetmezliğine ve hatta ölüme neden olabilir. Çalışmanın amacı sıçan böbrek dokusunda iskemi-reperfüzyona bağlı oluşan doku hasarına karşı gossypinin olası koruyucu etkilerini araştırmaktır. Çalışmada toplam 48 adet Wistar albino cinsi erkek sıçan kullanıldı. Sıçanlar randomize ve eşit olmak üzere 6 gruba ayrıldı (n=8). Kontrol (C), sham (S), istemi-reperfüzyon (I/R), I/R + DMSO, I/R + 400 µg/kg gossypin ve I/R + 4 mg/kg gossypin grupları oluşturuldu. Sham grubunda arka bölge açılarak sağ nefrektomi yapıldı. Gossypin reperfüzyondan önce intraperitoneal olarak uygulandı. Sol renal artere 1 saat iskemi sonunda 24 saat reperfüzyon uygulandı. Prosedürler tamamlandıktan sonra böbrek dokularında spektrofotometrik metodlarla TNF-α, IL-1β, IL-6 and IL-10 seviyeleri ölçüldü. Apoptozis ve inflamatuvar yolaklar kazpaz-3 ve NF-κB antikorları kullanılarak değerlendirildi. Gossypin grupları I/R grupları ile kıyaslanınca IL-1β and IL-6 seviyelerinde istatistiksel olarak önemli derecede azalma tespit edildi ($P<0.05$). I/R grubu ile kıyaslandığında gossypin uygulanan gruplarda istatistiksel olarak anlamlı olmasa da TNF-α miktarlarına azalma tespit edildi. Mevcut çalışmada gossypinin I/R modelindeki inflamasyon aşaması ve apoptosis üzerine etkisini ve I/R neden olan tüm durumlarda oluşacak hasarı azaltmada kullanılabileceğini göstermeye çalıştık. Ek olarak, bu çalışma bulanda ileride yapılacak olan çalışmalara ışık tutabilir.

Anahtar sözcükler: *Renal istemi reperfüzyon hasarı, Gossypin, Sitokinler, Kaspaz-3*

INTRODUCTION

Renal ischemic injury is a complex syndrome characterized by an accelerated cycle of inflammation, cell damage, and persistent local ischemia caused by many celluler anomalies [1]. Kidney is particularly sensitive to ischemia reperfusion (I/R) injury due to its high metabolism and vascular anatomy. Acute renal injury causes acute and

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chronic renal failure. The I/R injury is seen as secondary to trauma, shock, sepsis, renal transplantation, cardiovascular and urological surgery in intensive care units [2,3]. Renal I/R injury resulting in acute renal failure is a major clinical problem due to the high mortality rate [4,5]. Therefore, treatment strategies or therapeutics that prevent or reduce I/R-induced acute renal injury have clinical significance.

It was shown that increased production in reactive oxygen species (ROS) caused by I/R activates leukocyte infiltration in the kidney, and these infiltrated-leukocytes synergistically produce more ROS and cytokines that directly lead to renal damage [6]. ROS also inactivate antioxidant enzymes [7,8] and cause increase in activated neutrophils and cytokines [9,10] and contributes to the activation of apoptotic genes [11,12]. I/R increases level of pro-inflammatory cytokines such as nuclear factor kappa B (NF-κB) which plays a role in the regulation of various genes involved in the acute phase inflammatory reaction [13], tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and decreases level of anti-inflammatory cytokines such as interleukin-10 (IL-10) [14-18]. Gossypin (Gos) (gossypin-8-O glucoside, 3,5,7,3,4 -pentahydroxy-8-0-glucosylflavone) is a bioflavanoid naturally found in plants of the family Malvaceae, especially in Hibiscus vitifolius [19,20]. The ability of Gos to protect against various diseases has been proven in many studies [21-27]. In many studies, it was shown that Gos has antioxidant, antiinflammatory and analgesic properties [19,23,28,29]. The aim of this study is to investigate the possible renoprotective effects of Gos as a new alternative to this damage by examining inflammation markers and apoptosis process molecules in the experimental renal I/R injury model by histopathological methods.

MATERIAL and METHODS

Chemicals

Gossypin (Biovision, USA) was dissolved in dimethyl-sulfoxide (DMSO, Amresco, Canada) and administered intra-peritoneally at 400 µg/kg and 4 mg/kg doses.

Approval of Ethics Committee and Center of Research

The study with Atatürk University Animal Experiments Local Ethics Committee approval (dated 19.04.2016, with decision no: 2/71) was conducted at the Atatürk University Experimental Animal Production and Research Center. All the procedures in the study were performed in line with the ethics committee protocol. During the course of the experiment, rats were conserved in 12 h light/12 h dark cycle at 20-22°C, and the ad libitum feeding of standard chow and normal tap water was performed in rats.

Experimental Animals and Creating Groups

A total of 48 male Wistar albino rats (12-16 weeks, 240-260 g) were used in the study. 6 groups with 8 rats in each were randomly formed. Group 1 is the control group with performing no surgical intervention. The second group was sham group and back region of rats in this group were opened with the help of a bistoury, and the right renal pedicle was dissected by connecting with silk. The experimental model was performed over single kidney (left kidney). The third group was the I/R group. In third group, after similar application of the procedures of the second group, the left renal pedicle was clamped. After 1 h of ischemia, the clamp was opened and kidney was subjected to reperfusion for 24 h. The animals in the 4. (I/R + DMSO), 5. (I/R + 400 µg/kg gossypin) and 6. (I/R + 4 mg/kg gossypin) groups underwent surgical procedures. And then, 300 µL of DMSO to 4. group, 400 µg/kg of gossypin to 5. group and 4 mg/kg of gossypin to 6. group were administered intraperitoneally before starting the reperfusion. At the end of the study, renal tissues in all groups were taken for necessary analyses.

Collection and Storage of Tissue Specimens After Sacrification

The experimental model in rats was performed under anesthesia formed with intramuscular administration of 75 mg/kg ketamine, 8 mg/kg xylazine. The kidney tissues of the sacrificed rats were divided in two, and one of the pieces was placed in a 10% formaldehyde solution for histopathological procedures and the other was stored at -80°C for biochemical analyses.

Tissue Homogenization and Determination of Biochemical Parameters

For biochemical measurements, 10% homogenate was formed by adding phosphate buffer to kidney tissues and then homogenized by centrifuging at 12.000 rpm for 1-2 min. on ice (IKA, Germany). Homogenized tissue samples were centrifuged at 5000 rpm for 30 min at +4°C to obtain supernatant. In the biochemical analysis of the groups, IL-1β [Cat No: E-EL-R0012, Elabscience], IL-6 (Cat No: E-EL-R0015, Elabscience), IL-10 (Cat No: E-EL-R0016, Elabscience), TNF-α (Cat No:E-EL-R0019, Elabscience) levels were measured from supernatants using rat specific ELISA kits. Measurements were performed in accordance with kits' own protocols.

Histopathological Examination

Caspase-3, NF-κB antibodies were used to investigate apoptosis and inflammatory pathways in the kidney tissues of the groups. Hematoxylin-eosin staining method was used to determine the damage levels.

Statistical Analysis

The IBM SPSS 20.0 package program was used in the analysis. Using the One Way ANOVA method for statistical analysis, $P<0.05$ was considered statistically significant. Data were expressed as mean ± standard deviation.

RESULTS

Cytokine concentration was measured after renal reperfusion of the kidney for 24 h. The TNF-α levels for each group are shown in *Fig. 1A*, there was no difference among the groups. Renal I/R caused a marked elevation of proinflammatory cytokines, IL-1β and IL-6 in kidney tissue *(Fig. 1B,C*, respectively). In the groups treated with gossypin, the levels of these cytokines decreased. The level of IL-10, an anti-inflammatory cytokine, in renal tissue was significantly reduced in rats with renal I/R, and IL-10 levels were slightly increased in groups treated with Gossypin *(Fig. 1D)*.

In *Fig. 2*, it is shown that the staining of the groups by the hematoxylin-eosin method. Differences and similarities between the groups have been expressed in various symbols.

Caspase-3 immunohistochemical staining of the study groups are shown in *Fig. 3* and their evaluation is shown in *Table 1*. In *Table 1*, it is seen that there was a decrease in Caspase-3 immunopositivity of podocytes and tubule cells in the Gos groups compared to I/R group. NF-κB immunohistochemical staining of the study groups are shown in *Fig. 4* and their evaluation is shown in *Table 2*. In *Table 2*, it is seen that there was a decrease in NF-κB immunopositivity of podocytes and tubule cells in the gos groups compared to I/R group.

DISCUSSION

Renal I/R injury occurs by reperfusion after reduced or discontinuation of blood flow to the kidneys, and causes acute renal failure. It is a common condition in many surgical procedures [30-32]. Acute renal failure caused by I/R injury is a serious health problem and, unfortunately, there is no therapeutic or protective agent in this disease at the present. The aim of this study was to investigate the protective effects of GOS against I/R induced renal injury by some cytokine levels and histopathological analysis of NF-κB and caspase-3 immunopositivity. Many studies have shown that gossypin is a flavonoid with strong anti-inflammatory and immunomodulatory properties [20,21,29]. However, the factors that mediate the anti-inflammatory effect of GOS remain unclear. Therefore, we tried to understand the effects of GOS on I/R-induced renal injury and the underlying anti-inflammatory mechanisms.

Initiation of reperfusion of GOS in ischemic tissue causes inflammatory reactions [33]. One of the most known intracellular signaling pathways of inflammatory responses is the NF-κB signaling pathway [32]. In many studies, it was shown that NF-κB is an important transcription factor during inflammatory process and ischemia reperfusion and NF-κB activation is responsible for the activation of many proinflammatory cytokines such as interleukin-1β, interleukin-6, tumor necrosis factor-α [34-36].

In knockout mice, it is considered that NF-κB plays an important role in reducing sensitivity to I/R injury, and NF-κB-mediated inflammatory responses cause tissue damage [37]. In many I/R injury studies, levels of various proinflammatory cytokine such as TNF-α, IL-1 and IL-6 were reported to significantly increase during reperfusion [10,38,39]. IL-10, an anti-inflammatory cytokine [40,41], reduced the renal injury in mice by inhibiting inflammatory and apoptotic pathways [42]. In studies of gossypin, no information about IL-10 has been seen. In a nephrotoxicity model, it was shown that increased TNF-α, IL-1 and IL-6 levels in the kidney were reduced by gossypin administration [23]. It was shown that gossypin inhibited NF-κB, in a culture study [29]. In parallel with this study, we determined that NF-κB immunopositivity decreased in gossypin groups

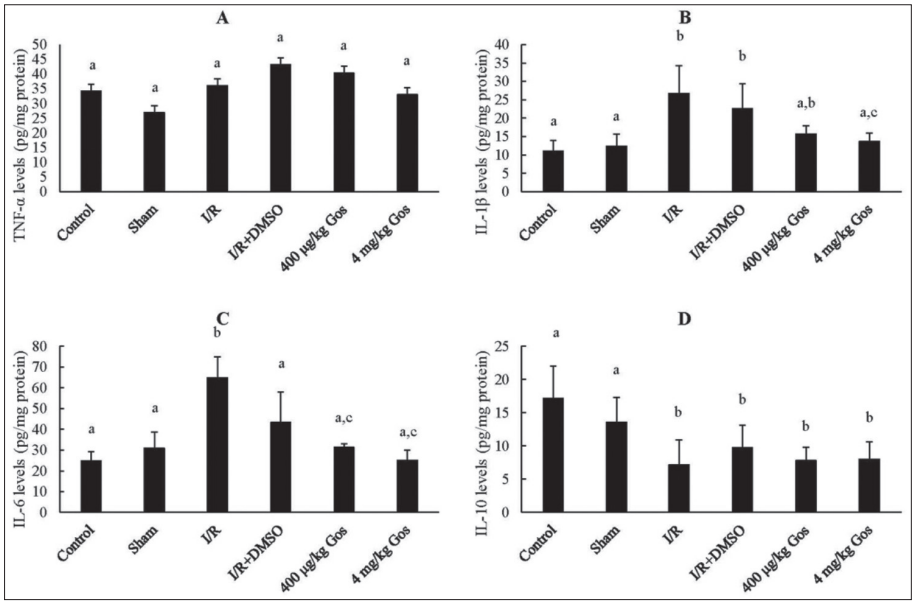

Fig 1. There was no significant difference between the groups in terms of TNF-α levels P>0.05, IL-1β and IL-6 levels b; P<0.05, c; P<0.05, IL-10 level b; P<0.05

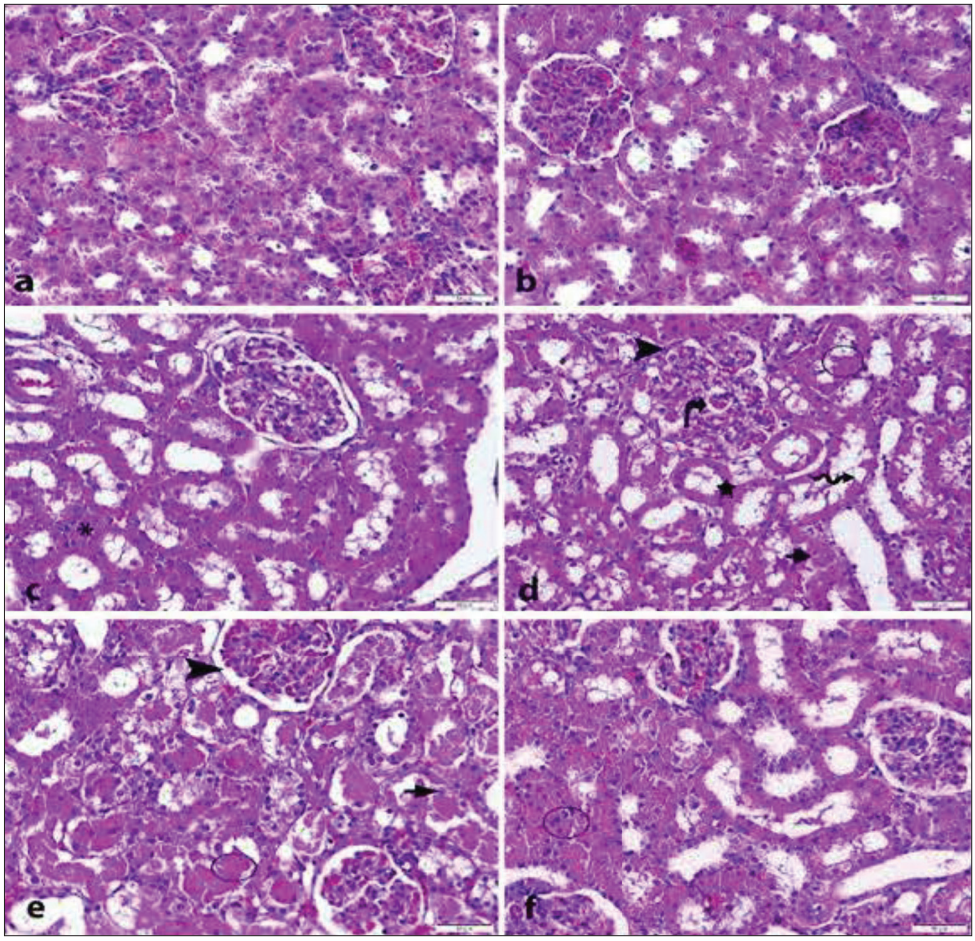

Fig 2. Hematoxylin-Eosin, **a:** Control Group; **b:** Sham Group; **c:** I/R + DMSO Group; **d:** I/R Group; **e:** I/R + 400 μg/kg Gossypin Group f: I/R + 4 mg/kg Gossypin Group. *Asterix:* Increase in connective tissue; *Arrowhead:* irregularities in Bowman's range; *Star:* Cellular loss in tubules; *Spiral arrow:* Tube basal membrane defect; *Thin arrow:* Hypertrophy in proximal tubule cells; *White arrow:* Distal tubule pyknotic nucleus; *Rotating arrow:* Capillary dilatation; *Circle:* Hyalinization in Tubules- 20X (scale bar 50 μm)

Table 1. *Caspase-3 immunopositivity*

Immunoreactivity	Control	Sham	I/R	I/R+DMSO	400 μg/kg Gos	4 mg/kg Gos
Podocyte cells	-	-	+++	-	+	+
Tubule cells	-	-	+++	+	++	+

Expression levels; -: No immunopositivity; + Mild immunopositivity; ++ Moderate immunopositivity; +++ Severe immunopositivity

Table 2. *NF-κB immunopositivity*

Immunoreactivity	Control	Sham	I/R	I/R+DMSO	400 μg/kg Gos	4 mg/kg Gos
Podocyte cells	-	-	+++	-	++	+
Tubule cells	-	-	+++	+	++	+

Expression levels; -: No immunopositivity; + Mild immunopositivity; ++ Moderate immunopositivity; +++ Severe immunopositivity

compared to I/R groups. In our study, the level of TNF-α decreased in the gossypin administered groups although not statistically significant, compared to the I/R group. It was shown that there was a statistically significant decrease in IL-1β level of the gossypin groups compared to the I/R group. Irfan et al. reported that NF-κB and some cytokine levels decreased in sepsis model similar to our results [21]. In statistical analysis, IL-10 levels were detected to be significantly decreased in the I/R group compared to the control group. IL-10 levels in gossypin administered groups increased, although not statistically significant, compared to I/R group. When viewed as a whole, it was

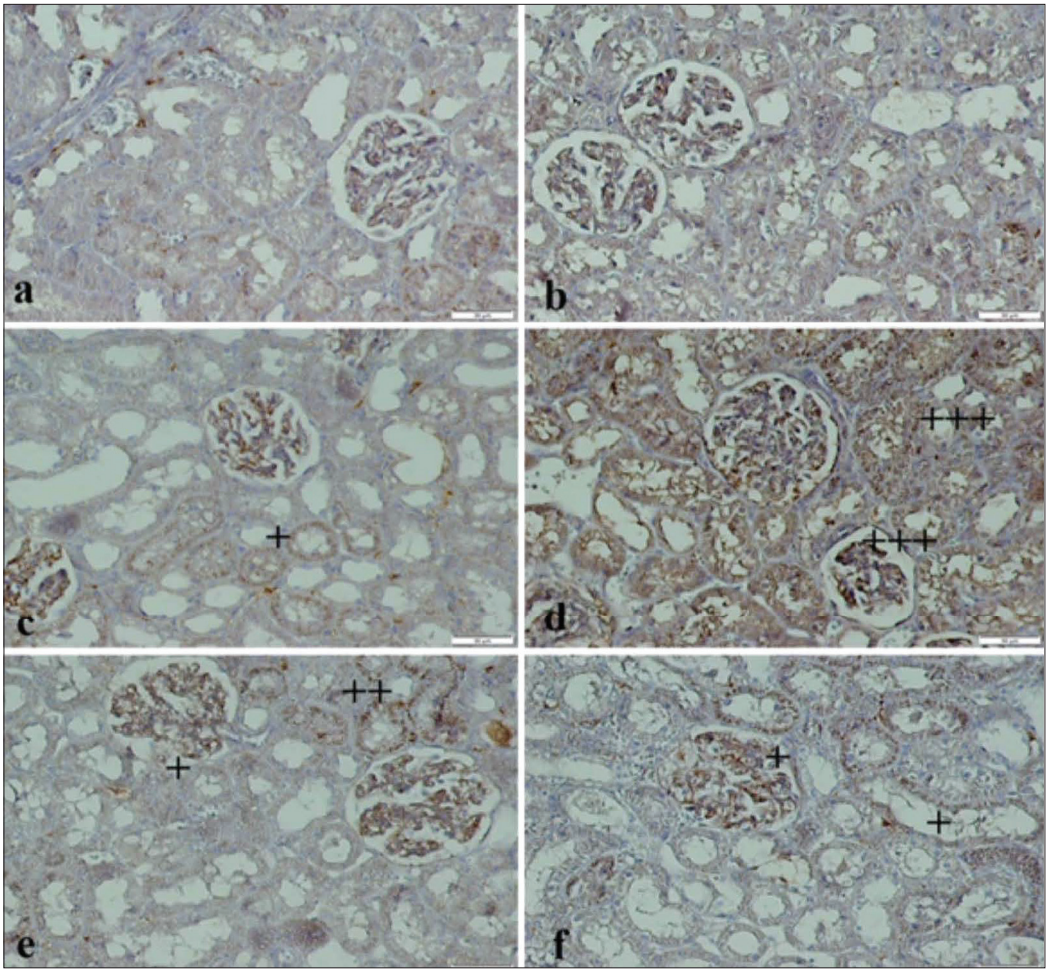

Fig 3. Caspase-3, **a:** Control Group; **b:** Sham Group; **c:** I/R + DMSO Group; **d:** I/R Group; **e:** I/R + 400 μg/kg Gossypin Group; **f:** I/R + 4 mg/kg Gossypin Group (scale bar: 50 μm)

shown that gossypin reduces the I / R injury by suppressing the inflammatory pathway.

Apoptosis is important for the development and homeostasis in many types of tissue [43]. Apoptosis is a programmed cell death caused by endogenous or exogenous factors. It eliminates abnormal or dead cells to maintain homeostasis. Apoptosis and necrosis are two main types of cell death during I/R injury, and more than half of the dead cells die of apoptosis during the first 24 h of reperfusion [44-46].

When the caspases that play a role in the later stages of the apoptosis pathway activated once, the effector caspase induces a series of hydrolysis reactions leading to the initiation of cell death [47]. Caspase-3 is an important marker of apoptosis [48-50] and leads to the initiation of cascades causing apoptosis [51]. It is widely accepted that Caspase-3 is an important protease and is an important effector substance involved in hydrolysis by acting alone or in association with apoptosis-related proteins [52,53]. We encountered only two cancer studies investigating the effect of gossypin on apoptosis in the literature and it was shown that gossypin increases apoptosis to destroy the cancer in these studies [29,54]. In the present study, we determined that there was a decrease that is in caspase-3 immunopositivity of podocytes and tubule cells in the gossypin groups compared to I/R group, and we showed that gossypin has a renoprotective effect due to antiapoptotic properties by reducing the level of Caspase-3 in contrast to the effect observed in cancer.

As a result, treatment with Gossypin significantly reduced the renal injury caused by renal I/R. However, both treatment doses used in the study reduced cytokine levels and oxidative stress, suppressed apoptosis in kidney tissues.

Conflict of Interest

None.

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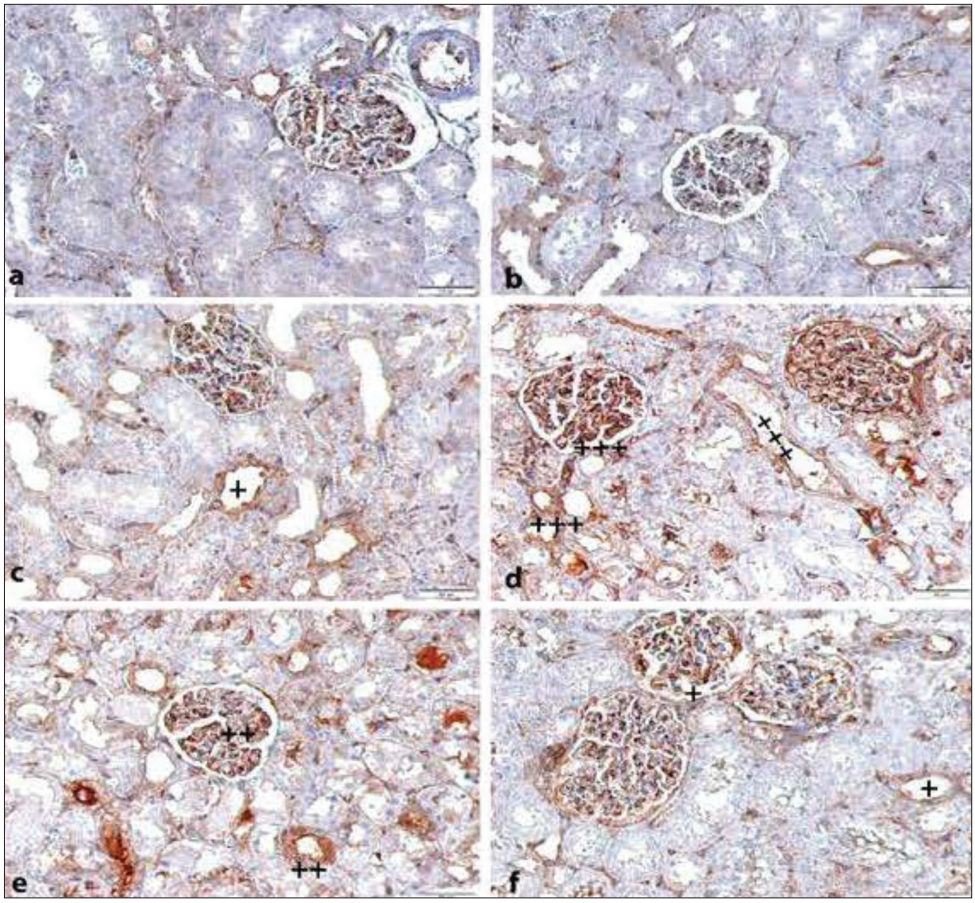

Fig 4. NF-κB, **a:** Control Group; **b:** Sham Group; **c:** I/R + DMSO Group; **d:** I/R Group; **e:** I/R + 400 μg/kg Gossypin Group; **f:** I/R + 4 mg/kg Gossypin Group

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Research Article

Determination of Kanamycin Residue in Anatolian Buffalo Milk by LC-MS/MS

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Abstract

The present study aimed to evaluate the persistence of kanamycin in lactating Anatolian buffalo milk followed by an intramuscular injection of kanamycin. The collection of milk samples was performed twice daily up to the 10th milking followed by kanamycin injection and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was employed for the residue analysis. The detection limit of the method was determined as 3.56 µg/kg. The highest concentrations of kanamycin were determined in the first milking after injection and mean concentration of this milking was found to be as 1473 µg/kg. Kanamycin residue in all buffalo milk samples was lower than the maximum residue limit (150 µg/kg) with the fifth milking. In addition, a monitoring study was conducted to determine whether buffalo milk marketed in Afyonkarahisar pose a public health risk regarding kanamycin residue. The results of the monitoring study showed that one (2%) of the 50 buffalo milk samples contained kanamycin at the concentration of 22.36 µg/kg. In conclusion, this study determined the persistence of kanamycin in Anatolian buffalo milks based on an LC-MS/MS method. In addition, results of the study showed that buffalo milks marketed in Afyonkarahisar Province pose a very low risk regarding kanamycin residue.

Keywords: *Kanamycin, Aminoglycosides, Anatolian buffaloes, Milk, LC-MS/MS*

Anadolu Manda Sütlerinde Kanamisin Kalıntısının LC-MS/MS İle Belirlenmesi

Öz

Bu çalışmada, laktasyondaki Anadolu mandalarına intramüsküler olarak kanamisin enjekte edilmesini takiben sütlerindeki kanamisin kalıntısının belirlenmesi amaçlandı. Süt numunelerinin toplanması, kanamisin enjeksiyonunu takiben günde iki kez 10'uncu sağıma kadar gerçekleştirildi ve kalıntı analizi için likit kromatografisi tandem kütle spektrometresi (LC-MS/MS) kullanıldı. Metodun tespit limiti 3.56 µg/kg olarak belirlendi. En yüksek kanamisin konsantrasyonları enjeksiyondan sonraki ilk sağımda tespit edildi ve sağımdaki ortalama konsantrasyon 1473 µg/kg olarak belirlendi. Manda sütü örneklerinde kanamisin kalıntı düzeyinin beşinci sağımdan itibaren maksimum kalıntı sınırından (150 µg/kg) daha düşük olduğu belirlendi. Ayrıca, Afyonkarahisar'da pazarlanan manda sütlerinin kanamisin kalıntısı konusunda halk sağlığı açısından risk oluşturup oluşturmadığını belirlemek amacıyla bir saha taraması yapıldı. Saha taraması sonucunda 50 manda sütü örneğinden birinin (%2) 22.36 µg/kg konsantrasyonunda kanamisin içerdiği saptandı. Sonuç olarak bu çalışmada kanamisinin Anadolu manda sütlerinde kalıcılığı LC-MS/MS metodu ile belirlendi. Ayrıca, çalışmanın sonuçları Afyonkarahisar'da tüketime sunulan manda sütlerinin kanamisin kalıntısı açından çok düşük risk taşıdığını gösterdi.

Anahtar sözcükler: *Kanamisin, Aminoglikozidler, Anadolu mandası, Süt, LC-MS/MS*

INTRODUCTION

Milk is an important source for human diet due to its major nutrients including fat, proteins, and carbohydrates. Also, it contains essential vitamins and minerals such as calcium, selenium, magnesium, riboflavin, pantothenic acid and

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vitamin B_{12} [1,2]. Buffalo milk also possesses rich nutrient content and it is the most produced milk source after the cow milk worldwide. In addition, this valuable milk source is employed in the production of many dairy products including cheese, cream, butter, and yoghurt [3,4].

Antibiotics are potent chemicals used for the treatment of various diseases in livestock animals [5]. Kanamycin as a broad-spectrum aminoglycoside antibiotic is widely used for the treatment of pneumonia, mastitis, and diarrhoea in veterinary medicine. This antibiotic exhibits its antimicrobial effect by interfering with ribosomal RNA of gram-negative bacteria [6]. Nevertheless, kanamycin may also induce several side effects such as allergic reactions, ototoxicity, nephrotoxicity, hematopoietic system toxicity, and neuromuscular blocking in human and animals [7]. Improper use of antibiotics can cause residue risk in foods of animal origin, which may cause food safety concerns [8,9]. To protect public health and food safety, the maximum residue limit for kanamycin in milk was established by the European Union as 150 µg/kg [10].

Several detection methods including immunoassays, capillary electrophoresis, high-performance liquid chromatography, gas chromatography, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were developed for aminoglycoside residue analyses with different sensitivities. However, LC-MS/MS is accepted as the most reliable confirmatory method based on its high sensitivity and accuracy [11,12].

Several studies were reported on pharmacokinetic features of aminoglycosides for farm animals [13-15]. However, the information about the persistence of this antibiotic in the milk of Anatolian buffaloes is lacking. The present work determined kanamycin persistence in Anatolian buffalo milk by means of LC-MS/MS method. Also, the developed method was employed to analyze 50 buffalo milk samples marketed in Afyonkarahisar, Turkey.

MATERIAL and METHODS

Material

Kanamycin sulfate and formic acid (LC-MS grade) obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and water (LC-MS grade) were purchased from Merck (Darmstadt, Germany). All other reagents and chemicals were analytical grade provided by commercial sources.

Five clinically healthy female Anatolian buffaloes, weighing 400-500 kg were selected for this study. The experimental animals were obtained from Afyon Kocatepe University, Veterinary Faculty Research and Application Farm. This study was approved by Afyon Kocatepe University Animal Experiments Local Ethics Board (Approval no. 49533702/77). All animals were kept under similar conditions having standard ration and free access to water. Each experimental animal was intramuscularly given a single dose of kanamycin 10 mg/kg with a commercial product (Kanovet, Vetaş, İstanbul, Turkey). Milk samples were collected during 5 days at the 0 (blank sample), 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 h. A blank sample was taken prior to drug administration from each animal. Collected milk samples were directly stored at -20°C for further analysis.

From August to December 2017, a total of 50 buffalo milk samples were collected in Afyonkarahisar province, Turkey. Milk samples were provided by producers and local markets. All samples were transported to the laboratory immediately after sampling under cold conditions and stored at -20°C in a deep freezer for further analysis.

The stock solution of kanamycin was prepared in distilled water (1 mg/mL) and stored at -20°C prior to use. Working solutions of kanamycin were also prepared in distilled water by serial dilution. To generate eight-point concentrations (0.5, 1, 2, 5, 10, 20, 50, 100 ng/mL) of the calibration curve, calibration standard samples were prepared in milk by spiking with an appropriate volume of serially diluted stock solution.

Methods

The extraction of milk samples was performed as previously described by Jank et al.[16] with some modifications. Briefly, each milk sample (2 mL) was transferred into a polypropylene centrifuge tube and then mixed with acetonitrile (4 mL). The purification of milk samples was completed by three centrifugation steps for 10 min at 4000 rpm and 4°C (except last one which was performed at 2°C). Then, the supernatant was kept in a water bath (≤45°C) that was evaporated under an N_2 stream until the reduction of the volume solvent to approximately 500 µL and the volume was adjusted to 1ml before transferred to HPLC vials.

The LC/MS/MS analysis of Anatolian Buffalo milk samples was carried out via Agilent Technologies 1200 series (Waldbronn, Germany), attached with a binary high-pressure gradient pump. Atlantis HILIC column (150 × 2.1 mm, 3 µm; Waters, Milford, MA, USA) was employed for LC separation at 40°C. The mobile phases consisted of solvent A (0.1% formic acid solution) and solvent B (acetonitrile containing 0.1% formic acid). The gradient of LC separation was as follows: 0.0 min, A/B (80/20); 3.0 min, A/B (10/90); 4.0 min, A/B (10/90); 4.10 min, A/B (80/20). The flow rate of the mobile phase was set at 0.4 ml/min and the injection volume of the sample was 10 µL.

Agilent 6460 LC/MS Triple Quadrupole instrument equipped with an ESI (Waldbronn, Germany) was used for mass spectrometry analysis. A nitrogen generator (Balston, Haverhill, MA, USA) was employed to produce nebulizer and drying gas (350°C). All MS parameters including sheath gas flow, nebulizer gas, capillary voltage sheath gas temperature, and Collision Energy were as 10 L/min, 40 p.s.i., 4000 V, 400°C, and 15 eV, respectively. Positive ion

mode was chosen for all MS analysis. Kanamycin retention time was found to be as 2.65. Its molecular weights, precursor ions (m/z), and product ions (m/z) were 485.0, 324.1, 162.8 respectively.

The method was validated by spiking raw milk samples. The quality parameters established were linearity range, limit of detection (LOD), limit of quantification (LOQ), recovery, and intra- and inter-day precisions. The limit of detection (LOD) was defined as the lowest concentration of kanamycin that the analytical process can reliably differentiate from background levels (signal-to-noise ratio≥3), while the limit of quantification (LOQ) was defined as the lowest concentration of kanamycin that can be quantified (a signal-to-noise ratio ≥10).

RESULTS

The typical chromatogram of kanamycin was shown in *Fig. 1*. The method was validated by determining linearity, recovery, precision and accuracy, LOD, and LOQ. The quantification of kanamycin in buffalo milk samples was performed by LC-MS/MS. Chromatographic separation was also performed using an LC technique in line with Kim et al.[17]. The linearity of the calibration curve (y=282.5x-192.03) showed an appropriate correlation (r^2=0.999) in the range from 0.5 to 100 µg/kg *(Fig. 2)*. Relative standard deviation (RSD%) was used to express the overall precision of the method and they were less than 5.79%. The accuracy expressed regarding intra-day and inter-day recoveries at three different levels of 80, 160, 400 µg/kg. Intra-day recoveries ranged from 102.55±5.20 to 108.70±5.96 µg/kg while inter-day recoveries ranged from 100.50±4.80 to 107.04±6.19 µg/kg, which confirms the method has a good recovery and precision *(Table 1)*. Some researchers also developed LC-MS methods for the detection of kanamycin residue in milk and their results were summarized in *Table 2*.

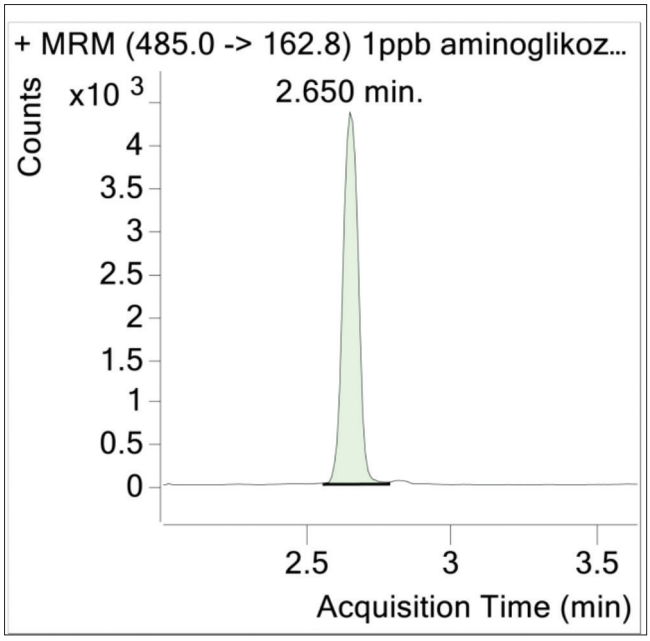

Fig 1. Chromatogram of kanamycin standard

For the experimental study, the highest concentrations of kanamycin were detected at the first milking ranging from 1231 to 1877 µg/kg with a mean concentration of 1473 µg/kg. Also, kanamycin milk concentration decreased consequently during milking period and kanamycin concentration was observed under the maximum residue limit (150 µg/kg) at the fifth milking. The mean kanamycin milk residual concentration measured at the 10 milking post-treatment was as high as 4.96 µg/kg *(Fig. 3)*.

For the monitoring study, a total of 50 buffalo milk obtained from Afyonkarahisar and analyzed for the presence of kanamycin residues. Kanamycin was not detected in 49 samples but only 1 buffalo milk sample contained 22.36 µg/kg kanamycin.

Table 1. *Intra- and inter-day precisions for kanamycin in buffalo milk samples*

Spiked (ppb)	Intra-day Assays (n=8)		Inter-day Assays (n=8)	
	Percentage Recovery±CV	RSD (%)	Percentage Recovery±CV	RSD (%)
80	108.70±5.96	5.49	107.04±6.19	5.79
160	102.55±5.20	5.07	100.50±4.80	4.78
400	103.80±4.27	4.11	102.02±2.79	2.73

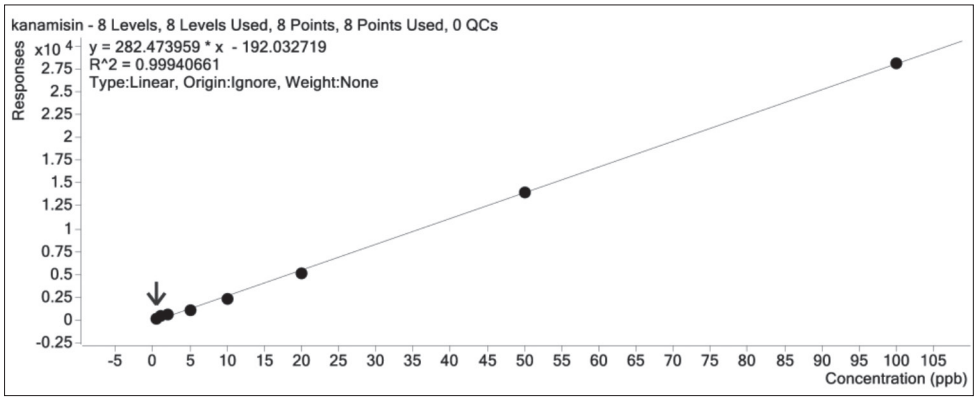

Fig 2. Calibration curve for kanamycin

Table 2. *Selective methods for the quantification of kanamycin in milk*

Method Type	Matrix	LOD µg/kg	LOQ µg/kg	Recovery (%)	Reference
LC-MS	Buffalo Milk	3.56	11.85	100-108	Current Study
LC-MS	Bovine Milk	15	37.5	105-106	Arsand et al.[13]
LC-MS	Bovine Milk	0.7	1.0	NA	Goutalier et al.[18]
LC-MS/MS	Bovine Milk	14	45	78-104	Bousova et al.[19]
LC-MS	Bovine Milk	33	33	92	Saluti et al.[20]
LC-MS	Bovine Milk	<11.5	<18.5	81–91	Tao et al.[21]
LC-MS	Bovine Milk	11	36	78-86	Yang et al.[22]

NA: *Not available*

	Kanamycin ± SD (µg/kg)
Control t=0	<LOD
Milking 1	1473.46±271.16
Milking 2	728.92±155.17
Milking 3	287.33±87.73
Milking 4	153.89±30.14
Milking 5	53.56±11.87
Milking 6	31.51±4.04
Milking 7	25.82±5.51
Milking 8	18.37±3.63
Milking 9	10.16±3.38
Milking 10	4.96±0.8

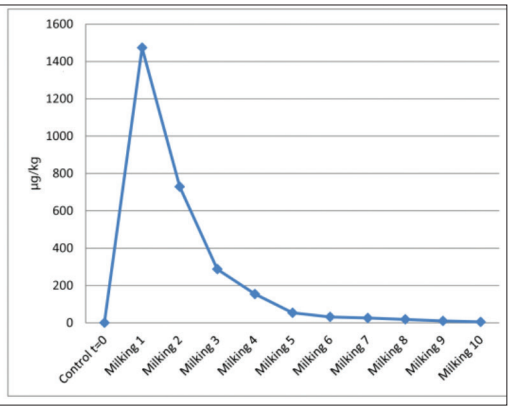

Fig 3. Persistence of kanamycin in milk of buffaloes

DISCUSSION

Acetonitrile was chosen for the extraction of kanamycin from milk matrix due to its protein precipitation capacity. Aminoglycosides have good stability under cold circumstances. Therefore, kanamycin was prevented from degradation by centrifugations at low temperatures (4°C). The LOD of kanamycin was determined as 3.56 µg/kg while its LOQ value was determined as 11.85 µg/kg. The validation parameters including LOD, LOQ, and recovery values were compatible with other studies [18-22].

In this experimental study, for the first time, the passage of kanamycin into buffalo milk was followed and its milk excretion kinetics was determined by LC-MS/MS. Kanamycin was detected in milk between the 0 and 10 milkings after intramuscular administration to dairy buffaloes. The highest concentrations of kanamycin were detected at the first milking and its milk concentration decreased under the maximum residue limit of 150 µg/kg [10] at the fifth milking. In this study, variations in kanamycin levels of each animal may be caused due to individual differences. There are limited studies evaluating the persistence of kanamycin in the milk of livestock animals. In one of these studies, it was reported that kanamycin was intramammary administered to four healthy Holstein lactating dairy cows. In this purpose, kanamycin monosulphate was infused to each quarter of cow with a single dose of 100.000 IU (=133 mg). The maximum concentration of kanamycin in the glandular tissue was determined as 44.3±10.7 µg/g at 2 h and kanamycin was still present in the udder at the concentration of 12.9±5.6 µg/g at 24 h. At the end of the experiment, they showed that large concentrations of this drug could be reached in the glandular tissue [18]. In another study, it was reported that withdrawal period of kanamycin determined as 4 milking after the intramuscular injection of kanamycin to ewes at the dose of 12 mg/kg twice daily for 3 days [23].

Milk is one of the most consumed foods of animal origin worldwide. However, misuse of antibiotics during treatment of milk-producing animals may cause antibiotic residues in their milk [7]. Aminoglycosides are potent antibiotics used in veterinary medicine [24]. This study also evaluated the food safety risk of buffalo milk marketed in Afyonkarahisar, Turkey regarding the presence of kanamycin residues. According to the results of the study, only 1 of 50 (2%) buffalo milk samples contained kanamycin at the concentration of 22.36 µg/kg. The level of the kanamycin residue in milk was under the established MRL level of 150 µg/kg for kanamycin in milk and this result can be considered as a positive sign regarding food safety. Similarly, Unusan [25] performed an field monitoring study based on an ELISA method specific for aminoglycosides (streptomycin) in Turkey and reported that only one (1.7%) of 60 ultra-heat-treatment milk samples contained aminoglycosides. de Oliveira et al.[26] analyzed a total of 299 pasteurized bovine milk samples collected from retail

markets in Brazil by ELISA kit and an LC–APCI–MS/MS QToF method and reported that none of the samples contained streptomycin and dihydrostreptomycin residues. Also, 192 raw milk samples were analyzed in Hebei Province of China for the presence of twenty-eight veterinary drug residues including kanamycin tested by ultraperformance liquid chromatography with tandem mass spectrometry method and they reported none of the milk samples was contaminated with kanamycin [27]. In a study from Italy, milk samples from 45 dairy farms were screened for the presence of antimicrobials during 12 months and reported that none of the milk samples contained aminoglycosides residues [28]. Vragovic et al.[29] reported that aminoglycoside (streptomycin) contamination ranged from 0 to 73.82 µg/kg for 75 milk samples collected from Croatian markets. In addition, Du et al.[30] analyzed a total of 198 milk samples collected from China and reported 15.1% of samples contained aminoglycoside antibiotic streptomycin with the highest value of 7.69 µg/kg. de Novaes et al.[31] surveyed a total of 961 milk samples for veterinary drug residues in Brazil and the results of their study showed that lower levels of neomycin and gentamicin were detected while no residues of streptomycin were detected in milk samples.

Consequently, we report here the milk kinetic of kanamycin for Anatolian buffaloes by a precise, reliable, and accurate LC-MS/MS method with simple sample preparation. The LOD and LOQ of the method were sufficient to detect kanamycin in milk under the maximum residue limits set by EU. The results of the study provide novel information for veterinarians regarding the withdrawal period of kanamycin in buffalo milk that is in line with the withdrawal time set for cattle by the EU. Also, the analysis of real milk samples indicated a very low residue risk regarding aminoglycoside antibiotic kanamycin in Afyonkarahisar Province of Turkey.

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Research Article

Expression Profile of Sox5 and Sox6 in Sertoli and Spermatogonial Cells in Growing Mice Testis

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Abstract

SRY box genes are peculiar to animal kingdom. They are involved in many processes particularly sex determination and testes development in male embryo. Although Sox family genes have been identified in various cells, their expression pattern and role is not entirely recognized in Sertoli cells. In this research, we focused on the expression of SoxD group Sox5 and Sox6 genes in Sertoli cells of mice during pre- and post-pubertal testicular development ranging from one-week-old to eight-week-old mice. The expression was studied by immunohistochemistry on whole testes, and qPCR to determine the mRNA level of all age groups, while immunocyto-chemistry was performed for localization in specific age groups. qPCR results of Sertoli cells from first week to eight week showed different levels of expression. The mRNA level of Sox5, during pre-pubertal age, was significantly high (P<0.001), but as the age progressed, the expression became low. Conversely, Sox6 was initially expressed faintly, but at the pubertal age, the expression rose significantly (P<0.001). Furthermore, the expression signals of both genes on spermatogonial cells were also found strong. The study shows that Sox5 and Sox6 are expressed during postnatal and pubertal periods and may play a vital role in the maturation of spermatozoa. In addition, they overlap to regulate multiple functions like spermatogenesis and steroidogenesis in testes.

Keywords: *Sertoli cells, Spermatogenesis, Sox5, Sox6, Testis*

Gelişme Dönemindeki Fare Testislerinde Sertoli Hücreleri ve Spermatogonial Hücrelerde Sox5 ve Sox6 Ekspresyonu

Öz

SRY gen grubu hayvanlar alemine özgüdür. Erkek embriyoda cinsiyet tayini ve testis gelişimi başta olmak üzere birçok sürece dahildirler. Sox familyası genleri çeşitli hücrelerde belirlenmiş olsa da, Sertoli hücrelerindeki ekspresyon özellikleri ve rolleri tam olarak tanımlanmamıştır. Bu araştırmada, puberta öncesi ve sonrası bir ila sekiz haftalık yaştaki farelerde testis gelişimi sırasında Sertoli hücrelerindeki SoxD grubu Sox5 ve Sox6 genlerinin ekspresyonu araştırıldı. Ekspresyon, tüm testis örneklerinde immünohistokimya ve tüm yaş gruplarının mRNA seviyesini belirlemek için qPCR ile incelenirken, spesifik yaş gruplarında lokalizasyonun belirlenmesi için immünositokimya yapıldı. Sertoli hücrelerinin qPCR sonuçlarında, ilk haftadan sekizinci haftaya kadar farklı ekspresyon seviyeleri gözlendi. Puberta öncesi yaşlarda Sox5'in mRNA seviyesi anlamlı derecede yüksekti (P <0.001), ancak yaş ilerledikçe ekspresyon azaldı. Tersine, Sox6 expresyonu başlangıçta zayıfken, puberta ile birlikte anlamlı şekilde arttı (P <0.001). Ayrıca, spermatogonial hücrelerde her iki genin ekspresyon düzeyleri de güçlü bulundu. Çalışma, Sox5 ve Sox6'nın postnatal ve pubertal dönemlerde eksprese edildiğini ve spermatozoanın olgunlaşmasında hayati bir rol oynayabileceğini göstermektedir. Bunun yanı sıra, söz konusu genler testislerde spermatogenez ve steroidogenez gibi birden fazla işlevin düzenlenmesinde rol oynamaktadır.

Anahtar sözcükler: *Sertoli hücreleri, Spermatogenez, Sox5, Sox6, Testis*

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INTRODUCTION

Sertoli cells (SC) are the primary cells to differentiate and play a crucial role in testis development and spermatogenesis. These cells differentiate in the male fetal gonads and have a task in mullerian duct regression with the help of Anti-mullerian hormone (AMH) [1]. These cells have originated from the early somatic ancestor cells that are considered to have grown from the coelomic epithelium. Furthermore, the coelomic epithelium develops from mesonephros which differentiates after thickening, a process implied to be resulting in the expression of Sry gene. Coelomic epithelium also enwraps gonadal primordium, the development of which depends on certain transcription factors [2,3]. As gonadal primordium thickens, the coelomic epithelium and the expression of Sry get enhanced to play a crucial role in the development of Sertoli cells. A quarter of Sertoli cells are required to express Sry for enabling the testes to differentiate in gonad [4].

Sox gene group is the Sry family-related group contained in HMG box, a DNA binding domain having features of the Sox family transcription factors. This group, which is found only in animal kingdom, consists of twenty genes which are further divided into eight groups ranging from A to H according to the degree and level of similarity or conserved regions [5]. Sox group of protein allocate a high degree of homology (usually 70-95%) within the same group both in and outside the HMG region, but the Sox proteins among different groups have limited homology (>46%) within HMG region and not any outside this region. While majority of Sox genes present up to three exons and combine to form a single protein, SoxD and SoxH genes are divided into compound exons and result in different variants having distinct characteristics. In mammals, the determination of male sex is determined by Sry gene expression [6,7]. These genes are briefly expressed in premature Sertoli cells during embryonic days ED (10.5) and (ED) 12.5. Their function in the Sertoli cells is yet unclear and they are supposed to either repress or nourish male sexual growth [8]. In addition, Sry gene expression also triggers the commencement of another Sox9 gene [9]. The history of Sry research shows that it belongs to a group of transcription factors having a role in the architectural cluster of steriospecific nucleuicprotein complex which are crucial for right gene expression [10]. It is observed that Sry related genes, sub grouped in DNA binding protein, are expressed in testis DNA having specific overlapping characteristics with Sry [11].

Sox D group transcription factors are the largest in all sox family. The members of this group are highly identical and occupy a large conserved region in HMG box domain. The HMG box is located at the C-terminal of the protein. The protein of this group shows a short extension outside from these domains. There is about 87% identity among this group and <60% with other Sox genes. SoxD has different characteristics of expression, that is, only the long form expresses N-Terminal, composed of Lucine zipper, coiled coil domain, and a glutamine containing regions called Q box, which shows their hemodimerization or herterodimerization quality with other SoxD members.

Due to common conserved region, Sox5 and Sox6 are closer to one another than Sox13. They have overlapping functions and seem to play an important role in gene expression during spermatogenesis in mice [12,13]. Both Sox5 and Sox6 are known for their lack of transactivation domain. The twin genes can be expressed in short form, i.e., 2kb and 3kb respectively. But they can also be expressed as long transcripts 6kb and 8kb in other tissues including testes. Sox6 is expressed in full length but Sox5 appears in short and long form in testis. Sox5 gene was reported earlier and later on its isoforms were also identified [13,14]. These genes are involved in different pathways including chondrogenesis and development of nervous system [13-15].

The DNA binding short form of Sox5 bears a specialized function in development and is expressed in testes, lungs and brain [16], while long form of Sox5 expression is found in different cells of lungs, kidney, brain and heart. Sox5, along with Sox6 genes, also play role in bone formation including tooth and muscle development. Due to its different isoforms, researchers have always shown more interest in Sox5 [17,18]. On the other hand, Sox6 has different expression patterns. It appears in the central nervous system, otic vesicle, somites, thymus, branchial arches, craniofacial mesenchyme, notochord, liver and limb buds [12,19-21]. Using blotting technique mRNA, Sox6 has been detected in tissues of heart, brain, lungs, spleen, pancreas, liver, kidney, skeletal muscles and whole testes [22,23]. The extensive expression of Sox6 has also been reported in adult tissues of rainbow trout [24]. Moreover, Sox6 is involved in the chondrogenesis and erythropoiesis and its absence in mice results in quick death after birth, most probably from deformed development of heart [25,26]. There are no recognized regions for trans-suppression or trans-activation, but several cofactors are involved in the above-mentioned process to regulate the proliferation, differentiation, and continuous existence in ectoderm, mesoderm and endoderm originated cell-lineage [27].

In a nutshell, this family has a distinctive and overlapping function in the development of the vertebrates which poses the main challenge. Nevertheless, the knowledge concerning the function of SoxD in the vertebrate development is improving with every passing day. Although soxD group is found in various tissues of mammals, twin genes expression and role in maturing testes is still unknown. Therefore, in this study we focused on the expression patterns of Sox5 and Sox6 in different cells of testes to make a gateway and to explore their roles in spermatogenesis and steroidogenesis.

MATERIAL and METHODS

Experimental Animals

Male pathogenic free Kunming mice ranging from week-1 to week-8 were obtained from Central Animal Laboratory of Hubei Province, Wuhan, China. All procedures were performed under the protocols approved by Ethical Committee of Hubei Research Centre and Huazhong Agriculture University bearing the ID HZAUMO-2017-042 and in accordance with the guidelines established by the NIH Lab. Animal Care Committee.

Isolation and Culture of Primary Sertoli Cells

Isolation and culturing of primary Sertoli cells (SC) were performed according to previous reports with slight modifications. The procedure normally results in >90% minimal contamination of Sertoli cells [28,29]. Briefly, testes from five Kunming mice were aseptically removed in petri dish having 1xHBSS. Testes were extensively washed with 1xHBSS 3-4 times. After removing the tunica albuginea, tubules were chopped and transferred to 15 mL tube containing 7-10 mL DMEM F-12 (Dulbecco's Modified Eagle's Medium/Nutrient F-12 medium) enriched 0.5 mg/mL Collagenase IA (Sigma-Aldrich) and 200 µg DNaseI (Sigma, USA) incubated at 37°C for 20 min. The suspension was layered over 5% percol (Pharmacia, Sweden). The supernatant was discarded in order to remove the Leydig cells and bottom layer was further digested by trypsin 0.25% and 0.02% EDTA (1:1) (Gibco, USA) for 15 min at 37°C including 200 µg DNaseI (Sigma-Aldrich, USA). The digestion process was halted by adding equal quantity of 10% FBS cultural media (Invitrogen, USA). The mixture was passed through 70 µm and 50 µm (BD Bioscience USA) cell strainer, centrifuged at 1500 rpm/5min and washed twice. Later on the cells were poured in Lectin DSA coated cell culture plate (Lectin DSA at 5 µg/mL in 1x HBSS lectin from Datura stramonium that selectively binds Sertoli cells; Sigma, USA) which was already prepared and incubated at 37°C in 5% CO_2 for 1 h. Lectin coated plates were washed twice before use. The cells were transferred to lectin coated plates and incubated for at least 1 h at 37°C to achieve maximum attachment of Sertoli cells. Cultural media was changed by hypotonic shock solution (0.3x HBSS) and the plates were maintained under standard cell culture conditions at 37°C with 95% air and 5% CO_2 in a humidified chamber to lyse the unnecessary germ cells. Hypotonic solution was changed and Sertoli cells were enriched by 10%FBS, 1%penicillin/streptomycin and fungizone at 0.5 µg/mL in DMEM F-12 by fresh culture media for 2 days.

Immunohistochemistry (IHC)

The regional localization of sox5 and Sox6 was examined by IHC method with little modification [30]. The expression of Sox5 and Sox6 were checked in the testes of two-week-old and adult (42 days) male mouse and three mice were used in each age group. A standard protocol of streptavidin biotin-peroxidase complex was followed. In short, testes from the respective ages were collected and dehydrated in different concentrations of alcohol. The paraffin embedded 4-5 µm tissue section was cleaned with xylene, dewaxed and incubated with blocking solution at room temperature for 30 min (1% BSA solution (bovine serum albumin), Wuhan, China). The sections were incubated with Sox5 and Sox6 anti-rabbit and anti-goat polyclonal antibodies (Cat. sc-20091, Sc-17332, Santa cruz, USA) overnight at 4°C. Next day, at room temperature, the sections were washed 3 times with PBST and incubated with HRP conjugated with anti-rabbit or anti-goat secondary antibody and peroxidase-conjugated streptavidin for 1 h (Boster, Wuhan China). In negative control, primary antibody was not used. Expression of Sox5 and Sox6 was defined with brown color.

Extraction of RNA and cDNA, qPCR

The cultured cells were rinsed with 1xHBSS twice and total RNA was extracted by Trizol kit from Sertoli cells of all ages of mice (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quantity and purity of RNA were measured using Nanodrop Spectrophotometer absorbance at optical density 260/280. cDNA was synthesized using first strand cDNA kit (Toyobo Co., Japan), the manufacturer's instruction was followed step by step. Primers for the respective genes were designed by primer 5 software and confirmed by UCSC Bioinfo. software. For qPCR, the implication was performed (Bio-Rad iQ5 Real Time PCR System, CA, USA), in reaction mixture consisting of specific primers *(Table 1)*, the master mix and RNA free water. The mRNA level was normalized with GAPDH and RNAse free water was used as control. For precision, melting curve analysis was used to check the PCR purity as prescribed previously [31]. Experiment was conducted in triplicates.

Immunocytochemistry (ICC)

The localization of Sox5 and Sox6 at cellular level were examined after 48 h in cultured mouse sertoli cells from five mice aseptically. The ICC results show the location of both proteins inside the Sertoli cells. The cells were transferred on cover-glass (in 6 well plates) and washed with PBS three times and fixed by 4% formaldehyde (Beyotimes,

Table 1. *Quantitative real time polymerase chain reaction primers*

S.No.	Gene Name	Primers	Tm °C
1	SOX5	ATGCTTACTGACCCTGATTTACCT TCCACTTTCTGTCTGCTTGTCAC	58
2	SOX6	TGGGCAAAGGACGAAAGGAG GCCTGTTCTTCATAGTAAGGTTGCT	58
3	GAPDH	TCAACGGCACAGTCAA CTCGCTCCTGGAAGAT	

China) at room temperature for 30 min. After rinsing, cells were exposed to 0.5% Triton X-100(PBS preparation) for 20 min. Cells were again washed with PBS thrice each time for three min. Normal goat and rabbit serum was used drop by drop on the cells before they were incubated for 30 min. at room temperature. Furthermore, each slide was saturated with sufficient primary antibodies of Sox5 and Sox6 (Santa Cruz Laboratories, CA, USA), diluted 1:150 in 5% BSA, in respected slide and incubated at 4°C overnight in dark moist chamber. Next day, cells were stained with Cy3 labeled secondary antibodies (goat anti-rabbit IgG, rabbit anti-goat IgG, Boster China at 1:100) in a wet box for 1 h at 37°C, then rinsed each time for 5 min. For nuclear staining, cells were stained with DAPI (1:5000) for 5 min, and extra staining was removed by washing. Cells were then dried with absorbent paper. In control, primary antibodies were not used, only PBS was added. The slides were finally mounted with 95% glycerin. Each plate was finally analyzed with confocal laser scanning microscope (LSM 510 Meta instruments Zeiss, Germany).

Statistics Analysis

All the experiments were repeated three times for each section and the data were presented as ±SEM. Multiple group data were analyzed byone way ANOVA followed by LSD test using SPSS-16 system software. For individual comparison P<0.001 was considered as highly significant.

RESULTS

In order to check the expression of Sox5 at both stages, i.e., pre-pubertal and post-pubertal age of mice testes, the immunohistochemistry result showed that the expression of both genes in post-pubertal age was higher as compared to pre-pubertal age only shown by brown color. The expression was also high in spermatogonial cells (black arrows) followed by Sertoli cells (red arrows) *(Fig. 1)*. So this indicates that this gene is closely related to the spermatogenesis. In adult age, the spermatogonial cells are more active in the way of sperm development. Sox6 gene expression is clear in both per pubertal and post pubertal Sertoli cells (red arrows) as well as spermatogonial cells (black arrows). Spermatogonial cells have stronger expression *(Fig. 1)*. The same finding was also reported that the sox5 expressed in the postmeiotic and round spermatids and sox6 (LZ) was found in adult testes [6].

In order to observe the research potential of Sox5 and Sox6 in various developmental ages of Sertoli cells and spermatozoa production, we checked the expression level by qPCR. The results showed variation among the expression level in different ages of mice. At second, third and fourth week of postnatal age, the level of Sox5 was significantly high (P<0.001) but in adult age the expression level was low *(Fig. 2A)*. But in case of Sox6, expression level in adult age was significantly high (P<0.001) *(Fig. 2B)* as compared to pre pubertal age which indicates that sox5 and sox6 seem to have overlapping characteristics. It means that they boost the role of one another and helps in functions of Sertoli cells. Furthermore, it also refers to the role that Sox5 and Sox6 may have in the spermatogenesis.

Immunocytochemistry shows the localization of Sox5 and Sox6 in Sertoli cells at one week of age. As Sox5 (strong signals in nucleus) and Sox6 (nucleus and cytoplasm) are transcription factors so this experiment showed that they localized in the nucleus of Sertoli cells. The immunocytochemistry results show the translation of gene and indicated by red color (Cy3 stained), show the presence of Sox5 and Sox6 protein in the nucleus and cytoplasm as in *Fig. 3*. The SoxD proteins previous information has shown that they critically modulate cell fate in major lineages [13].

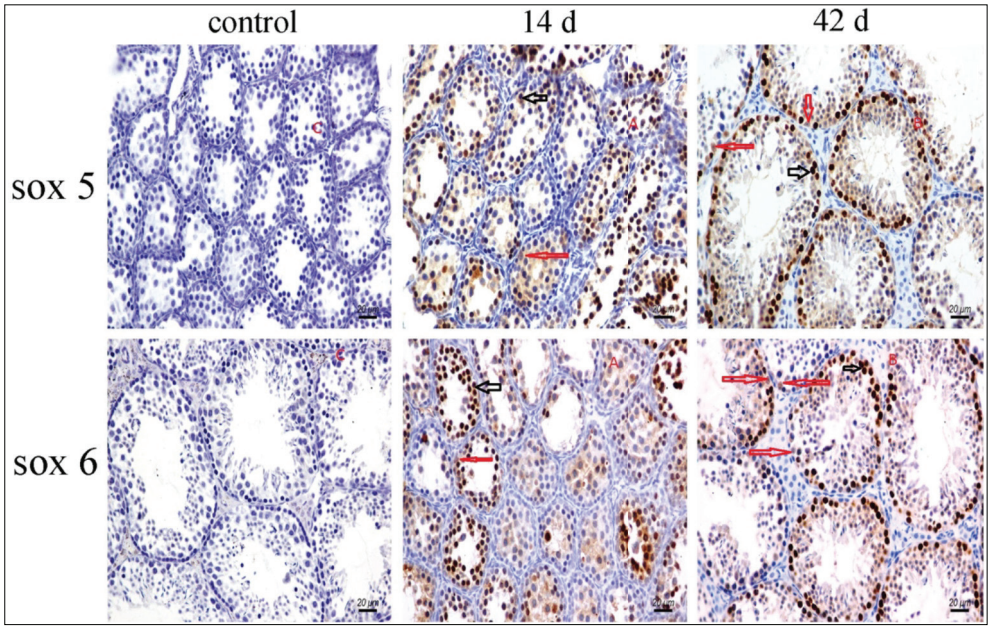


Fig 1. The expression of Sox5 and Sox6 in seminiferous tubules of mouse testis. Red arrows indicate positive Sertoli cells while black arrows indicate spermatogonial cells. Control is indicated by C without antibody. The letters A & B indicates 14 & 42 days mouse testes sections, respectively. Bar is 20 µm for each slide and brown color shows the positive cells

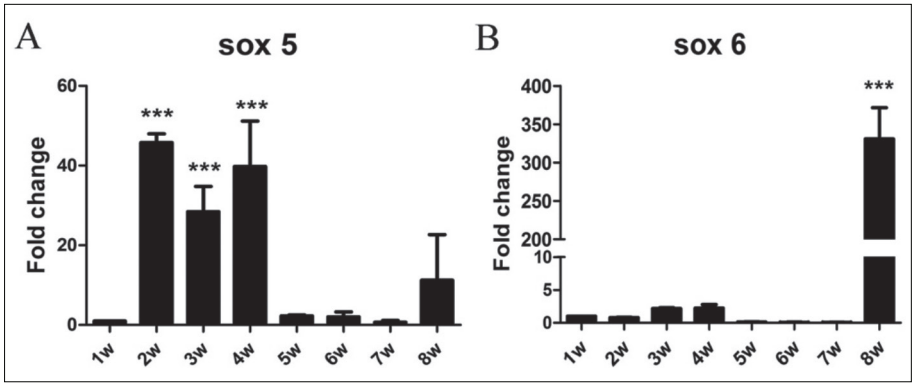
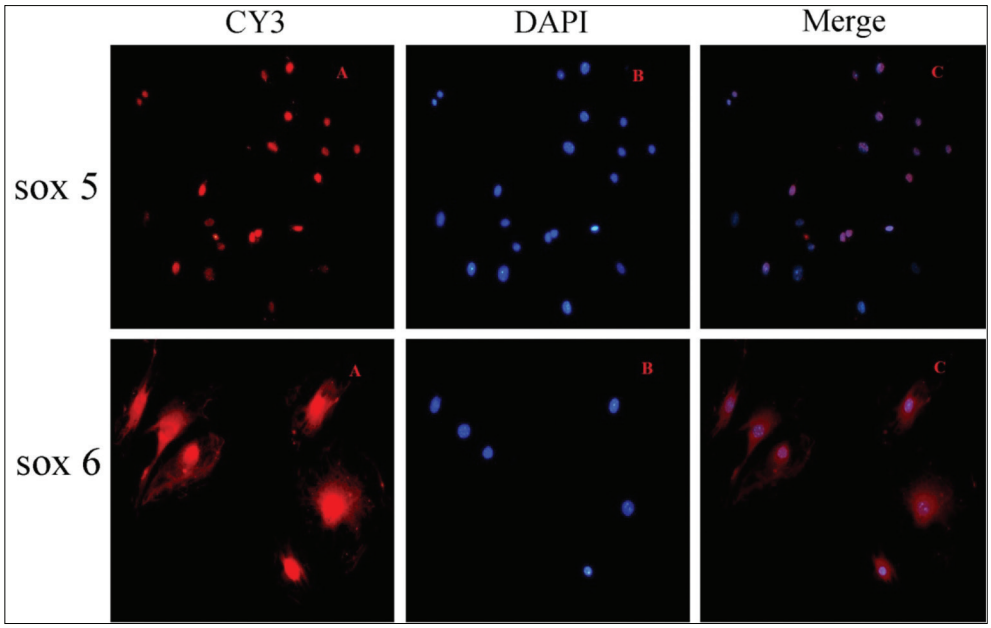


Fig 2. Relative expression (by quantitative polymerase chain reaction) of Sox5 and Sox6 in Sertoli cells of mouse in different growth stages. Both pre- and post-pubertal ages showed highly significant difference *** (P<0.001) in both genes

Fig 3. Localization of Sox5 and Sox6 by immunocytochemistry analysis. Cy3(A) stained cytoplasm is shown by red color whereas the DAPI stained nucleus (B) is indicated by blue color and (C) shows the merged image. The images are taken by laser microscope using 200 and 400 folds magnification



DISCUSSION

This study shows the expression of Sox5 and Sox6 at mRNA and protein level in mice testes both in pre- and post-pubertal periods. Although the expression of Sox5 and Sox6 is reported vastly in other organs, the area concerning its expression and roles in the testes has relatively received less attention. The immunocytochemistry result showed that both Sox5 and Sox6 transcription factors are expressed at the pre pubertal age in Sertoli and spermatogonial cells. As they are transcription factors, so they give stronger signals in the nucleus *(Fig. 3)*. The mRNA levels in Sertoli cells showed that the expression of Sox5 and Sox6 varies at different developmental stages. At the pre pubertal level, Sox5 expression was significantly high (P<0.001) *(Fig. 2A)*, but as the adolescence neared, its expression became fainter and this finding is similar to the previous works [16]. On the contrary, Sox6 expression at the pre pubertal age was significantly low. But at the 8th week, its expression became highly significant (P<0.001) *(Fig. 2B)* all in line with the previous findings [12]. In addition, immunohistochemistry results showed that Sox5 and Sox6 are strongly expressed in spermatogonial cells of both the respective ages. Their expression exists in Sertoli cells but fainter than spermatogonial cells. These results are consistent with the previous researches [12,14,16]. It means that these genes at adult age are more functional and assists in sperm maturation.

The expression of SoxD family genes are reported in various tissues and cells in testis, neuron, oligodendrocytes, chondrocytes and palatogenesis [16,32,33]. A member of this family (Sox5) has been reported in the postnatal period in testes [14], which concede with our findings in spermatogonial cells. The S-Sox5, a short transcript, in human and mouse having 48kD size was also found in testes in ciliated/flagellated cells; the short isoform of Sox5 is deficient in N-terminus and have a length of half of the long transcript (6kb). The long form was discovered earlier than the short form [34]. The expression of L-Sox5 is high in chondrocytes and muscles which prove that it has function in the cartilage and development of muscles [35]. Sox5, besides in testes, is also strongly expressed in human brain as high expression of S-Sox5 was found in brain and some ciliated tissues, which indicates that it might have role in the sperm development. Long Sox5 84kD, on the

other hand, is expressed in brain, kidney, heart and skeletal muscles; meanwhile, it has also been reported that another short Sox5 of 25kD is expressed in spleen, liver and testis which seems to be a new translated protein reported by some databases [34]. At mRNA level from the results of Sox5 expression varies in Sertoli cells (2A), as age increases its expression level also increases, reaching the highest level at the adult age. According to immunohistochemistry, which show the presence of its protein and it exists at both pre pubertal and adult age but the expression in Sertoli cells is fainter than spermatogonial cells. The molecular structure of sox protein is unique and shows various motifs. They are flexible and versatile in DNA binding sequencing are capable of using various mechanisms to either enhance or repress transcription [13]. The immunocytochemistry result at the same age shows that Sox5 is localized in the nucleus and show the expression.

Sox6 is also a member of SoxD group family. It appears in variety of mammalian tissues. The role of Sox6 in testicular development is not known, the large transcript of Sox6 is expressed in skeletal tissues but its short form, about 3kb, appears in the testis. It is also observed in central nervous system during embryogenesis. The expression of Sox6 in cardiac and skeletal muscles shows crucial role in the muscle development [26]. The Sox6 ortholog is also found in the fish (i.e. trout) in both forms about 10kb and 3kb form. This indicates that there are conserved regions in the pattern so it might have a significant function in different tissues [36]. Sox5 and Sox6 along with sox9 are expressed during embryogenesis at early stage and have functional role in embryo development. These transcripts are fully detectable in the mesenchymal cells at early stage of cartilage formation up to the bone formation. Sox5 and Sox6 are homologous proteins and the persistent presence of sox transcripts shows that they have crucial role in the development of bone formation and cell differentiation pathway and they also take part in the activation of a group of gene matrix. The initial data also shows that the twin (Sox5 and Sox6) genes are paramount for proper cartilage and bone formation in vivo [37]. The short form of Sox5 and Sox6 are detectable in the adult testes which may have function in the spermatozoa maturation pathway [13]. Sox genes are mostly involved in the architectural structure, so their mutation or deletion may interfere with birth or cause developmental defects [23]. Sox6 is important transcription factor for cell endurance as it contributes to cell survival. The deficiency of Sox6 causes inhibition of neural differentiation in P19 cells [38]. Sox6 has also been identified to have a unique tumour suppression role, as it has HMG domain using p53 stabilization mediated channel [39].

LSox5 and Sox6 are highly similar and are co-expressed in many types of cells. Mice born with both null Sox5 and Sox6 gene die soon after birth because of generalized chondrodysplasia while mice with single null gene had been born with mild abnormality in skeletal. Moreover, deficiency of both genes causes poor development in skeletal tissues [40]. Sox5 and Sox6 have been reported to be only expressed at adult age and restricted to post meiotic spermatogonial cells [12], but our experiment showed that these genes are expressed in other cells like Sertoli cells, which are crucial for sperm development. Several published research have observed that these structurally unique proteins (sox) are biologically very important in the cellular development. They are highly flexible in selecting DNA-binding sequences and are capable of using various mechanisms to either enhance or repress transcription. They thereby modulate such varied processes as cell proliferation, survival, differentiation, and terminal maturation in a number of cell lineages [13].

In conclusion the expression of soxD genes shows that their role is important in the development in cells and tissues. From our study, it is clear that Sox5, Sox6 and their different isoforms exist at various stages of testicular development, and the maturation of spermatozoa, although the functions of these two genes in testes have still not been elucidated. After this identification, further pathways studies through RNAi or overexpression experiments are needed to know their potential function in spermatogenesis or steroidogenesis. Moreover, our study has implications for the further role which Sox6 may play in the sustenance of tissue. The area has been left rather undiscovered, though ready for further explorations.

Conflict of Interest

All authors do not have any potential conflict related to this research.

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Research Article

The Effects of Presynch-10 and Ovsynch on Some Endometrial Toll- and Nod-like Receptor Gene Expressions in Repeat Breeder Cows [1] [2]

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Abstract

Subclinical endometritis (SE) is one cause of repeat breeder syndrome (RBS). When endometrial cells come into contact with antigens during inflammatory events like endometritis, they act like innate immune system cells. Ovsynch-based therapies stimulate the innate immune system, which is how they may be able to cure SE and then RBS. This study aimed to assess the effects of presynch-10 and ovsynch applications on mRNA expressions of some endometrial Toll- and Nod-like receptors (TLRs and NLRs) in cows with RBS. A total of 40 repeat breeder cows were used in the study. The presynch-10 and ovsynch protocols were used in group 1 (G1, n=20), and no protocol was used in group 2 (G2, n=20). Endometrial samples were collected before and after the synchronization protocols in G1. In G2, samples were collected, and after waiting time up to the duration of synchronization protocol in G1, samples were taken again. The expression profiles of the genes being investigated were examined using RT-qPCR. NLRC4, NALP3 and TLR4 were basically expressed in all of the samples while NOD2 expression was completely undetectable. There was a statistical difference when NOD1 and TLR2 expressions in the second samples were compared between G1 and G2 ($P<0.05$). It was concluded that presynch-10 and ovsynch administration in cows may have a positively effect on the innate immune response and so be effective in the treatment of SE.

Keywords: *Endometritis, Cow, Innate immunity, Pattern recognition receptors*

Repeat Breeder İneklerde Presynch-10 ve Ovsynch Uygulamalarının Bazı Endometriyal Toll ve Nod Benzeri Reseptör Genlerinin Ekspresyonları Üzerine Etkileri

Öz

Subklinik endometritis (SE), repeat breeder sendromunun (RBS) nedenlerinden biridir. Endometriyal hücreler, endometritis gibi yangısal olaylar sırasında antijenlerle karşılaştıklarında, doğal bağışıklık sistemi hücreleri gibi davranırlar. Ovsynch temelli tedaviler doğal bağışıklık sistemini uyararak SE ve sonra RBS'yi tedavi edebilmektedirler. Bu çalışma presynch-10 ve ovsynch uygulamalarının RBS'li ineklerde bazı endometrial Toll- ve Nod- benzeri reseptörlerin (TLR'ler ve NLR'ler) mRNA ekspresyonları üzerindeki etkilerini değerlendirmeyi amaçlamıştır. Çalışmada toplam 40 repeat breeder inek kullanılmıştır. Grup 1'de (G1, n=20), presynch-10 ve ovsynch uygulanmış, Grup 2'de herhangi bir uygulama yapılmamıştır (G2, n=20). G1'de senkronizasyon uygulamaları öncesi ve sonrasında endometriyal örnekler alınmıştır. G2'de örnekler alındıktan sonra G1'de uygulanan senkronizasyon protokolü süresi kadar beklenerek örneklemeler tekrarlanmıştır. İncelenen genlerin ekpresyon düzeyleri RT-qPCR ile belirlenmiştir. NLRC4, NALP3 ve TLR4'ün temel olarak tüm örneklerde eksprese edildiği, NOD2 ekspresyonunun ise belirlenemeyecek seviyelerde kaldığı saptanmıştır. G1 ve G2'den alınan ikinci endometriyal örnekler arasında NOD1 ve TLR2 ekspresyonları arasında istatistiksel bir fark oluşmuştur ($P<0.05$). İneklerdeki presynch-10 ve ovsynch uygulamalarının doğal bağışıklık yanıtını olumlu yönde etkileyebileceği ve SE tedavisinde etkili olabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Endometritis, İnek, Doğal bağışıklık, Kalıp tanıyan reseptörler*

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INTRODUCTION

Repeat breeding syndrome (RBS) is one of the most important causes of infertility in dairy herds and prolongs the calving interval, which results in economic losses [1]. Repeat breeding syndrome is defined as cows younger than 10 years of age that fail to conceive after 3 consecutive inseminations while exhibiting normal estrus every 18 to 24 days, have no clinical disorders or pathological discharge in the genital organs [1,2]. The most common causes of RBS are subclinical endometritis (SE) [3], poor estrus detection [4], delayed increase in LH that delayed a rise in progesterone level [5], embryonic losses [6], environmental factors [7] and malnutrition [8]. When the microorganisms that play a role in the etiology of SE, which one of the most important causes of RBS, reach the uterus, they come into contact with the endometrium, which is the frontline of the genital tract defense. Endometrial cells have pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) on the surface of microorganisms. This allows them to function like a member of the innate immune system [9]. Studies have shown that PRRs are expressed in endometrial cells in humans [10] and many animal species [11]. TLRs and NLRs are important members of the PRR family. TLR2 is very important receptor involved in the recognition of the cell surface components of Gram-positive bacteria [12]. The interaction of TLR2 and its ligand triggers an inflammatory response. Similar to TLR2, activation of TLR4 also leads to an inflammatory response. TLR4 is responsible for recognizing Gram-negative bacteria, such as *Escherichia coli* [9]. NLRs are also known as the second line in the natural defense system after TLRs. Responses to microorganisms were generated by NLRs when TLR response was inadequate or microorganisms escaped TLRs [13]. NLRs recognize endotoxin, protein or RNA of many microorganisms [14-18], viral agents and fungi [19].

This interaction acts as a signal that initiates inflammatory reactions [20]. Luteal function deficiencies are another important factor that causes RBS. In this context, bacteria found in the uterus or inflammation in the endometrium may inhibit the release of luteinizing hormone (LH) and adversely affect follicular development, ovarian functions and the ovulation mechanism [21]. Therefore, one approach to resolving RBS related to ovarian dysfunctions or sub-clinical endometrial inflammation is administering ovulation synchronization protocols. In this way, the compromised ovulation mechanism can be made functional by hormonal intervention. Studies revealed that if the first injection of gonadotropin-releasing hormone (GnRH) is given when the diameter of the dominant follicle is 10 mm, the ovsynch protocol will be more successful [22]. Therefore, the success of ovsynch can be improved by administering two doses of prostaglandin F2-alpha (PGF2α) at an interval of 14 days before starting the ovsynch protocol. As a result, the dominant follicle can be brought to the proper size. It is further hypothesized that PGF2α administration is beneficial in the treatment of SE as it induces local immunity of uterus [23,24].

This study aimed to assess the effects of administering presynch-10 and ovsynch to change mRNA expressions of some TLRs and NLRs in the endometrial tissue of cows with RBS. Cytological and bacteriological samples were also investigated.

MATERIAL and METHODS

All procedures were approved by the Local Ethical Committee of the Experimental Research on Animals (No: 2013/14). Cows were housed in a free stall barn from November 2013 through November 2014 and fed *ad libitum* total mixed ration diet and fresh water.

Cows

Forty multiparous Holstein-Friesian cows with RBS were enrolled in this study. Cows with RBS were identified by reviewing dairy farm records and gynecological examinations. For this purpose, multiparous cows that had been inseminated three consecutive times but did not get pregnant were selected. Rectal, vaginal and ultrasonographic examinations were performed to determine whether or not there were any pathological conditions in uterus or ovarium. Body condition scores (BCS) were determined using a 5-point scale (1= thin to 5= obese) [25]. The BCS's of the cows in the present study were between 3.25 and 3.5 and, were clinically healthy and there was a corpus luteum (CL) in the ovaries.

Study Design

Cytological, bacteriological and biopsy samples were collected from the endometrium for microbiological identification, polymorph nuclear leucocyte rates and detection of gene expressions from all cows. Blood samples were also collected from the tail vein to identify the blood levels of estradiol and progesterone. The cows were randomly divided into two groups.

In group 1 (G1, n=20), rectal, vaginal and ultrasonographic examinations were performed and then endometrial samples were collected 10 days before synchronization began. Then presynch-10 and ovsynch protocols [26] were administered. Blood samples were collected from the tail vein on injection days. Endometrial samplings and clinical examinations were repeated 10 days after the last injection of ovsynch, and blood samples were collected *(Table 1)*. The cows in group 2 (G2, n=20) were not given any medications. First, clinical examinations were performed, and endometrial and blood samples were taken. Following the waiting period as long as the synchronization protocol used in G1, estruses were followed-up. Corpora lutea were identified by rectal examination 10 days after estrus, after which clinical examinations were performed and all of the samples were collected again in G2.

Table 1. *Applications of synchronization, gynecological examination and endometrial sampling in G1*

Syncronization Protocols	Days	Applications
Presynch-10	-34	VI, RE, R-USG, ESS-1, EBS-1, ECS-1, BIS
	-24	1st Clo
	-10	2nd Clo
Ovsynch	0	1st Buse, BIS
	7	Clo
	9	2nd Buse, BIS
	10	Ovulation, BIS
	20	VI, RE, R-USG, ESS-1, EBS-1, ECS-1, BIS

***VI:** vaginal inspection, **RE:** rectal examination, **R-USG:** rectal ultrasonography, **ESS:** endometrial swab sampling, **EBS:** endometrial biopsy sampling, **ECS:** endometrial cytology sampling, **BIS:** blood sampling, **Clo:** cloprostenol [estrumate, 500 µg, im], **Buse:** buserelin acetate [receptal, 10 µg, im]*

Bacteriologic and Cytologic Examination

Special swabs (Uterus Culture Swab with AMIES transport medium, Ref. 17214/2951, Minitube, Germany) and endometrial brushes (Cytobrush®, Ref.17214/2960, Minitube, Germany) were used for bacteriological and cytological sampling. The swab and cytobrush were inserted inside a stainless-steel catheter about 50 cm long to reach the uterine lumen without any contamination.

First, cytological samples were collected by rotating the cytobrush while in contact with the uterine wall. Cytological slides were prepared by rolling the cytobrush onto clean microscope glass. Slides were dried for 1-2 min in the air and then placed in special labeled transport containers. Samples were dyed with the May Grünwald Giemsa Method, and 200 cells were counted per slide. Bacteriologic samples were collected by swabs and, immediately placed in the AMIES medium and transported to the laboratory within 24 h. Samples were plated onto blood, Brucella, Campylobacter, SDA and MacConkey agars.

RNA Extraction and cDNA Synthesis

Endometrial biopsy samples were collected from the dorsal wall and intercaruncular area of the uterus with a bovine biopsy instrument (Kruuse, 63 cm, Kat no: 141700, Denmark). Collected samples were placed into microcentrifuge tubes, and snap-frozen in liquid nitrogen. Samples were transported to the laboratory in a nitrogen tank, transferred to a refrigerator (-86°C) and stored until analysis. Total RNA extracted from endometrial samples was performed using the Trizol method.

Purity and concentration evaluations were assessed with Thermo Scientific NanoDrop 2000®, and integrity was evaluated by electrophoresis in 0.8% agarose gel. DNase I (#EN0521) was used for DNA digestion, and the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit was used according to manufacturer recommendations for cDNA synthesis.

Real Time PCR

Primer sequences were designed using PrimerQuest and primer-BLAST (idt/PrimerQuest and ncbi/tools/primer-blast) software programs *(Table 2)*. Housekeeping genes (HKGs) (B2-GAPDH, Beta-Aktin, HPRT1, YHWAZ, SDHA, SUZ12, TUBA1, 18S) were examined in order to select those that were the most stable and suitable.

Hormone Analysis

Blood samples were collected on injection days and when the 1st and 2nd endometrial samples were collected. They were then centrifuged for three min at 3000 g to obtain blood serum. Serum samples were stored at -20°C until analysis. Serum estradiol and progesterone assays were performed using the ECLIA® method (Electrochemiluminescence Immunoassay) in an internationally certified laboratory (TURKAK, TS EN ISO/IEC 17025:2005, DUZEN Laboratory Group, Turkey).

Statistical Analysis

Normality tests were conducted for all of the data obtained from the study. The paired t test was used to compare cytological cell count data. The Chi-square test was used to compare microbiological data among groups. Statistical comparison of serum estradiol and progesterone levels were performed using the paired t test and Wilcoxon test, respectively.

Quantification of the RT-qPCR results was performed using the ROCHE Nano Lightcycler® software. The most stable and suitable gene was identified as YHWAZ and selected as a reference gene. Subsequently, this reference gene was used to normalize qPCR data. The normalization procedure was performed on the mathematical model of $2^{-\Delta\Delta Ct}$ reported by Livak and Schmittgen [29]. The Wilcoxon test was performed for data from the same cow, and the Mann-Whitney U test was performed to identify differences among the groups. Minitab 16.0® software was used for statistical analysis and box plot graphics.

RESULTS

The calving-to-first-service interval for cows in G1 and G2 was 85.95±19.79 and 90.05±21.77 respectively. The mean number of days in milk (DIM) was 205.6±55.60 in G1 and 236.25±54.51 in G2 (min: 150, max: 349). Since the mean DIM of the cows is 205.60±55.60 (min: 150, max: 349), >3% polymorphonuclear leukocyte (PMNL) was accepted as a threshold value for SE in the cytological evaluation [30].

There were 38 cows that had SE according to the first endometrial cytology samples (ECS1) taken from 40 cows, and this number constitutes 95% of all cows. On the

Table 2. *Primer sequences of genes used in this study*

Gene	Primer (5'-3') Forward, Reverse	PCR	Gen Accession Number	Reference
TLR2	5'-GGTTTTAAGGCAGAATCGTTTG-3' 5'-AAGGCACTGGGTTAAACTGTGT-3'	190bp	NM_174197	[27]
TLR4	5'-CTTGCGTACAGGTTGTTCCTAA-3' 5'CTGGGAAGCTGGAGAAGTTATG-3'	153bp	NM_174198	[27]
NLRC4 (Ipaf)	5'-CCAGGAAGTGCTGAGAAAGG-3' 5'-CCCTTTACGTTTGTGCCTGT-3'	214bp	NM_001192323.2	Designed using Primer3
NOD-1	5'GCTTATCCAGAACCAGATCAC-3' 5'CCTCTTCTCATCTTCAAAGACC-3'	142bp	NM_001256563.1	Design using idt/PrimerQuest and ncbi/tools/primer-blast
NOD-2	5'GTAGACTTCACTGAATCCCAAC-3' 5'GCTCTCCACACCCATAAATAC-3'	168bp	NM_001002889.1	Design using idt/PrimerQuest and ncbi/tools/primer-blast
NALP3	5'CAGAATCTCACCCACCTTTAC-3' 5'GTCTAACTCCAACACCTGAAG-3'	114bp	NM_001102219.1	Design using idt/PrimerQuest and ncbi/tools/primer-blast
YHWAZ	5'CTGAGCAAGGAGCTGAATTATC-3' 5'CTCTGTATTCTCGAGCCATCT-3'	162bp	NM_174814.2	Design using idt/PrimerQuest and ncbi/tools/primer-blast
B2-GAPDH	5'-GGCGTGAACCACGAGAAGTATAA-3' 5'-CCCTCCACGATGCCAAAGT-3'	119bp	NM_001034034.2	[28]
β-actin	5'-ATCGGCAATGAGCGGTTCC-3' 5'-GTGTTGGCGTAGAGGTCCTTG-3'	143bp	BT030480.1	[27]
HPRT1	5'GCTACTGTGTGCTTAGGAAAG-3' 5'CTACCGAAACCTACTGAAACAC-3'	114bp	NM_001034035.2	Design using idt/PrimerQuest and ncbi/tools/primer-blast
SDHA	5'- CGTTGTATGGAAGGTCTCTG-3' 5'- GATGGACCCGTTCTTCTATG-3'	126bp	NM_174178.2	Design using idt/PrimerQuest and ncbi/tools/primer-blast
SUZ12	5'-GAACACCTATCACACACATTCTTGT-3' 5'- TAGAGGCGGTTGTGTCCACT-3'	130bp	XM582605	Designed using Primer3
TUBA1	5'-GCCCTACAACTCCATCCTCA-3' 5'- ATGGCCTCATTGTCTACCA-3'	78bp	NM_001166505.1	Designed using Primer3
S18	5'-ATGCGGCGGCGTTATTCC-3' 5'-GCTATCAATCTGTCAATCCTGTCC-3'	204bp	NR_036642.1	Designed using Primer3

B2-GAPDH: *B2-glyceraldehyde-3-phosphate dehydrogenase,* ***β-actin:*** *beta actin,* ***HPRT1:*** *hypoxanthine phosphoribosyl transferase-1,* ***YHWAZ:*** *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta,* ***SDHA:*** *succinate dehydrogenase complex, subunit A,* ***SUZ12:*** *suppressor of zeste 12,* ***TUBA1:*** *tubulin alpha 1a, S18: ribosomal protein S18, bp: base pairs*

Table 3. *Groups of cows according to PMNL and SE rates*

Groups	Percentage of PMNL (SE status)	ECS1		ECS2	
		n	%	n	%
G1	>0% - <3% (not SE)	0	0	1	5
	≥3% (SE)	20	100	19	95
G2	>0% - <3% (not SE)	2	10	0	0
	≥3% (SE)	18	90	20	100

PMNL: *polymorphonuclear leukocyte;* ***ECS:*** *endometrial cytology sample;* ***SE:*** *subclinical endometritis*

Table 4. *PMNL rates between groups*

Groups	PMNL (X±S)		
	ECS1	ECS2	P
G1	21.85±9.50	18.70±8.08	>0.05
G2	13.85±8.54	16.15±7.13	>0.05
P	>0.05	>0.05	

X: *mean;* ***S:*** *standard deviation;* ***P:*** *significance value;* ***ECS:*** *Endometrial cytology sample*

other hand, all of the cows in G1 had SE, and this number decreased to 19 after administering the presynch-10 + ovsynch protocol.

Eighteen cows had SE according to the evaluation of ECS1, and all cows had SE when the second endometrial cytology samples (ECS2) were evaluated in G2 *(Table 3)*. There was no statistical difference between the PMNL cell numbers in the ECS1 taken from G1 and G2 (P>0.05). Also, there were no statistical differences between the PMNL counts of ECS1 and ECS2 in both G1 and G2 (P>0.05) *(Table 4)*.

When the first endometrial swabs (ESS1) taken from all cows were evaluated, no pathogenic microorganisms were identified in 30 of 40 samples. According to the microbiological analyses, no pathogens were identified in 18 samples while *Citrobacter* sp. was identified in the other 2 samples in G1. In the second endometrial swabs (ESS2) taken after the synchronization protocol, there was no pathogen in 17 endometrial swabs while *Citrobacter* sp. and *E. coli* were isolated in the other 3 samples. In G2, no pathogenic agent was isolated in 12 of the ESS1 while *E. coli*, *Streptococcus* sp., *Candida* sp., *Klebsialla* sp. and *Aspergillus* sp. were isolated in the other samples.

Table 5. *Isolated microorganism rates of G1 in and G2*

Isolated Microorganism	G1 (n=20)				G2 (n=20)			
	ESS1		ESS2		ESS1		ESS2	
	n	%	n	%	n	%	n	%
NPI	18	90	17	85	12	60	10	50
Citrobacter sp.	2	10	2	10	-	-	-	-
E. coli	-	-	1	5	4	20	7	35
CNS	-	-	-	-	-	-	1	5
Streptococcus sp.	-	-	-	-	1	5	1	5
Candida sp.	-	-	-	-	1	5	-	-
Klebsiella sp.	-	-	-	-	1	5	1	5
Aspergillus sp.	-	-	-	-	1	5	-	-

NPI: *no pathogen was isolated;* ***CNS:*** *coagulase negative staphylococci;* ***ESS:*** *endometrial swab sample*

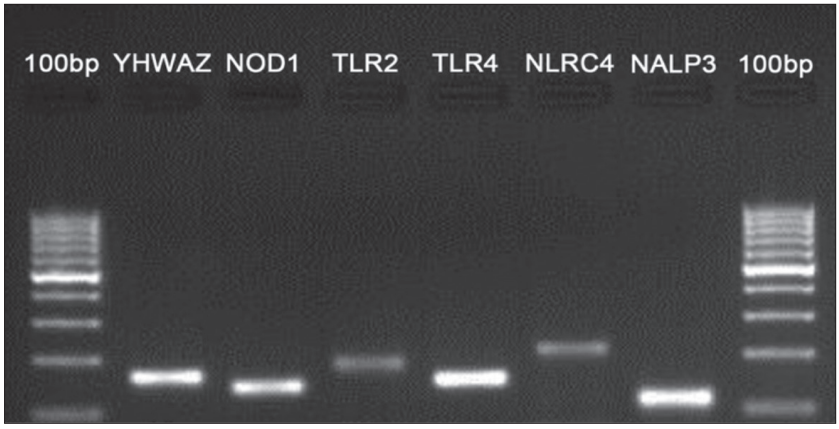

Fig 1. Agarose gel electrophoresis of RT-qPCR products from endometrial tissues. YHWAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide, NOD1: nod like receptor 1, TLR2: toll like receptor 2, TLR4: toll like receptor 4, NLRC4: NLR family CARD domain-containing protein 4, NALP3: NACHT-, LRR-, and pyrin domain-containing protein 3, bp: base pair

When ESS2 were examined, no pathogen was isolated in 10 samples. However, *E. coli, Streptococcus* sp., *Coagulase negative staphylococci* (CNS) and *Klebsiella* sp. Were isolated in the other 10 samples in G2 *(Table 5)*.

The resulting PCR products from YHWAZ, NOD1, NOD2, TLR2, TLR4, NLRC4 (Ipaf) and NALP3 were separated with electrophoresis on 2% agarose gel *(Fig. 1)*. The qPCR test results, melting curve analyzes and agarose gel images were evaluated together.

The geNorm®, NormFinder® and BestKeeper® programs were used to select the reference gene. YHWAZ was identified as the most suitable and stable reference gene. Expression levels of the genes being examined were determined based on statistical evaluations.

Data show that NLRC4, NALP3 and TLR4 were basically expressed in all samples while NOD2 expression was undetectable. There was a significant statistical difference when NOD1 expressions of EBS2s were compared between G1 and G2 ($P<0.05$). Similarly, there were differences in TLR2 expression between EBS1 and EBS2 in G2 ($P<0.05$) and between EBS2s in G1 and G2 ($P<0.05$) *(Fig. 2)*.

Serum progesterone levels were 8.52±3.83 ng/mL in G1 and 6.26±2.20 ng/mL in G2 in the first samples ($P>0.05$). In addition, progesterone and estradiol levels were identified in G1 on the days indicated in *Fig. 3* and *Fig. 4*. No statistical difference was observed between the estradiol levels while progesterone levels showed significant differences between the first (6.56±1.67) and second (3.20±0.03) samplings ($P<0.05$).

DISCUSSION

RBS is one of the most important problems affecting fertility, causing economic loss in dairy farming [31,32]. SE is one of the major etiologic factors of RBS in high milk-yielding cows, and it causes infertility, delayed onset of postpartum ovarian activity, prolongation of the luteal phase and a decline in the rate of conception [33]. The most commonly used technique to diagnosis SE is endometrial cytology using the cytobrush method [34]. Diagnosis is made by counting the PMNLs in the slides prepared from samples taken from the endometrium. But, the threshold number of cells required to diagnose SE is still a matter of debate. Various studies have been conducted on this subject, and different PMNL cells ratios have been accepted as a threshold based on DIM values [23,35,36]. Salasel et al.[30] defined a threshold of 3% PMNL for samples

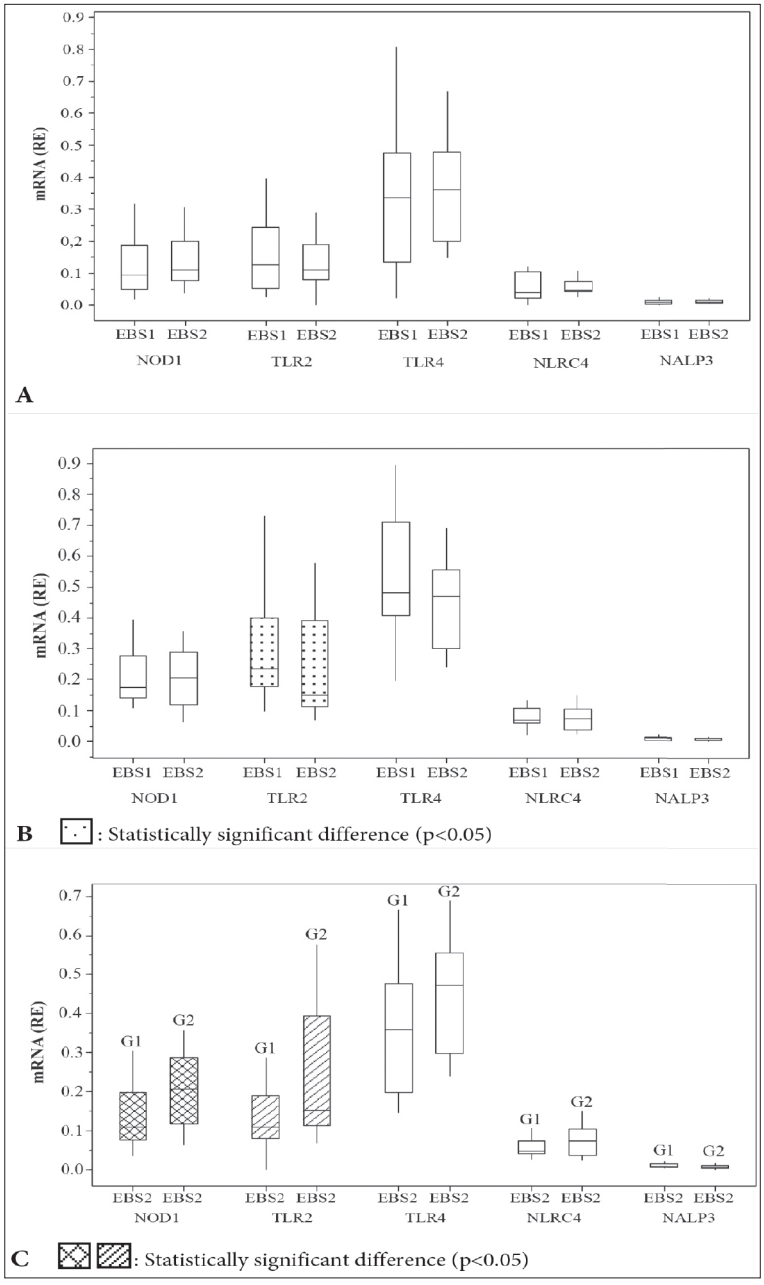

Fig 2. Box plot graphics of gene expressions. **A-** Transcription levels of NOD1, TLR2, TLR4, NLRC4 and NALP3 between EBS1 and EBS2 in G1, **B-** Transcription levels of NOD1, TLR2, TLR4, NLRC4 and NALP3 between EBS1 and EBS2 in G2, **C-** Transcription levels of NOD1, TLR2, TLR4, NLRC4 and NALP3 between EBS2 in G1 and EBS2 in G2

collected from cows at 190 DIM based on the results of the ROC analysis. The 3% PMNL threshold for diagnosis of SE was used in this study since the mean value of DIM was 205.6±55.6. Many studies have shown that the incidence of SE varies between 12-94% in the etiology of RBS [34,35]. The 95% SE rate calculated in this study is consistent with these studies.

One of the treatments of RBS caused by SE is the administration of presynch-10 and ovsynch. However, the use of PGF2α or GnRH in the treatment of endometritis on cytological recovery is controversial. For example, it has been reported in various studies that PGF2α stimulates the release of proinflammatory cytokines from the endometrium, induces local immunity and for these reasons can be used for SE treatment [37]. Nevertheless, there are also some studies which indicate that PGF2α administration has no statistically significant effect on the treatment of cytologic SE [23,38,39]. Similarly, cows with active CL cytologically diagnosed with SE were administered PGF2α for treatment, the cytological examination was performed again after treatment and there was no decrease in the prevalence of cytological SE [23]. In another study, GnRH administration was performed to stimulate ovulation in 128 cows at postpartum days 17±3. No significant differences were found between the control and the SE group based on the cytological examination on postpartum day 35±3 [39]. Histological studies have shown that leukocytes and neutrophils accumulate in the deep layers of the endometrium, such as stratum compactum [40]. Therefore, PMNL cells may not be found in the cytological samples taken from endometrium in

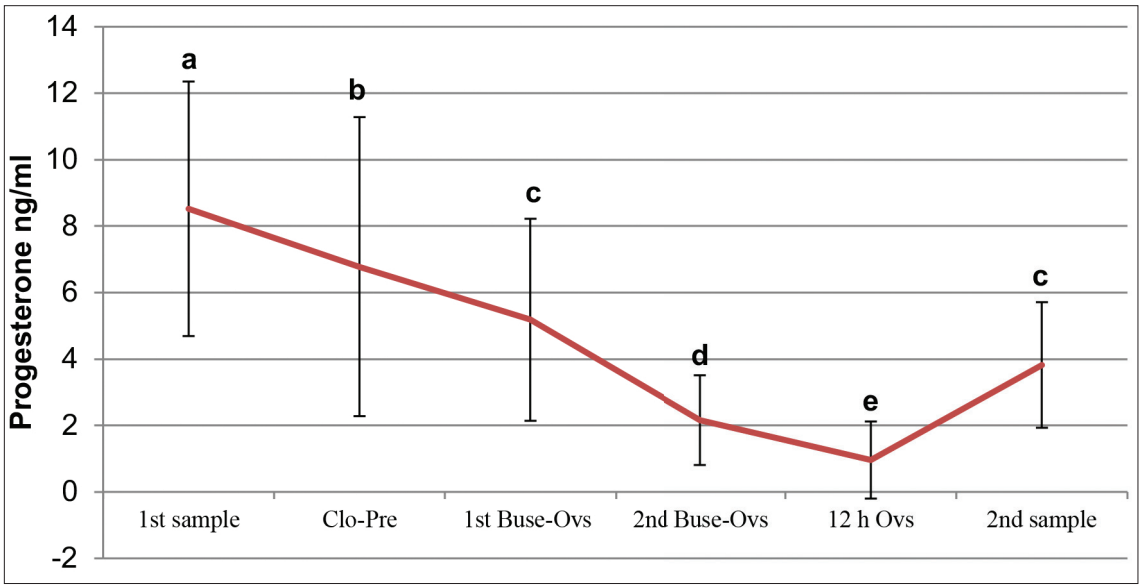

Fig 3. Progesterone levels by administration days in G1. a:b:c:d:e, P<0.05, **Clo-Pre:** 1st cloprostenol injection of presynch-10; **1st Buse-Ovs:** 1st buserelin acetate injection of ovsynch; **2nd Buse-Ovs:** 2nd buserelin acetate injection of ovsynch; **12 h Ovs:** 12 h after ovsynch

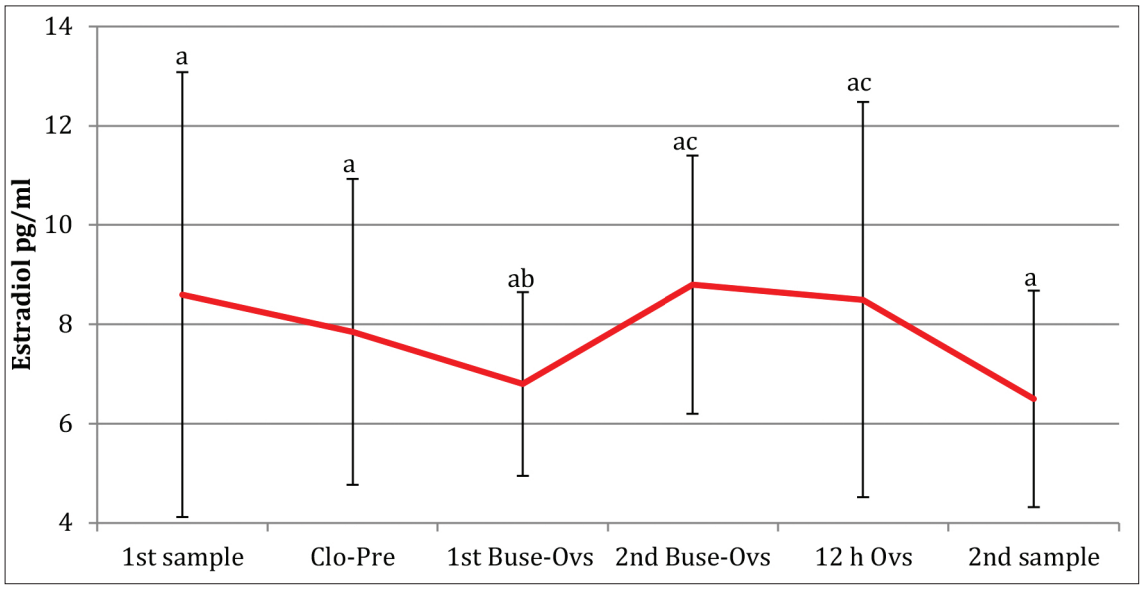

Fig 4. Estradiol levels by administration days in G1. b:c, P<0.05, **Clo-Pre:** 1st cloprostenol injection of presynch-10; **1st Buse-Ovs:** 1st buserelin acetate injection of ovsynch; **2nd Buse-Ovs:** 2nd buserelin acetate injection of ovsynch; **12 h Ovs:** 12 h after ovsynch

chronic SEs [41]. The reason that there is no cytological difference in the samples before and after presynch-10 and ovsynch administration in G1, or between the two samples in G2 may be that the cells are located in the deep layers of the endometrium. In addition to cytology, bacteriological examination is also important to evaluation of SE. Many different bacterial strains can be identified in clinical or subclinical endometritis in cows. For example, *Trueperella pyogenes* is the most prevalent microorganism in clinical endometritis [42] and is less common in SE [43]. In addition, it has been shown that the increase in the PMNL count is associated more with *T. pyogenes* than other pathogens [44]. *T. pyogenes* was not be identified in any microbiological cultures made from the 40 endometrial samples. However, *Coagulase negative staphylococci,* which is considered an opportunistic microorganism of the genital tract, and *E. coli, Bacillus* sp. and *Citrobacter* sp. which are classified as uterine pathogens in cows, and fungi, such as *Aspergillus* sp. were identified at various concentrations. The microbiological results indicate that there was no microbiological improvement in the samples whether taken before or after prescynch-10 and ovsynch. In this context, it can be assumed that the synchronization protocols have no microbiological healing effect in

cows with RBS. However, the statistical difference found between the first and second samples in G2 may be coincidental. The results of microbiological analyses have revealed that a wide variety of microorganisms are isolated in cows with RBS and SE. This situation suggests that endometrial microbiological findings are coincidental in RBS with SE [3]. Moreover, serum estradiol and progesterone levels were also monitored to determine whether the presynch-10 and ovsynch administrations were effective or not. And, as a result of the statistical analysis; hormone levels in the first serum samples of all cows in G1 and G2 were revealed to be consistent with progesterone and estradiol levels in the late diestrus of cows [45]. This also shows that they were in the same stage of the estrus cycle. In addition, the hormone levels in the other blood samples collected from G1 were compatible with the measured hormone levels with applications similar synchronization protocols in a different study [46]. On the other hand, progesterone levels measured during ovsynch were found to be different from our findings in another study. When the study examined, it was determined that as presynch, PGF2α was administered 2 days before ovsynch and the CLs were lysed [47]. Therefore, the measured progesterone levels were less than our findings. Because the last PGF2α injection was administered to 10 days before starting ovsynch. Namely, it was an active CL when ovsynch started. These results suggest that there was no abnormality in the ovarian functions of these cows and, administered synchronization protocols were effective.

After all these evaluations, the effects of synchronization methods administered on expression levels of some TLR and NLR genes were assessed. TLR2 and 4 are members of the TLR family that contribute to the innate immune response by stimulating the release of proinflammatory cytokines, and it has been determined that they were expressed in the endometrial cells of cows. In addition, the expression profiles of these receptors reportedly do not change according to the region where the samples are taken from in the uterus [27]. Recent studies revealed that TLR4 expression was higher in healthy and fertile cows than infertile ones with chronic endometritis at approximately 200 days postpartum [48]. Lower TLR4 expression in chronic endometritis has been associated with repeated or prolonged antigen stimulation [49]. There was no difference in TLR4 gene expression levels between or within groups, which is probably due to the fact that subclinical infections were chronic at that time. When the NLR expression profile was examined; it was concluded that the level of NLRC4 expressions did not change in all biopsy samples because the *Salmonella* could not be isolated from any endometrial swab. The activation of NLRC4 occurs when Salmonellar flagellin bind to NLRC4 [20]. The NOD2 and NALP3 are other members of NLRs and they are intracellular receptors. Muramyl dipeptide (MDP), which is a minimal structure of the bacteria's peptidoglycan cell wall, is a ligand that activates NOD2 and NALP3. MDP must be in cytosol to activate NOD2 and NALP3 [50]. NALP3 expression levels were low, and there were no differences between the samples. Similarly, NOD2 expression remained at undetectable levels. The low and undetectable expression levels of these genes may be due to the absence of activators in the cytosol. Likewise, this may be due to these receptors not being affected by prolonged inflammatory stimulus or synchronization protocols.

Tissue stress or malfunction could induce the innate immune response, which is referred to as para-inflammation. In this context, the reason for higher TLR2 and NOD1 expressions in EBS1 than EBS2 in G2, may be due to immune system stimulators, such as poor environmental conditions or malnutrition of the endometrial cells due to their malfunction and prolonged inflammation [51]. The increases seen in both TLR2 and NOD1 in the EBS2s of G1 supported the hypothesis that synchronization protocols may have immunomodulatory effects. These findings may be due to decreased progesterone level in EBS2 than EBS1 or administered PGF2α injections during presynch-10 and ovsynch. Because it is known that progesterone suppresses local immunity of uterus and, the synthesis of prostaglandins. PGF2α administered during synchronization caused luteolysis and, decreased progesterone concentration. Besides, PGF2α has also immunomodulatory effects independent of progesterone concentration [52]. However, these administrations did not affect the expression profiles of other receptor genes. These data showed that the mechanisms linking the steroids to immunity are yet to be fully elucidated and many studies need to be done in reproductive immunity.

Our data show that except for NOD2 all of the receptor genes examined were expressed in the endometrial cells of cows with SE. All of the other gene expressions were identified in all groups. These gene expressions could indicate that the mucosal innate immune system of endometrium plays various roles in RBS caused by SE. Furthermore, this study revealed that expressions of TLR-2 and NOD1 genes could be affected by ovulation synchronization methods related to GnRH and PGF2α.

Hence, a potential reason for the low success rate of implantation is subclinical endometritis in cows that may damage tissues. This damage could induce an inflammatory process by releasing some cytokines that are also stimulated by the activation of TLR and NLRs, thus initiating an inflammatory process and leading to embryo implantation failure, which is one of the causes of RBS. In this context, it was concluded that presynch-10 and ovsynch administration in cows positively affected the innate immune response and may be effective in the treatment of subclinical endometritis. Finally, the future potential scope of our research is to investigate the effects of TLR2 and/or NOD1 on the etiology of RBS.

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Research Article

Stereological and Histomorphological Assessment of New Zealand Rabbit Kidneys

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Abstract

The objectives of this study were to determine renal volume, volume ratios in New Zealand rabbits by stereological methods, reveal the histomorphological properties of tubulus proximalis, tubulus distalis, collecting tubule, Henle's loop and number of glomerulus. Besides, it is to investigate the possible differences between the functional subcomponents of the right and left kidneys and the effects of gender discrimination on them. The study was carried out on 9 males and 9 females healthy New Zealand rabbits' kidneys. After weighing kidneys, diameters and lengths were measured with a digital caliper. Total kidney volume and volume fractions of subcomponents of left and right kidneys were estimated by Cavalieri's method. The histological section was taken from the sampled kidneys and kidney structures in the unit area were counted. After all values of each component were expressed as ratios with in kidney, they were analyzed statistically to reveal differences between sexes. There was no statistical difference between the renal densities. The right dorsoventral and mediolateral diameters of the females and males were found to be greater than the left ($P<0.05$). No statistical difference was found in volume measurements with Archimedes' principle and Cavalieri's method ($P>0.05$). It was determined that the number of left collecting tubules in female rabbits was higher than males and it was statistically significant ($P<0.05$). Obtained data by making sexual dimorphism will contribute to the existing anatomical knowledge accumulation.

Keywords: *Kidney, Histomorphometry, Cavalieri's principle, Stereology, Rabbit*

Yeni Zellanda Tavşanlarında Böbreğin Stereolojik ve Histomorfometrik Değerlendirilmesi

Öz

Çalışmanın amacı Yeni Zelanda tavşanlarında böbrek hacim ve hacim oranlarını stereolojik yöntemlerle belirlemek, tubulus proximalis, tubulus distalis, toplayıcık borucuk, Henle kulpu ve glomerulus sayılarının histomorfolojik özelliklerini ortaya koymak, sağ ve sol böbreklerin fonksiyonel alt bileşenleri arasındaki olası farkları ve cinsiyet farkının bunlara etkisini araştırmaktır. Çalışma 9 erkek ve 9 dişi sağlıklı Yeni Zelanda tavşanı böbreği üzerinde gerçekleştirildi. Böbrekler tartıldıktan sonra, çapları ve uzunlukları dijital kumpas yardımıyla ölçüldü. Sol ve sağ böbreği oluşturan alt bileşenlerinin toplam böbrek hacmi ve hacim oranları Cavalieri metodu kullanılarak hesaplandı. Örneklenen böbreklerden histolojik kesitler alındı ve birim alandaki böbrek yapıları sayıldı. Böbreği oluşturan bileşenlerin değerleri oransal olarak ifade edildikten sonra, cinsiyetler arasındaki farklılıkları ortaya çıkarmak için istatistiksel analiz gerçekleştirildi. Böbrek yoğunlukları arasında istatistiksel bir fark tespit edilemedi. Dişi ve erkek tavşanlarda sağ dorsoventral ve mediolateral çapların sol taraftan daha büyük olduğu tespit edildi ($P<0.05$). Arşimed prensibi ve Cavalieri metodu ile yapılan hacim ölçümlerinde istatistiksel bir fark bulunmadı ($P>0.05$). Dişi tavşanlarda sol toplayıcı borucuk sayısının erkek tavşanlardan daha yüksek olduğu ve istatistiksel olarak anlamlı olduğu tespit edildi ($P<0.05$). Cinsiyet ayrımı yapılarak elde edilen verilerin mevcut anatomik bilgi birikimine katkıda bulunacağı düşünülmüştür.

Anahtar sözcükler: *Böbrek, Histomorfometri, Cavalieri prensibi, Stereoloji, Tavşan*

INTRODUCTION

Morphometric features of kidneys and the relative organ weights are clinically important. Kidney volume and volume fractions have been used to predict overall renal function in a normal individual and in those with chronic renal

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disease [1,2]. Renal cortex thickness and area have been shown to be useful for the prediction of the presence of unilateral renal artery stenosis with far greater sensitivity and accuracy than renal bipolar length in patients with the early atherosclerotic renovascular disease or fibromuscular dysphasia [1,3]. Also these parameters can be used in pharmacological and toxicological studies in addition to the chemical and food industries [2,4].

Structural parameters, such as cortical volume and glomerular number, are significantly and positively correlated with glomerular filtration rate [5]. The volume of the renal cortex is considered to be an important factor in the prognosis of patients with chronic kidney disease [6]. Volumetry of the renal parenchymal, the cortex volume of the anticipated remnant renal volume and the number of subcomponents that make up the kidney provide essential information before renal surgery [6,7]. Therefore, volume estimation and histomorphometric property are necessary to evaluate normal or pathological conditions. These morphometric parameters in the healthy animal can be used to elucidate the relation between a structure and its function [8].

Although the rabbit kidney is similar to other rodent kidneys, it is preferred because it is more sensitive to nephrotoxicity studies, so New Zealand rabbit is increasingly used as an experimental model [9,10].

In studies on the rabbit kidney, biochemical parameters are generally examined, and the morphology and histology of the kidney are not mentioned. A few studies have reported on the morphological and morphometric features of the kidneys in various rodent species, including the rat [11], rabbit [12,13], guinea pig [14]. However, histomorphometry of the kidney has not been mentioned in rabbit studies.

The objectives of this study were to determine renal volume and volume ratios in New Zealand rabbits by stereological methods, and to reveal the histomorphological properties of tubulus proximalis, tubulus distalis, collecting tubule, Henle's loop and number of glomerulus. Besides it is to investigate the possible differences between the functional subcomponents of the right and left kidneys and the effects of gender difference on them.

MATERIAL and METODS

Materials

In this study, 18 (9 male, 9 female) healthy New Zealand Rabbits aged 14 months were used and the approval for investigation was obtained by Karamanoglu Mehmetbey University Faculty of Health Sciences Ethics Committee (No:09-2018/36). All the rabbits were given standard rabbit diet and *ad libitum* water, and the animals were housed individually under the same conditions. Animals were anesthetized by administration of xylazine hydrochlorure (10 mg/kg, IM) plus ketamine hydrochloride (30 mg/kg, IM) [15]. Abdominal cavity of the animals in the supine position was entered an incision along abdominal wall and was given 10% formalin saline into abdominal aorta. Euthanasia was carried out by an incision made on the vena cava caudalis. The left and right kidney were removed after euthanasia.

Morphometric Measurements

After removing the perirenal adipose tissue and connective tissues, the right and left kidneys were individually weighed and total volumes of kidneys were measured with a graded cylinder applying the Archimedes' principle. The dorsoventral and mediolateral diameters at the hilus renalis level and craniocaudal lengths were measured using a digital caliper. The density of each kidney was calculated by dividing the weight to the volume.

Estimation of Total Volume and Volume Fractions by Application of the Cavalieri's Method

In order to be able to apply the Cavalieri's method and to avoid disintegration of the kidneys during sectioning, the kidneys were plated with agar (Blood Agar Base LABM-LAB028). After boiling for 10 min, the solution was cooled to 60°C and poured into special containers containing kidneys and the blocks were prepared [16]. The blocks were stood at room temperature for 24 h. Kidneys were cut with an electric salami slicing machine (SINBO SMS-5601) and depending on the size of the kidneys, 10 to 12 sections were obtained for volume estimation *(Fig. 1)*. The mean slice thickness was 4.03 mm in the left kidney and 4.01 mm in the right kidney. The slicing process was carried out perpendicular to the craniocaudal length. The same faces of the sections were scanned at 600 dpi in JPG format using a horizontal scanner (hp Scanjet G4010).

In the volume calculations (kidney, renal cortex, renal medulla and renal pelvis), ImageJ program was used. The point counting frame with different point frequencies was discarded on the section images with the grid command

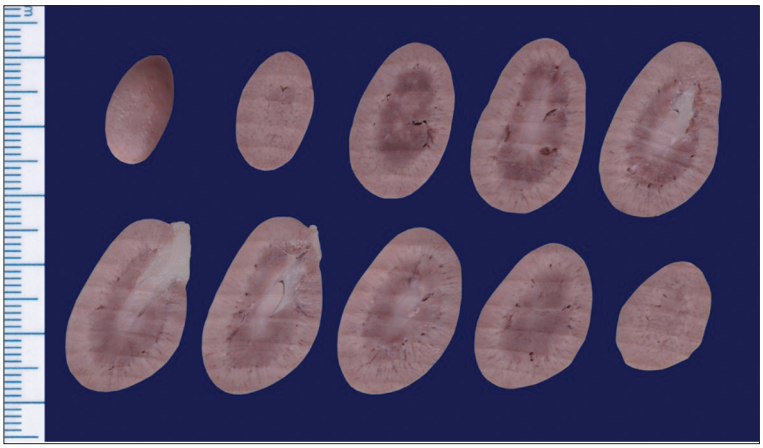

Fig 1. An example of consecutively sectioned kidney with slicer

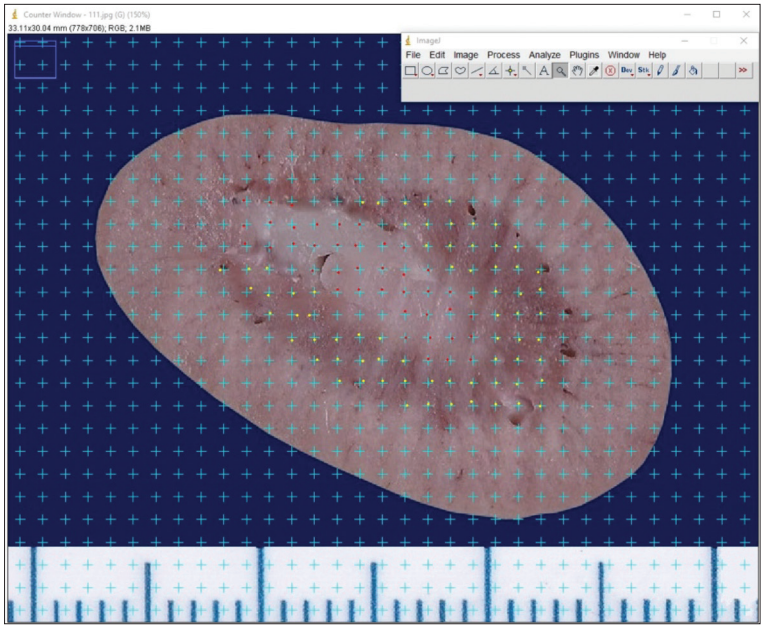

Fig 2. Renal cortex, renal medulla and renal pelvis counting on kidney with ImageJ program (area per point = 1 mm^2)

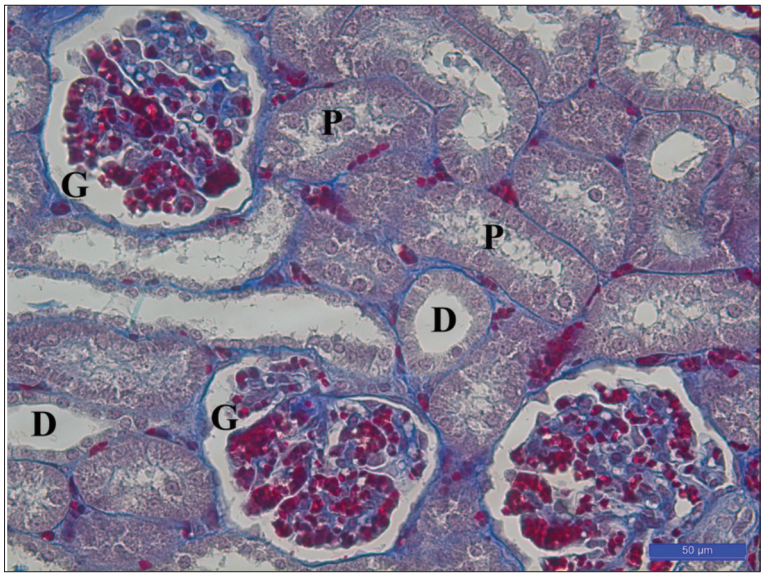

Fig 3. Histological appearance of the kidney in New Zealand Rabbits, D: Distal tubules, G: Glomerulus, P: Proximal tubules, Crossman's trichrome staining

of the software. In this counting frame, the area per point was set 1 mm^2 for kidney, the cortex and medulla and 0.1 mm^2 for the renal pelvis to reach a reliable coefficient of error (CE) *(Fig. 2)*. For each area of interest, a different marker was chosen and the points falling into the areas were counted separately. CE was calculated according to the relevant literature [17].

The volumes of the structures of interest in the sections were calculated separately using the formula V= a(p) xx t. In this formula, V refers to the volume of interest region a(p) is the area of the one point on the grid, Σp is the sum of the points on the structure of interest and t is the section thickness [17,18]. Renal cortex, renal medulla and renal pelvis volume ratios were obtained by dividing related kidney section to the volume of total kidney.

Histological Analysis

After the volume calculations, the kidneys were sampled at a rate of ½. The tissue samples were fixed in 10% buffered formaldehyde-saline solution, dehydrated, and embedded in paraffin blocks. The tissue sections taken from paraffin blocks in 6 µm thick were stained with Crossman's trichrome staining. The cross-sections of the corpusculum renis, tubulus proximalis, tubulus distalis, Henle's loop and number of glomerulus on the sections taken from the blocks were determined using light microscopy in the unit area *(Fig. 3* and *Fig. 4)*.

Statistical Analysis

Statistical analysis was performed using SPSS software version 21.0. The results of this study were compared by two sample t test. The values were expressed as mean and standard error (mean±SE). P<0.05 was considered statistically significant.

RESULTS

The weights of female and male New Zealand rabbits were 3254.4±169.9 g and 2714.2±77.6 g, respectively. The weight of the left kidney measured in female rabbits was 12.66±0.69 g and the right kidney weight was 12.19±0.54 g. In the male rabbit, these weights were 11.19±0.41 g and 10.86±0.37 g, respectively. It was found that the density of the left kidney was 1.04±0.06 g/mL and of the right kidney was 1.05±0.02 g/mL in the female rabbit. In the male rabbit, density measurements of left and right were 1.05±0.02 g/mL and 1.04±0.02 g/mL, respectively. There was no statistical difference between the renal densities.

Measurements of length and diameter of kidneys in female and male rabbits were given in *Table 1*. The dorsoventral and mediolateral diameters of right kidneys in females and males were found to be greater than those of the left ones. It was determined that the left and right mediolateral diameters of female rabbits were larger than those of the males, and the difference was statistically significant (P<0.05).

In the volume measurements of female rabbits performed with Archimedes' principle, the left kidney was 12±0.76 mL and the right kidney was 11.11±0.68 mL. In males,

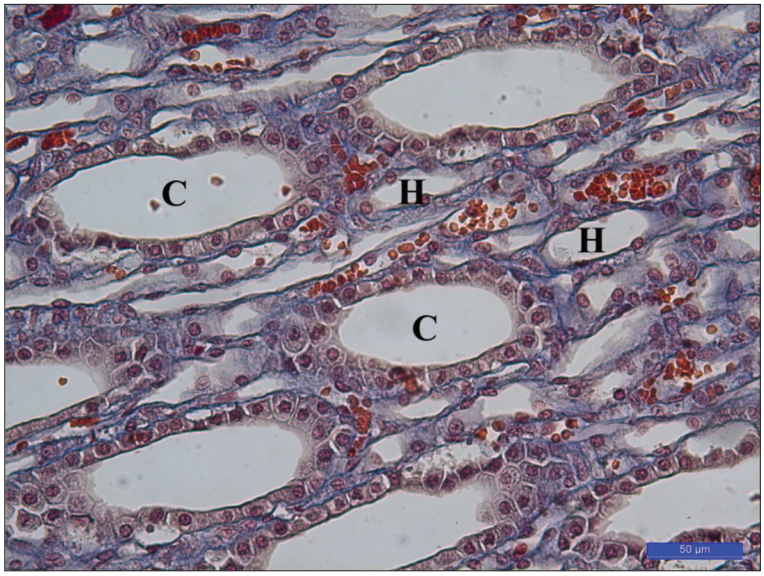

Fig 4. Histological appearance of the kidney in New Zealand Rabbits, C: Collecting tubules, H: Henle's loops, Crossman's trichrome staining

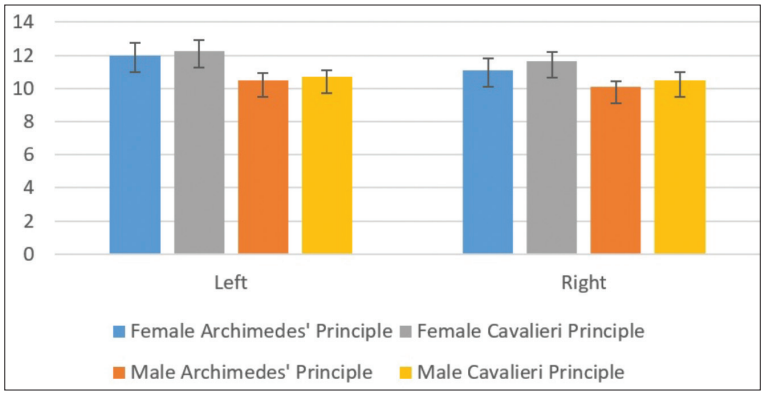

Fig 5. The volume of kidneys obtained using Archimedes' principle and the Cavalieri's method

kidneys and subcomponents of kidneys in male and female rabbits were given in *Table 2*. It was determined that in female rabbits, 69.67% of the left kidney was composed of renal cortex, 29.35% of the renal medulla, 0.98% of the renal pelvis. On the right side, 77.98% of the kidney was renal cortex and 20.87% of the renal medulla 1.15% renal pelvis. In male rabbits, these rates were 77.58%, 21.57% and 0.85% in the left kidney, 77.13%, 22.03% and 0.84% in the right kidney, respectively. The left renal medulla was found to be larger than the right in male and female rabbits (P<0.05). No statistical difference in the volume of kidney and in the subcomponents of kidney was found between the female and the male New Zealand rabbits (P>0.05). The error coefficients were below 5% *(Fig. 6)*.

The average counts of the glomerulus, proximal tubule, distal tubule, Henle's loop, collecting tubule counts in the unit area of male and female New Zealand rabbit kidney were given in *Table 3*. It was determined that the number of left collecting tubules in female New Zealand rabbits was higher than that of males and it was statistically significant (P<0.05). There was no difference between counted histological kidney structures in the left and right kidneys (P>0.05).

DISCUSSION

In diagnosis of renal diseases, parameters such as volume and volume ratios, histomorphometric structure, and relative organ weight of kidneys are of great importance [4,19]. Changes in cortex and medulla of the kidney indicate pathological

Table 1. *Kidney length and diameter measurements*

Parameter	Female		P Value	Male		P Value
	Left (Mean±SE)	Right (Mean±SE)		Left (Mean±SE)	Right (Mean±SE)	
Craniocaucaudal length (mm)	38.32±0.62	38.66±0.79	0.549	36.83±0.51	37.14±0.54	0.462
Dorsoventral diameter (mm)	21.37±0.56	19.57±0.47	0.005*	20.89±0.47	19.56±0.50	0.044*
Mediolateral diameter (mm)	24.99±0.54	25.92±0.46	0.009*	23.52±0.34	24.35±0.26	0.049*

** P<0.05*

these measurements were 10.5±0.41 mL in the left and 10.11±0.34 mL in the right. In the measurements made with the Cavalieri's method, in female rabbits the left kidney was 12.26±0.66 mL and the right kidney was 11.66±0.54 mL, in male rabbits the left kidney was 10.69±0.42 mL and the right kidney was 10.49±0.49 mL. No statistical difference was found in volume measurements with Archimedes' principle and Cavalieri's method (P>0.05) *(Fig. 5)*.

The calculated volumes with Cavalieri's method for the

changes. Kidney morphometry and the amount of nephron structures are influential on the potential functional capacity of the organ [10,20]. The knowledge of the volumes of structures of the healthy kidney is required for diagnosis of pathologies that alter renal volume and its structure. In this study, morphometric properties of kidney were determined in detail by making sexual dimorphism. In addition, a study showing the numbers of the glomerulus, proximal tubule, distal tubule, Henle's loop and collecting tubule in New Zealand rabbits could not be determined in

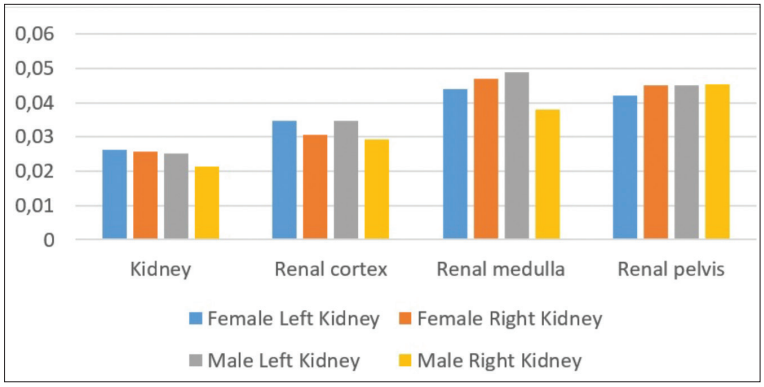

Fig 6. Coefficient of Error values kidney and its structures

is asymmetrical. The right kidney is located more cranial than the left kidney [12]. Therefore, differences in right and left kidney's diameters and lengths are expected. In the present study, dorsoventral and mediolateral diameters of left kidneys were found to be larger than those of the right kidney in females and males New Zealand rabbit, and females were found to be larger than males. However, no difference was detected in craniocaudal length. In the study conducted by Dimitrov et al.[21], the difference was thought to be due to the age difference of the rabbits used. The difference with Eken et al.[22] was thought to be due to the methodological difference.

Table 2. *Volume measurements of left and right kidneys*

Item	Female		P Value	Male		P Value
	Left (mL) (Mean±SE)	Right (mL) (Mean±SE)		Left (mL) (Mean±SE)	Right (mL) (Mean±SE)	
Kidney	12.26±0.66	11.66±0.54	0.291	10.69±0.42	10.49±0.49	0.520
Renal cortex	8.54±0.61	8.63±0.52	0.856	7.28±0.34	7.42±0.27	0.674
Renal medulla	3.59±0.12	2.93±0.18	0.005*	3.31±0.27	2.97±0.26	0.044*
Renal pelvis	0.12±0.02	0.10±0.01	0.285	0.10±0.01	0.10±0.01	0.477

** P<0.05*

Table 3. *The number of glomerulus, proximal tubule, distal tubule, Henle's loop, collecting tubule in per unit area (Mean±SE)*

Item	Female		P Value	Male		P Value
	Left	Right		Left	Right	
Glomerulus	19.89±1.79	17.44±1.81	0.281	20.00±1.69	17.33±1.44	0.110
Proximal tubule	262.22±14.17	270.11±17.66	0.723	266.44±6.24	257.33±9.48	0.259
Distal tubule	117.33±7.12	132.89±10.48	0.300	107.78±6.05	105.33±8.77	0.863
Henle's loop	433.44±41.54	456.56±35.79	0.733	468.44±26.18	473.00±49.44	0.942
Collecting tubule	184.78±15.26	157.56±16.85	0.303	119.22±11.66	140.11±13.21	0.208

** P<0.05*

literature search. Therefore, this data will contribute to the existing anatomical knowledge.

In a study with 50 adult male and female rabbits without macroscopic renal pathology, Santos-Sousa et al.[2] was found no significant difference in any of the renal dimensions between the right and left kidneys in either sexes. In another study performed on 12 mature healthy rabbits, Dimitrov et al.[21] reported that the left kidney's craniocaudal length and mediolateral diameter were larger than that of the right kidney and the right kidney's dorsoventral diameter was larger than that of the left. In a study with eight adult healthy rabbits of both sexes which used three dimensional reconstructions of multidetector computed tomography images, Eken et al.[22] reported that the dorsoventral diameter, mediolateral diameter and craniocaudal length of the left kidney were larger than those of the right. Kidneys begin their development near the sacral region and move forward. The posture of the two kidneys

In a study with nine male rabbits comparing fresh and fixed kidneys in formalin solution, Bolat et al.[12] did not detect any difference between the right and left kidney volumes. Renal cortex, renal medulla and renal pelvis volume ratios were 59.8%, 36.4%, 3.8% in left kidney and 61.8%, 34.7%, 3.4% in right kidney, respectively. The left kidney's dorsoventral diameters were also found to be larger than that of the right. In the present study, it was found that the left renal medulla was larger than that of the right in male New Zealand rabbits. Volume fractions of left and right renal cortex, renal medulla and renal pelvis were estimated to be 77.58%, 21.57%, 0.85% and 77.13%, 22.03%, 0.84%, respectively. In the study, dorsoventral diameters of the left kidney as well as the left mediolateral diameter were found to be larger than right. It is thought that the difference between the two studies is due to the age differences of the rabbits used.

Bolat et al.[12] reported that left renal density of male New

Zealand rabbits was 0.97, and the right renal density was 1. There was no statistical difference between left and right renal density. In present study, renal density was found to be 1.04±0.06 for the left kidney and 1.05±0.02 for the right kidney in female rabbit. In male rabbit, left and right was 1.05±0.02 and 1.04±0.02, respectively. No statistical difference was found in the presented study (P>0.05). In the literature search, data on the renal density of female New Zealand rabbits could not be found.

One of the most important steps of stereological studies is the determination of the error coefficient. The quality of the numerical measurements made by stereological studies and the accuracy of the sampling plan can be observed by calculating the error coefficient (CE). Although the error coefficient in stereological studies does not correspond to a real biological value, it is a value indicating the quality of the sampling strategy [23]. In order for the results of stereological studies to be considered as reliable, the error coefficient should be 5% or less [17]. In the present study, the error coefficients were below 5% *(Fig. 6)*.

The morphometric data of the New Zealand rabbits' kidney and its subcomponents determined by using stereological methods and the data obtained by counting in unit area will provide insight for the investigation and comparison of renal hypertrophy, atrophy and tumor formation. Furthermore, in order to complete our study, the structures of functional subcomponents as a result of diseases should be examined by electron microscopy in New Zealand rabbits. It is thought that this study will guide the future studies methodologically.

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Research Article

Effects of Ozone and L-Carnitine on Kidney MDA, GSH, and GSHPx Levels in Acetaminophen Toxicity

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Abstract

This study aimed to determine the therapeutic effects of medical ozone and L-carnitine therapy on acetaminophen (APAP)-induced kidney damage by evaluating malondialdehyde (MDA), glutathione (GSH), and GSHPx levels. In this study, 56 rats were randomized into 8 groups with 7 rats in each group. Kidney injury was induced by the administration of a single dose N-acetyl-p-aminophenol (1 g/kg) orally. Therapeutic ozone (0.7 mg/kg) and L-carnitine (500 mg/kg) were administered intraperitoneally. After the therapy, the rat kidneys were homogenized, and the tissue MDA, GSH, and GSHPx levels were measured. Compared to the control groups, there were higher MDA levels in the kidney tissues only in the "APAP," "APAP + Ozone," and "APAP + Ozone + L-carnitine" groups ($P<0.001$). Besides, the decrease in the GSH and GSHPx levels of the kidney tissues in the study groups were significant compared to the control groups, and the highest decreases were observed in the "APAP," "APAP + Ozone" and "APAP + Ozone + L-carnitine" groups ($P<0.001$). Findings obtained from this study revealed that acetaminophen toxicity caused oxidative damage in the examined kidney tissues, and L-carnitine and/ or ozone applications for protective purposes decreased MDA levels, a product of lipid peroxidation, and increased tissue GSH levels thru GSHPx antioxidant enzyme activity. In this context, the most important protective effect was observed in the group where L-carnitine and ozone were administrated together.

Keywords: *Acetaminophen, Ozone, L-Carnitine, Nephrotoxicity*

Asetaminofen Toksisitesinde Ozon ve L-Karnitinin Böbrek MDA, GSH ve GSHPx Düzeylerine Etkisi

Öz

Bu çalışmada, malondialdehit (MDA), glutatyon (GSH) ve GSHPx seviyelerini değerlendirerek tıbbi ozonve L-Karnitin tedavisinin asetaminofen (APAP) kaynaklı böbrek hasarı üzerindeki terapötik etkilerinin belirlenmesi amaçlanmıştır. Çalışmada 56 adet rat her bir grupta 7 tane olmak üzere toplam 8 gruba ayrılmıştır. Böbrek hasarı N-acetyl-p-aminopenol'ün (1 g/kg) oral yolla tek doz uygulamasıyla oluşturulmuştur. Terapotik olarak kullanılan ozon (0.7 mg/kg) ve L-karnitine (500 mg/kg) intraperitoneal yolla uygulanmıştır. Daha sonra ratların böbrekleri homojenize edilerek doku MDA, GSH ve GSHPx değerleri ölçülmüştür. Çalışma gruplarında böbrek dokusu MDA düzeylerinin yalnızca APAP, APAP + Ozon ve APAP + Ozon + L-karnitin uygulanan gruplardaki artışlarının kontrol amaçlı kullanılan tüm gruplara göre istatistiksel olarak anlamlı ($P<0.001$) olduğu saptanmıştır. Ayrıca çalışma gruplarında böbrek dokusu GSH ve GSHPx düzeylerinin yalnızca APAP, APAP+Ozon ve APAP + Ozon + L-karnitin uygulanan gruplardaki düşüşlerin kontrol amaçlı kullanılan tüm gruplara göre istatistiksel olarak anlamlı ($P<0.001$) olduğu ve en yüksek düşüşlerin ise yalnızca APAP verilen grupta olduğu belirlenmiştir. Çalışmadan elde edilen bulgular asetaminofen toksikasyonun incelenen böbrek dokularında oksidatif hasarlara yol açtığı, koruyucu amaçla L-karnitin ve/veya ozon uygulamalarının dokularda lipit peroksidasyon ürünü olan MDA düzeylerini düşürdüğü ve antioksidan enzimlerden GSHPx aktivitesi ile doku GSH düzeylerini artırdığı tespit edilmiştir. Bu bağlamda en önemli koruyucu etkinin L-karnitin ve ozonun beraber uygulandığı grupta izlendiği dikkati çekmiştir.

Anahtar sözcükler: *Asetaminofen, Ozon, L-karnitin, Nefrotoksisite*

INTRODUCTION

Acetaminophen (paracetamol-APAP) is widely used for its analgesic and antipyretic effects. Although it is a trusted medication, frequent cases of intoxications [1], as well as, suicide attempts are observed due to its easy accessibility. The most important toxic effects of APAP occur on the liver and kidneys [2]. Elimination of this molecule occurs from the

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liver via sulfation (20-46%) and glucuronidation (40-67%) [3]. When an overdose of APAP is taken, rapid glucuronidation and sulfation mechanism ensue, swiftly depleting glutathione (GSH) levels. Under normal conditions, this intermediate product is detoxified by conjugation with GSH and is metabolized to mercapturic acid and excreted by the kidneys (<5%). However, if toxic doses are taken, hepatic GSH, an important factor in the antioxidant defense of the body, decreases by more than 70%. When the hepatic GSH reserves decrease by more than 30%, the reactive intermediate N-acetyl p-benzoquinoline (NAPQI) binds to hepatic macromolecules by covalent linkage and inhibits the function of enzymatic systems in the liver. As a result, the increased amount of active metabolite NAPQI exceeds the binding (detoxifying) capacity of glutathione and cannot be excreted via GSH, and thus, it binds to cytosol proteins in the tissues and leads to cell necrosis [2,4,5]. Although 1-2% renal insufficiency was reported after high-dose APAP use [6], the exact mechanism of nephrotoxicity could not be clearly elucidated. It is thought that inflammation and GSH decrease in the kidneys may be related to oxidative stress [7]. It is claimed that in most cases of APAP toxication, nephrotoxicity develops after hepatotoxicity.

Free radicals are electron acceptor molecules in biological systems that arise as a degradation product in the aldehyde structure by the breakdown of carbon bonds during the peroxidation of lipids [8]. As a result, they cause mutagenic, genotoxic, and carcinogenic damage in the cells by affecting the structure and function of molecules, cell membrane, genetic materials such as DNA, RNA, and various enzymatic events. Malondialdehyde (MDA), one of the most important end products of lipid peroxidation, is known to be one of the most sensitive indicators of lipid peroxidation during oxidation [9,10].

Reduced GSH, ceruloplasmin, transferrin, and ascorbic acid (vitamin C) used in reducing hydrogen peroxide as well as antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx), glutathione S-transferase (GST), and antioxidants such as alpha-tocopherol are used in the cells for reducing and neutralizing the harmful effects of free radicals in the organism [11]. GSH is a compound that can easily release hydrogen ions. Because of the sulfur groups it contains, GSH plays a significant role in the protection of the tissues from peroxidative effects. GSH can also serve as a reductant by reducing oxidized glutathione (GSSG) and reducing hydrogen peroxide and lipid hydroperoxides directly to H_2O. Intracellular GSH is consumed under continuous intracellular oxidative stress conditions. The depletion of intracellular GSH causes oxidation and damage of lipids, proteins, and DNA by reactive oxygen species (ROS) [12]. Additionally, also the glutathione peroxidase enzyme (GSHPx) is involved in the elimination of peroxidative damage. The increase of free oxygen radicals in tissues disrupts the balance between the prooxidant and antioxidant system and causes oxidative stress by affecting the enzymatic and non-enzymatic antioxidant defense mechanisms in the cells [11]. The level of oxidative stress is determined by evaluating the decreases in the quantity of antioxidants or increases in their metabolites [13].

L-carnitine is an amino acid-like compound that is synthesized in liver and kidneys and plays an important role in the β-oxidation of long-chain fatty acids in the mitochondria. L-carnitine, however, helps the organism by acting as a buffer for excess acyl-Co A, which may be harmful to the cells [14]. Many studies have demonstrated that L-carnitine, which is an important cofactor in fatty acid metabolism, can improve the antioxidant status in mice and rats by accelerating the removal of free radicals from cells and has a strong antioxidant effect on lipid peroxidation [15-17].

The application of the ozone/oxygen gas mixture is defined as ozone therapy [18]. In clinical studies, ozone therapy has been found to be useful in cases such as peritonitis, infected wounds, chronic skin ulcers, burns, ischemic diseases, necrotic enterocolitis, and acute necrotic pancreatitis [19-22]. Ozone is readily soluble in biological fluids such as plasma, lymph, and urine, and reacts immediately with polyunsaturated fatty acids, antioxidants, reduced GSH, and albumin. These compounds act as electron donors and undergo oxidation resulting in the formation of hydrogen peroxide (H_2O_2) and lipid oxidation products. H_2O_2, an essential ROS molecule, can act as an ozone messenger to reveal its various biological and therapeutic effects [22]. Free radicals are important for the emergence of the biological effects of ozone because free radicals are sometimes starting agents in chemical reactions, sometimes interfering in the intermediate steps, or resulting from the reaction of reagents. It is evident that ozone activates the antioxidant defense system in living beings against the effects of free radicals as a result of increasing the free radicals by creating an oxidative effect [23]. Studies have shown that ozone increases the activity of antioxidant enzymes such as GSHPx, SOD and CAT in physiopathological conditions in which ROS occur [24]. In a study conducted in 2010 Demirbag et al.[25], reported that high-dose APAP applications lead to renal damage and ozone therapy has a curative effect on renal damage caused by APAP toxicity.

In this study, we aimed to determine whether L-carnitine and ozone applications have protective and/or therapeutic properties in kidneys with experimental APAP toxication, and tried to measure the tissue levels of GSHPx, a potent antioxidant enzyme, MDA an end product of lipid peroxidation, and reduced GSH.

MATERIAL and METHODS

Ethical Approval

All procedures performed in studies involving animals were

in accordance with the ethical standards of the institution or practice at which the studies were conducted (Ethical approval: KAÜ-HADYEK- 2018/42).

Animals

Fifty-six female Wistar Albino Rats aged 4-6 months and weighing 190-250 g were used in this study. The rats were obtained from the Atatürk University Experimental Animals Breeding Unit, and housed individually in plastic cages in a ventilated and temperature-controlled room at 25°C with a 12 h light-12 h dark cycle, and fed as *ad-libitum*.

Experimental Design

Animals were randomized into eight groups according to their weights making seven rats in each group.

The groups were;

Control group: Received only 0.9% NaCl orally.

Ozone group: O_3 (0.7 mg/kg) administered intraperitoneally [21].

L-carnitine group: L-carnitine (500 mg/kg) administered intraperitoneally [17].

Ozone + L-carnitine group: O_3 (0.7 mg/kg) intraperitoneally and L-carnitine (500 mg/kg) administered intraperitoneally.

APAP group (2nd Control): Damage caused by a single dose N-acetyl-p-aminophenol (1 g/kg) administered orally [7].

APAP + Ozone group: Damage caused by a single dose N-acetyl-p-aminophenol (1 g/kg) administered orally followed by intraperitoneal O_3 (0.7 mg/kg).

APAP + L-carnitine group: Damage caused by a single dose N-acetyl-p-aminophenol (1 g/kg) administered orally followed by intraperitoneal L-carnitine (500 mg/kg) administered one h later.

APAP + Ozone+ L-carnitine group: Damage caused by a single dose N-acetyl-p-aminophenol (1 g/kg) administered orally followed by intraperitoneal O_3 (0.7 mg/kg) and L-carnitine (500 mg/kg) administered one h later.

At the end of the study, the animals were not fed overnight. 24 h after the last administration sacrification was performed with cervical vertebra dislocation under ethical rules. Blood and tissue samples were obtained after sacrification. The renal tissue samples were collected in 10% formaldehyde for pathological examinations.

Ozone Administration

Ozone used in the study was obtained from Kafkas University Health Research and Application Hospital Medical Ozone Generator (Blue Medical Ozone Generator, Turkozone/TR). The ozone gas (oxygen 95%, ozone 5%), which was taken into the 20 mL injectors just before the application, was transferred to the application area with the ajutage part up to prevent ozone from voletile. During the application, 26 Gauge (13 mm) injector tip was used.

Tissue Biochemical Analysis

Malondialdehyde, GSH, and GSHPx levels were analyzed from the renal tissue samples. Kidneys were harvested, and the tissues were rinsed with 0.9% NaCl. Tissues were homogenized in phosphate buffer (pH 7.4) in 0.1 M KCl, and the homogenates were centrifuged at 1500 rpm for 5 min. All samples were stored at -25°C until analysis. Analyses for GSH and MDA concentrations were carried out by the methods described byBeutler et al.[28] and Yoshoiko et al.[29], respectively.

Histopathological Examination

For histopathological examinations, fixation was achieved by keeping the kidney tissues in 10% formaldehyde for 24 h. Concomitantly, the kidneys were put into histology cassettes and left for tissue follow-up. Using a microtome, sections of 4-micron thickness were taken from the paraffin blocks, stained with hematoxylin-eosin, and evaluated under a light microscope. For the evaluation, a 4-point scoring system (0=none, 1=mild, 2=moderate, 3=severe) was used for vascular congestion, glomerular damage, tubulointerstitial inflammation, and tubular damage.

Statistical Analysis

Statistical analysis of all data obtained from the study was performed with the SPSS for Windows, version 10.0 program. Statistical differences between the groups were tested by analysis of variance (ANOVA) and Tukey's test. Data were presented as mean±standard errors, and P values less than 0.05 were considered significant. As the significance level was accepted as $P<0.05$ a total of 7 subjects in each group corresponds to a power of about 80%. We conducted a normality test with the data and subsequently conducted ANOVA analyses when no evidence of deviation from the normality. We did not detect any deviations from the normality.

RESULTS

The levels of MDA, GSH, and GSHPx in the kidney tissues are shown in *Table 1*.

Statistically significant difference was observed in the MDA levels in the APAP, APAP + Ozone, and APAP + Ozone + L-carnitine groups compared to the control groups (control, Ozone alone, L-carnitine, and Ozone + L-carnitine) ($p<0.001$), and the highest increase was seen in the APAP-only group *(Table 1)*.

Statistically significant difference was observed in the GSH and GSHPx levels in the APAP, APAP + Ozone, and APAP + Ozone + L-carnitine groups compared to the control groups (control, Ozone alone, L-carnitine, and Ozone + L-carnitine) ($P<0.001$), and the highest decrease was seen in the APAP-only group *(Table 1)*.

Table 1. *The levels of MDA, GSH, and GSHPx in the kidney tissues*

Parameters	Control	Ozone	L-Carnitine	Ozone + L-Carnitine	APAP	APAP + Ozone	APAP + L-Carnitine	APAP + Ozone + L-Carnitine	P
MDA (nmol/g)	1.58±0.13^{c}	1.60±0.11^{c}	1.54±0.10^{c}	1.50±0.13^{c}	2.30±0.17^{a}	1.96±0.12^{b}	1.90±0.03^{b}	1.83±0.09^{b}	<0.001
GSH (nmol/g)	4.39±0.13^{a}	4.42±0.11^{a}	4.45±0.09^{a}	4.40±0.14^{a}	3.09±0.13^{c}	3.61±0.18^{b}	3.75±0.12^{b}	3.80±0.17^{b}	<0.001
GPx (U/g)	0.49±0.05^{a}	0.43±0.06^{a}	0.51±0.06^{a}	0.47±0.02^{a}	0.26±0.01^{c}	0.37±0.04^{b}	0.39±0.03^{b}	0.34±0.05^{b}	<0.001

MDA: *malondialdehyde;* ***GSH:*** *glutathione;* ***GSHPx:*** *glutathione peroxidase;* ***APAP:*** *acetaminophen;* ^{a,b,c,d} *show statistical significance between groups per line (P<0.05)*

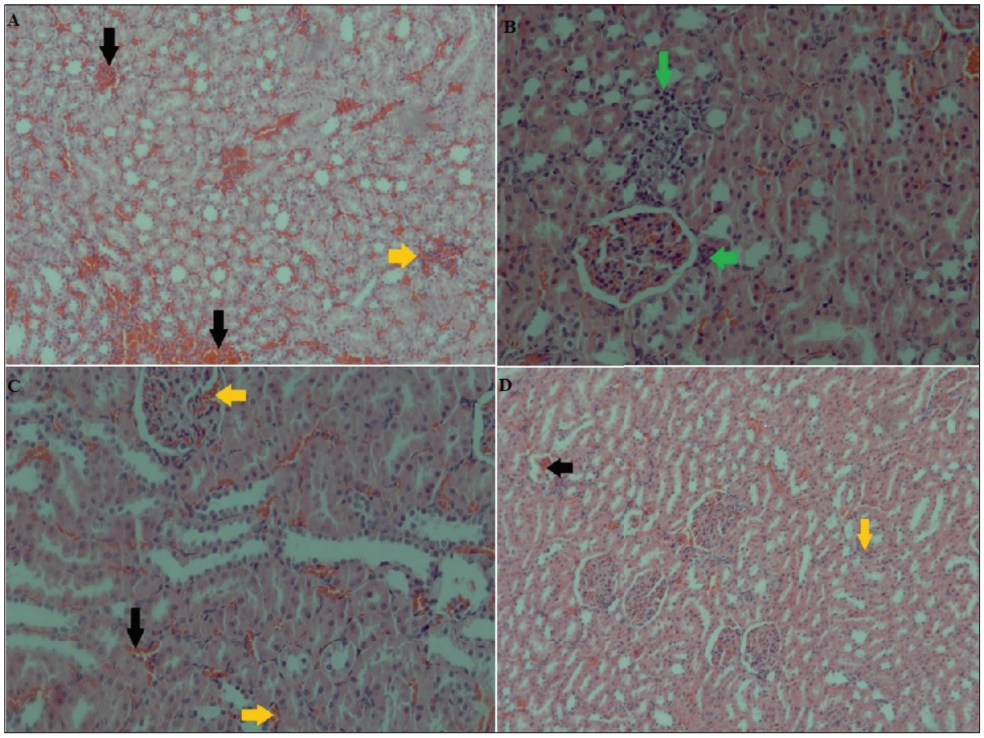

Fig 1. A: Control group with moderate congestion and erythrocyte extravasation (H&E, 100x); **B:** Ozone group with mild tubulointerstitial inflammation (H&E, 200x); **C:** L-carnitine group with mild congestion and erythrocyte extravasation (H&E, 200x); **D:** Ozone + L-carnitine group with mild congestion and erythrocyte extravasation (H&E, 100x); *Black arrows:* congestion, *Yellow arrows:* erythrocyte extravasation, *Green arrows:* tubulointerstitial inflammation

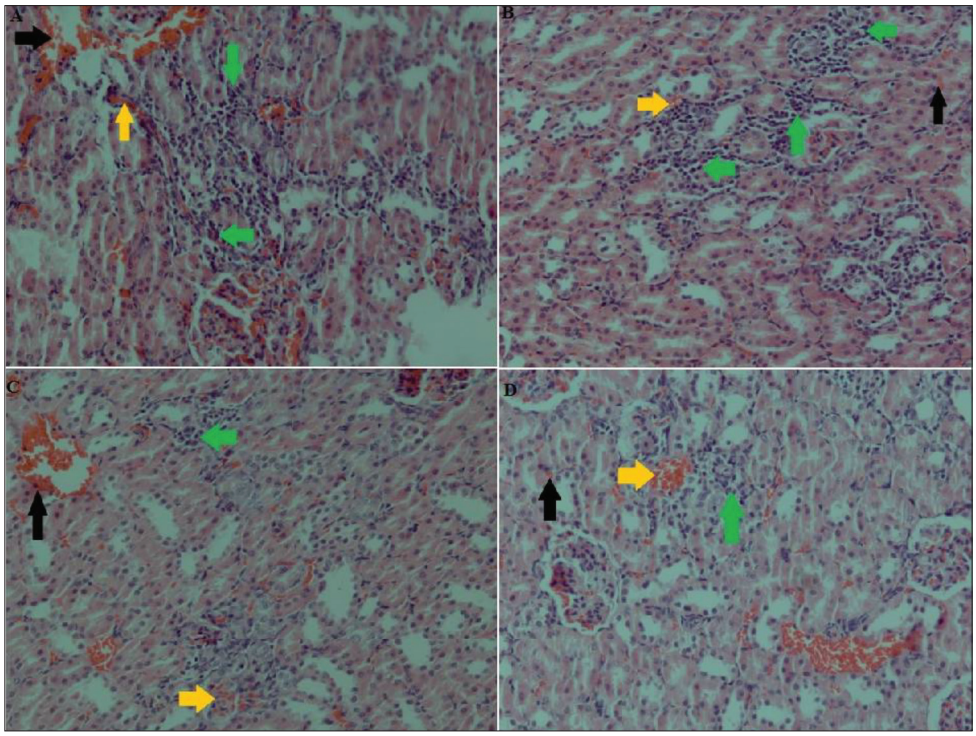

Fig 2. A: APAP group with moderate tubulointerstitial inflammation, mild congestion and erythrocyte extravasation (H&E, 200x); **B:** APAP + ozone group with mild-moderate tubulointerstitial inflammation, mild congestion and erythrocyte extravasation (H&E, 200x); **C:** APAP + L-carnitine group with mild tubulointerstitial inflammation, mild congestion and erythrocyte extravasation (H&E, 200x); **D:** APAP + ozone + L-carnitine group with mild tubulointerstitial inflammation, mild congestion and minimal erythrocyte extravasation (H&E, 200x); *Black arrows:* congestion, *Yellow arrows:* erythrocyte extravasation, *Green arrows:* tubulointerstitial inflammation

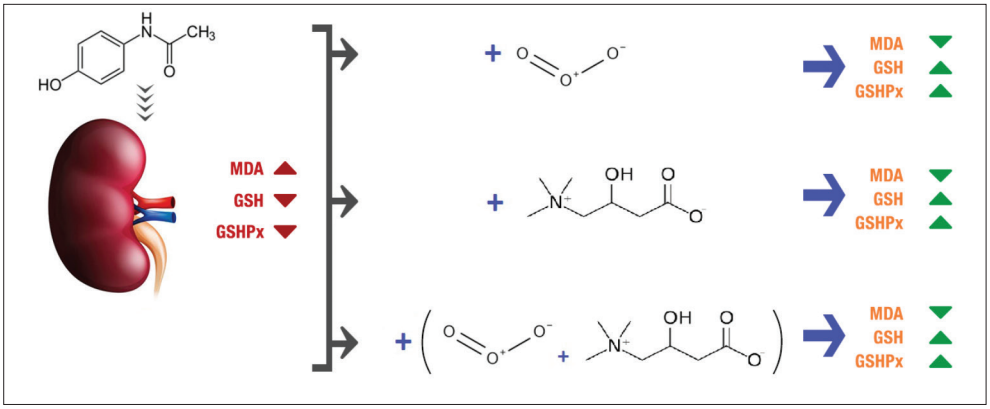

Fig 3. Summary of the results

A significant difference was found between APAP group and APAP + Ozone, APAP + L-carnitine and APAP + Ozone + L-carnitine groups in terms of MDA, GSH and GSHPx levels ($P<0.001$). Considering this analysis the mean of MDA levels were observed as: APAP> APAP + Ozone> APAP + L-carnitine> APAP + Ozone + L-carnitine; the mean of the GSH levels were APAP + ozone+ L- carnitine> APAP+L-carnitine> APAP + ozone> APAP; and lastly the mean levels of the GSHPx were APAP+L-carnitine> APAP+ Ozone > APAP + Ozone + L- carnitine> APAP respectively.

Histopathological examinations revealed mild vascular congestion, which was attributed to the procedure. While tubular damage was not observed in any of the cases *(Fig. 1A-D)*, the APAP group was noticed as having the most prominent tubulointerstitial inflammation *(Fig. 2A)*. Concerning the frequency and extent of the tubulo-insterstitial inflammation, the "APAP" group had intermediate-level tubuloinsterstitial inflammation in all cases, followed by "APAP + Ozone", "APAP + Ozone + L-carnitine," and "APAP + L-carnitine" groups *(Fig. 2B-D)*. In the evaluation of the control groups, mild tubulointerstitial inflammation was observed only in one case in the ozone group *(Fig. 1B)*.

Summary of the results is shown in *Fig. 3*.

DISCUSSION

This study was designed to evaluate the effects of L-carnitine and ozone on kidney tissues exposed to APAP-induced oxidative damage. Single-dose APAP (1 g/kg) administration is used according to Ucar et al.[7] in order to induce renal damage. APAP, L-carnitine (500 mg/kg), and ozone (0.7 mg/kg) were given to rats, and at the end of the experiment, kidney tissues were collected for the evaluation of oxidative damage and histopathological examination. Oxidative stress caused by APAP toxicity has been evidenced by increased levels of MDA and decreased levels of GSH and GSHPx activity following APAP administration. Single dose of ozone administration is shown to be effective for APAP induced hepatotoxicity [26] and L-carnitine for oxidative damage [27].

Long-chain unsaturated fatty acids are involved in a series of reactions with free radicals, resulting in lipid peroxidation and the degradation of membrane lipids into lower molecular weight moieties such as hydrocarbons, ketones, and epoxides. Determination of lipid peroxidation is a key marker of oxidative stress, and MDA formation is the most important oxidation by-product of lipid degradation, which can indicate the degree of lipid peroxidation in many organs [30]. In this study, the levels of MDA increased significantly in tissue samples of rats treated with APAP. These findings are similar to the results of other studies where increased tissue MDA levels were reported [31,32] after the application of some toxic substances in mice and rats.

Glutathione peroxidase, SOD, CAT, and GST are cellular antioxidant enzymes playing essential roles in the destruction of free radicals and the prevention of oxidative damage. These enzymes have a balance under normal physiological conditions and give an initial response to oxidative damage in tissues. Reduced GSH is a vital member of the antioxidant system and contributes to the cellular defense against oxidative damage through the formation of creatinine S-substituted GSH by reacting with toxic substances [31]. Because GSH is an important antioxidant with a significant cleansing function against ROS, it acts as an important redox buffer to stabilize the cellular redox state. It has been reported that GSH acts as an electron donor in protecting toxic substances from oxidative damage by promoting methylation of toxic substances to less toxic metabolites [31] and making toxic substances less harmful [33], which can significantly improve the production of hydroxyl radicals [17]. In our study, it was observed that APAP administration caused a significant decrease in GSH activity in the renal tissue samples. These findings are consistent with previous studies [31,34], which reported significant reductions of GSH levels in mice and rats exposed to various toxic substances. We share the opinion that decreases in GSH levels in animals exposed to toxicity may be due to the use of GSH as a cofactor by GST and by the use of GSH during cellular protection against oxidative stress to prevent oxidative

damage and to sustain the cellular redox state due to the suppression of this antioxidant enzyme [35].

In this study, it was shown that L-carnitine administration decreases MDA levels in renal tissue samples and increases reduced GSH and GSHPx levels. These results are in good agreement with other study results [34,36] in which L-carnitine as a preservative in various toxicity studies has been shown to prevent liver, heart, and brain damage caused by some toxic substances. It was also reported that GSH reduction was prevented when L-carnitine was administered before APAP; an increase in GSH level was also important for GSHPx, and it increased the activity of GSHPx [37]. We consider that L-carnitine significantly reduces the elevated levels of MDA in different organs [36], it keeps the GSH content of various organs close to the normal values in rats exposed to toxication, and possibly play a role as free radical scavengers [15], and these effects minimize oxidative damage in tissues by reducing NAPQI levels.

As shown in *Table 1*, L-carnitine administration has been found to significantly reduce high levels of MDA in tissues of animals exposed to APAP toxicity and to maintain almost normal values of GSH. These results are consistent with the previous findings, reporting antioxidant properties of L-carnitine. This effect of L-carnitine plays an important role in the oxidation of fatty acids, acting as a possible free radical scavenger and preventing the depletion of GSH [36]. Although there may also be some role of the stimulation of glutathione production, we suggest that the enhancement of the antioxidant enzyme activity by L-carnitine [34,36] may have contributed to the elimination of the oxidative stress due to APAP induced ROS.

Toxic exposure can lead to damage in most vital organ systems and especially in the kidneys, which are most sensitive to toxicity [38]. Increases in the end-products of lipid peroxidation and decreases in antioxidant parameter levels support our findings. It has been reported that oxidative stress and lipid peroxidation play an important role in the pathophysiology of nephrotoxicity in cisplatin-induced nephrotoxicity cases and that L-carnitine administration in rats normalizes renal function, decreases tissue MDA levels, and increases GSH levels [36]. Besides, the protective effects of L-carnitine have been demonstrated in the methamphetamine-neurotoxicity mediated by peroxynitrite roots [39].

The mechanism of APAP toxicity is well defined in the liver, but less understood in the kidney. Available evidence suggests that intracellular GSH plays an important role in the detoxification of APAP and in the prevention of toxicity caused by APAP in the liver and kidneys [40]. ROS production is seen as an early event that begins before intracellular GSH depletion and cell damage in APAP nephrotoxicity [4]. GSH can also serve as a reductant by the oxidation of glutathione (GSSG) and reducing hydrogen peroxide and lipid hydroperoxides directly to H_2O. Intracellular GSH is consumed under continuous intracellular oxidative stress. The depletion of intracellular GSH causes oxidation and damage of lipids, proteins, and DNA by ROS [4]. In this study, application of nephrotoxic-dose APAP to rats caused oxidative stress damage in renal tissues. We suggest that this effect occurs by increasing the degree of lipid peroxidation via inhibiting enzymatic antioxidants (e.g., GSHPx) in the kidney.

We found that tissue GSHPx enzyme activity and GSH levels decreased in the APAP treated rats, but increased in "APAP+ozone" group. Furthermore, a significant increase in the levels of tissue MDA (an indicator of tissue damage) was observed in the group receiving no ozone, while a decrease was observed in the ozone treated group. Although this condition is in accordance with different studies reporting that APAP induced renal damage is consistent with acute tubular necrosis [41,42], we conclude that our findings may be due to the beneficial effects of ozone in increasing tissue antioxidant enzyme activity and reducing tissue damage [37]. These results are also in agreement with the findings of a study investigating the protective effect of ozone on the kidneys and reporting its beneficial effects on the regulation of the antioxidant defense system [43]. The main finding of this study is that ozone alone or in combination with L-carnitine decreases tissue MDA levels due to APAP toxicity and increases tissue antioxidant parameter levels.

Results of this study showed that APAP toxicity caused oxidative damage in the examined renal tissues, and L-carnitine and ozone applications for protective purposes decreased MDA, a product of lipid peroxidation and increased tissue GSH levels via the antioxidant GSHPx enzyme activity. As a result, it has been concluded that L-carnitine and ozone can be used for treatment and protective purposes to prevent organ and tissue damage in APAP toxicity. Further studies are necessary to determine possible side-effects of ozone treatment.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Research Article

Socio-Economic Analysis of Dairy Cattle Enterprises in Urban Sprawl [1]

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Abstract

Dairy cattle are an activity that provides sustainability in the livestock sector and consequently in the agricultural sector. Therefore, socio-economic structures of dairy cattle farms are important. On the other hand, the location of an enterprise is very important both in terms of proximity to raw materials and marketing opportunities, and urban sprawl have high potential in this respect. In this study, the socio-economic structures of 91 dairy cattle farms in the province of Konya are investigated. Since milk is a perishable product, the marketing process must be carried out in a healthy way and the importance of urban sprawl has been determined in the study. The most important feature of enterprises in urban sprawl is that their capital structures are different. As these areas are in the process of urbanization, land and building capital is perceived as non-agricultural investment. This situation has a negative effect on the rantability of agricultural enterprises and the unit cost of milk. In the calculation of unit cost of milk the amortization, interest of capital and repair and maintenance costs of the building capital are considered as fixed costs and the average unit cost of milk is $0.33/kg. The cost of milk is determined as $0.29/kg when the building capital is subtracted from the cost calculations by considering the structural characteristics of urban sprawl. This difference in unit cost of milk is interpreted as the location rent of the dairy enterprises in the urban sprawl. In addition, transportation costs are low due to being close to the market. And it is also because of location rent. In this case, the management of dairy in the urban sprawl is evaluated economically and it is recommended to plan the organized livestock regions in the regions close to the cities.

Keywords: *Dairy cattle, Cost of milk, Location rent, Urban sprawl*

Kent Saçaklarında Süt Sığırcılığı Yapan İşletmelerin Sosyo-Ekonomik Analizi

Öz

Süt sığırcılığı, hayvancılık sektöründe ve buna bağlı olarak tarım sektöründe sürdürülebilirliği sağlayan bir faaliyettir. Bundan dolayı süt sığırcılığı yapan işletmelerin sosyo-ekonomik yapıları önem arz etmektedir. Öte yandan bir işletmenin kuruluş yeri gerek hammaddeye yakınlık gerekse pazarlama olanakları açısından oldukça önemlidir ve kent saçakları bu açıdan potansiyeli yüksek alanlardır. Bu çalışmada, Konya ili örneğinde kent saçağında faaliyetlerini sürdüren 91 adet süt sığırcılığı işletmelerinin sosyo-ekonomik yapıları incelenmiştir. Süt çabuk bozulabilen bir ürün olmasından dolayı pazarlama sürecinin sağlıklı bir şekilde yürütülmesi gerekmekte olup, kent saçaklarının önemi çalışmada belirlenmiştir. Kent saçaklarındaki işletmelerin en önemli özelliği sermaye yapılarının farklı olmasıdır. Bu alanlar kentleşme sürecinde olduğundan, arazi ve bina sermayeleri tarım dışı yatırım olarak algılanmaktadır. Bu durum tarım işletmelerinin rantabilitesine ve birim süt maliyetine olumsuz yönde etki yapmaktadır. Birim süt maliyeti hesaplamasında sabit masraf olarak işletmenin bina sermayesinin amortismanı, sermaye faizi ve tamir-bakım masrafları ele alınmakta olup, birim süt maliyeti incelenen işletmeler için ortalama 0.33 $/kg bulunmuştur. Kent saçağının yapısal özelliği dikkate alınarak konut sermayesi maliyet hesaplamalarından çıkarıldığında ise süt maliyeti 0.29 $/kg olarak belirlenmiştir. Birim süt maliyetindeki bu farklılık, kent saçağındaki süt işletmelerinin mevki rantı olarak yorumlanmıştır. Ayrıca pazara yakın olmasından dolayı ulaşım masraflarının da düşük olması mevki rantı içerisinde yer almaktadır. Bu durumda kent saçaklarında süt işletmeciliğinin yapılması ekonomik olarak değerlendirilmekte ve kentlere yakın bölgelerde organize hayvancılık bölgelerinin planlanması önerilmektedir.

Anahtar sözcükler: *Süt sığırcılığı, Süt maliyeti, Mevki rantı, Kent saçakları*

INTRODUCTION

Animal production has a significant share in the rural economy and contributes to the employment of the family labor force [1,2]. However, there are some problems in the marketing of agricultural products because of small-scale family businesses of Turkish agricultural enterprises and the specific structural characteristics of the agricultural sector.

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Due to the organizational problems of producers, the high number of intermediaries in marketing agricultural products and the high marketing margin cause ineffective marketing organization [3]. The lack of a well-functioning market organization reveals marketing problems and hinders production increase especially in perishable products such as milk [4]. İncrease in the milk production of Turkey largely depends on the elimination of marketing problems and the establishment of a well-functioning market organization in milk. With the organization, producers should put in an effective position in both input markets and product markets [5]. Structural reforms are needed to resolve the technical and economic problems of the livestock sector, and to realize the production and industry integration, an organization should be provided in marketing and production as in developed countries. It is also necessary to constitute input and price policies that will encourage the producer to produce quality products [6]. In addition, increasing the number of milking animals will contribute to the increase of animal capacity in enterprises [7].

Although there is a great potential in Turkey for the development of animal husbandry and increasing the amount of animal products, it has not been developed at the desired level [8]. Approximately 40% of the milk produced is delivered to the consumer as raw milk, 18-20% is processed in modern enterprises and 40% is processed in enterprises that are not compatible with hygiene conditions [9,10]. Because milk is a perishable product, the marketing organization needs to be well planned. Especially in the marketing of raw milk, if the transportation and storage criteria are not taken into consideration, elements that threaten human health take place. In this context according to "raw milk supply" prepared by the Ministry of Agriculture and Forestry in 2017, the supply of raw milk to the final consumer must be carried out within 24 h after milking. For this reason, being located in areas close to the market is very important for dairy cattle farms. In addition to this, considering the rantability of the enterprise, and evaluating the principle of minimization of production cost, the importance of position rent is revealed.

According to the theory of location rent which build by Von Thunen, considering the fact that transportation expenditures have a significant share in production cost, it is determined that the products will be more economical to grow as they move away from the city center where the market is located. According to this theory, dairy products should be located in the closest region to the city center due to its perishable feature [11]. Dairy cattle farms in Turkey continue to operate as commercial or family business especially in areas close to large cities [12]. In the enterprises which are far from the city center, the income of the producer falls as far as transportation costs as it moves away from the market. Although it is close to the city center, the areas showing the characteristics of rural areas are defined as urban sprawl. Together with the increase in population, the most important feature of the urban sprawl which formed by the acceleration of urbanization activities is the sustainability of agricultural activities despite the transition to urban area [13]. Since urban sprawl is transition areas to urban areas, in these zoning plan applications, ranch facilities are not allowed. These practices cause them not to be able to operate in areas close to the city and cannot benefit from location rent. In this case, the investment expectations of animal husbandry enterprises in urban sprawl are shifting to non-agricultural areas and this affects the livestock sector negatively. In fact, the product, price, location and promotion [14], which are the marketing mix that is considered in marketing of a product, have great importance and urban sprawl have an important role in bringing these components together in marketing of milk. In this study, structural features and location problems of dairy cattle farms in urban sprawl have been analyzed. The effects of the location rent to the profit of enterprise and milk marketing were investigated.

MATERIAL and METHODS

The main material of the study is the surveys conducted with the owners of agricultural enterprises in the neighborhoods which show urban sprawl characteristics in the province of Konya. In addition, previous studies on this subject were utilized.

The agricultural enterprises in the research area are determined as the main framework and the land widths of the agricultural enterprises in the research area are taken from the Farmer Registration System of the Provincial Directorate of Agriculture and Forestry. According to the stratified random sampling method, the number of samples is calculated using the following formula [15]. The method is used when the Coeffient of Variation is greater than 75%, and this coefficient has been great because of heterogenic data.

$$n = \frac{\Sigma\,(Nh.\,Sh)^2}{N^2.\,D^2 \;+\; \Sigma\,(Nh.\,Sh^2)} \qquad D^2 = d^2/z^2$$

In formula;

n: Number of samples, N: Number of enterprises in population, Nh: Number of enterprises in h category, Sh: The variance of h category, d: Allowed margin of error from population average, z: z value refers to the standard normal distribution table according to the error rate.

In the determination of the sample volume studied in a margin of error of 5% and within 99% confidence limits. The following formula is used to distribute the specified sample volume to the categories [15]. As a result of sampling, 91 sample enterprises are identified. It is determined as 1. group (0-30 decare)16, 2. group (31-120 decare) 41 and 3. group (121-+decare) 34. Taking into account the coefficient of variation, it was divided into layers to group the data homogeneously.

$$n = \frac{N_h S_h * n}{\Sigma N_h S_h}$$

In the analysis of the annual operating results of the enterprises; Indicators such as Gross Production Value (GPV), Gross Product (GP), operating costs, gross profit, agricultural income, and rantability are calculated. The gross production value in enterprises is determined by multiplying the amounts of plant and animal products obtained as a result of agricultural activity with product prices for farmers and then we added the increasing productive value of plant and animal capital to this value [16]. Financial rantability is obtained by dividing net profit into equity capital. In addition, the profitability of the investment capital is calculated and the profitability of the enterprises is determined. The profitability of investment capital is expressed by economic profitability. Conversion of cattle to the bovine unit (BBHB) is performed in order to examine the existing cattle on the same basis [17].

RESULTS

The average population per enterprise is determined as 4.76 in enterprises examined, 12.93% of the population is 0-6 years old, 12.01% was 7-14 years old, 21.48% is 50 years of age and 53.58% are active population between the ages of 15-49. The high rate of active population shows that labor force and income generating population are high.

In examined enterprises 52.41% of the population is identified as primary school graduates, 19.79% as secondary school graduates, 19.79% as high school graduates and 8.02% as university graduates. It is determined that 1.60% of the population is not literate. It has been determined that the rate of university graduates has grown as the scale of the examined enterprises grows.

The production pattern in the examined enterprises is concentrated in order to meet the feed requirement, which constitutes the most important expense item of animal

Table 1. *Production pattern of examined agricultural enterprises*

Product Groups	Products	Enterprise Groups							
		0-30		31-120		121-+		Average	
		da	%	da	%	da	%	da	%
Field Crops	Wheat	4.38	34.83	13.49	16.73	116.87	31.98	50.51	25.61
	Silage corn	3.66	29.10	20.24	25.10	35.51	9.72	23.03	20.06
	Barley	0.00	0.00	18.90	23.44	60.44	16.54	31.10	16.74
	Grain corn	1.25	9.95	12.21	15.15	50.07	13.70	24.43	13.69
	Clover	2.06	16.42	5.68	7.05	15.59	4.27	8.75	7.66
	Sugar beet	0.00	0.00	4.91	6.09	34.43	9.42	15.08	6.27
	Sunflower	0.00	0.00	1.71	2.12	13.09	3.58	5.66	2.29
	Vetch	0.00	0.00	1.27	1.57	7.79	2.13	3.48	1.51
	Haricot bean	0.00	0.00	0.00	0.00	4.56	1.25	1.70	0.47
	Chickpea	0.00	0.00	0.00	0.00	4.26	1.17	1.59	0.44
	Fallow	0.00	0.00	0.00	0.00	5.88	1.61	2.20	0.60
	Total	11.35	90.3	78.41	97.25	348.49	95.37	167.53	95.34
Vegetable	Tomato	0.45	3.61	0.30	0.38	0.74	0.20	0.49	0.88
	Pepper	0.34	2.74	0.13	0.17	0.74	0.20	0.40	0.63
	Lettuce	0.00	0.00	0.00	0.00	5.88	1.61	2.20	0.60
	Carrot	0.00	0.00	0.00	0.00	4.41	1.21	1.65	0.45
	Green bean	0.09	0.75	0.29	0.36	1.47	0.40	0.70	0.45
	Eggplant	0.20	1.62	0.12	0.15	0.74	0.20	0.37	0.43
	Cabbage	0.00	0.00	0.00	0.00	2.94	0.80	1.10	0.30
	Cucumber	0.13	1.00	0.00	0.00	0.00	0.00	0.02	0.17
	Total	1.21	9.72	0.84	1.06	16.92	4.62	6.93	3.91
Fruit	Walnut	0.00	0.00	0.67	0.83	0.00	0.00	0.30	0.37
	Cherry	0.00	0.00	0.43	0.53	0.00	0.00	0.19	0.24
	Almond	0.00	0.00	0.20	0.24	0.00	0.00	0.09	0.11
	Apple	0.00	0.00	0.07	0.09	0.00	0.00	0.03	0.04
	Grape	0.00	0.00	0.00	0.00	0.07	0.02	0.03	0.01
	Total	0	0	1.37	1.69	0.07	0.02	0.64	0.77
TOTAL		12.56	100.00	80.63	100.00	365.49	100.00	175.09	100.00

Table 2. *Animal assets and capital*

Animal	Enterprise Groups							
	0-30		31-120		121-+		Average	
	N	$	N	$	N	$	N	$
Bull	1.00	2.096,27	0.12	378,73	0.03	97,43	0.24	575,61
Cow	5.06	10.623,71	10.83	26.021,31	5.41	12.714,65	7.79	18.342,32
Heifer	2.31	4.179,61	3.39	5.951,12	3.91	7.158,08	3.40	6.090,60
Bullock	1.94	3.325,57	2.49	4.413,47	5.59	10.004,87	3.55	6.311,29
Female steer	1.06	2.089,80	1.68	4.269,55	1.50	2.039,95	1.51	3.053,26
Male steer	0.75	834,63	2.73	3.572,69	1.97	2.706,73	2.10	2.767,73
Female calf	0.69	420,55	1.24	838,26	0.59	414,08	0.90	606,33
Male calf	0.75	446,43	1.20	813,01	0.62	423,21	0.90	602,92
Ram	0.00	0.00	0.39	95,94	0.71	179,03	0.44	110,12
Sheep	0.00	0.00	16.59	3.297,48	12.79	2.804,17	12.25	2.533,39
Lamb	1.44	178,57	3.76	580,22	6.47	954,51	4.36	649,44
Goat	0.00	0.00	0.12	25,25	4.82	1.164,29	1.86	446,39
Bee	0.00	0.00	0.00	0.00	3.38	598,28	1.26	223,53
Total cattle	13.56	24.016,56	23.68	46.258,14	19.62	35.559,01	20.38	38.350,06
Total small ruminant	1.44	178,57	20.85	3.998,89	24.79	5.102,00	18.91	3.739,34
Total	15.00	24.195,13	44.54	50.257,03	47.79	41.259,29	40.56	42.312,93

(1 USD = 4.83 Turkish Liras in the study time-July-2018)

Table 3. *Active capital of examined enterprises*

Capital Groups		Enterprise Groups ($)							
		0-30		31-120		121-+		Average of Enterprises	
		$	%	$	%	$	%	$	%
Land capital	Land	126.294,00	58.67	826.559,11	83.12	3.546.918,77	93.21	1.719.833,69	90.15
	Land reclamation	9.937,89	4.62	16.992,37	1.71	44.336,87	1.17	25.968,65	1.36
	Building	41.304,35	19.19	67.873,55	6.83	88.150,04	2.32	70.777,88	3.71
	Plant	391,18	0.18	3.305,20	0.33	12.317,39	0.32	6.160,04	0.32
	Total	177.927,41	82.65	914.730,24	91.98	3.691.723,06	97.01	1.822.740,25	95.55
Fixed enterprise capital	Animal	24.195,13	11.24	50.257,03	5.05	41.259,29	1.08	42.312,93	2.22
	Machine tool	11.391,05	5.29	26.670,20	2.68	55.168,37	1.45	34.631,42	1.82
	Total	35.586,18	16.53	76.927,23	7.74	96.427,66	2.53	76.944,35	4.03
Revenue assets capital	Material and munitions capital	0.00	0.00	705,58	0.07	2.140,57	0.06	1.117,67	0.06
	Money capital	1.759,83	0.82	2.080,49	0.21	15.168,68	0.40	6.914,20	0.36
	Total	1.759,83	0.82	2.786,07	0.28	17.309,25	0.45	8.031,88	0.42
Total enterprise capital		37.346,01	17.35	79.713,30	8.02	113.736,91	2.99	84.976,22	4.45
Total active capital		215.273,42	100.00	994.443,54	100.00	3.805.459,97	100.00	1.907.716,47	100.00

production. While silage corn (20.06%), barley (16.74%), alfalfa (7.66%) and vetch (1.51%) are produced directly from animal feed, by-products of wheat and grain are also used, 85% of the production pattern contributes to animal production *(Table 1)*.

As a matter of fact, in the study conducted by Boz [12], it has been determined that the enterprises that produce feed in their own land are more resistant to the risk of instability in feed prices. In addition, in the study conducted by Aktürk et al.[8] it is found that silage corn and barley showed the highest effect on milk yield.

In examined enterprises; 20.38 head of cattle and 18.91 head of small cattle are determined per enterprise *(Table 2)*. The animal potential of urban sprawl is located in the cattle farms that want to benefit from retail milk sales at high prices by using urban proximity. In addition, small animal husbandry is carried out in the urban sprawl in order to meet the needs such as sacrifice. As a matter of fact, it is possible to say that the urban sprawl is more advantageous in terms of rural areas for both milk and meat marketing.

Animal capital in the examined enterprises is given in table

Table 4. *Annual activity results of examined enterprises*

Economic Success Criteria	Enterprise Groups			
	0-30	31-120	121-+	Average
Animal production value	14.480,36	28.599,96	19.512,85	22.722,21
Plant production value	3.788,69	19.404,16	96.961,38	45.636,01
Gross production value	18.269,05	48.004,13	116.474,23	68.358,22
Variable cost value	15.672,87	31.863,70	51.767,10	36.453,40
Gross profit	2.596,18	16.737,27	65.405,89	32.434,80
Operating costs	22.420,08	44.370,57	73.325,89	51.329,62
Gross income	20.584,46	52.323,64	125.193,63	73.969,27
Net income	-1.835,63	7.953,06	51.867,74	22.639,66
Production costs	25.434,01	51.088,78	85.252,92	59.342,68
Net profit	-4.849,55	1.234,85	39.940,71	14.626,59
Agricultural revenue	466,72	10.816,39	55.649,86	25.747,64
Financial rantability	-8.05	0.94	17.12	9.32
Economic rantability	-3.02	5.93	20.94	13.84

2 and total animal capital is determined as $42.312,93. $38.350,06 of this capital is from cattle and $3.739,34 of this capital is from small ruminant per enterprise. Cows get the highest share in animal capital. Although animal capital varies according to the size of the enterprise, cattle capital is the highest in medium-sized enterprises, while small animal capital is much more in large-scale enterprises. Dairy cattle are the key to sustainable production from cattle [4]. Therefore, for the development of the livestock sector and consequently agricultural sector, the success of dairy cattle breeding enterprises is very important.

The land capital gets the highest share in the active capital with 90.15%, land reclamation capital is 1.36%, the building capital is 3.71%, the plant capital is 0.32%, the animal capital is 2.22%, the instrument machinery capital is 1.82%, material ammunition capital is 0.06% and money capital is determined is 0.36% *(Table 3)*. The active capital asset is effective on enterprise success both qualitatively and quantitatively. As a matter of fact, the asset capital includes the whole capital asset of the enterprise and its high value affects the business success although it varies according to the enterprise income. The lack of sufficient income compared to the value of the active capital in the enterprise composes a risk for the sustainability of the enterprise. Animal production is an important activity for the sustainability of enterprises.

It is determined that the value of decare vegetative production and the value of animal production per animal are high in urban sprawl agricultural enterprises compared to rural agricultural enterprises. This can be explained by rent theory. As a matter of fact, location rent can be defined as, getting high income because of the location of the land, production and marketing facilities. 66.76% of the gross production value per enterprise is realized in crop production and 33.24% in animal production. Dairy cattle breeding is not common in urban sprawl, although animal production marketing opportunities and advantages in urban sprawl agricultural enterprises are high. As a matter of fact, dairy cattle shelters require high investment and modern animal shelters cannot be allowed in urban sprawls in metropolitan areas such as Konya. This is one of the most important factors in the non-prevalence of dairy cattle production activity.

The total variable cost of the examined enterprises is determined as $36.453,40 per enterprise and varies according to the enterprise groups *(Table 4)*. 52.33% of enterprise expenses are determined as animal production changing cost and 47.57% as plant production changing cost.

It is observed that agricultural income is lower in agricultural enterprises in urban sprawl compared to agricultural enterprises in rural areas per decares. It can be shown as a reason that agricultural enterprises in the urban sprawl get more income from non-agricultural activities than rural areas, so they get less agricultural income.

Financial and economic rantability shows the success of the capital used by the enterprise in its production activities. Financial rantability shows the success of the enterprise's own capital, while economic rantability shows the success of total capital [18]. The rates of financial and economic rantability in dairy farms in the urban sprawl were higher (2.02% and 2.04%) [19] than the rates of dairy cattle farms in the rural areas of Konya province. The high demand for non-agricultural capital for building and land capital in urban sprawl agricultural enterprises give these capital components the ability to be an investment tool. As a matter of fact, the reasons for long-term possession by the owners of the agricultural lands located in the urban sprawl are for the income that will be derived from the value increase which will realize in the future not for agricultural activity, whether it is the renter or the owner. It would be more appropriate not to include land capital and building capital in the total capital in the balance sheet

Table 5. *Distribution of production costs*

Costs		Enterprises Groups			
		0-30	31-120	121-+	Average
Compound feed	$	8.043,51	13.464,00	10.035,32	11.229,90
	%	58.06	57.10	62.32	59.22
Barley grits	$	633,09	1.942,48	602,85	1.211,74
	%	4.57	8.24	3.74	5.91
Bran	$	204,35	142,65	157,53	159,06
	%	1.48	0.61	0.98	0.90
Grain corn	$	67,29	65,14	265,80	140,49
	%	0.49	0.28	1.65	0.83
Cornflakes	$	0,00	45,90	91,34	54,81
	%	0.00	0.19	0.57	0.30
Beet pulp	$	232,32	1.249,31	351,72	735,13
	%	1.68	5.30	2.18	3.50
Straw	$	854,13	1.869,05	993,79	1.363,58
	%	6.17	7.93	6.17	6.96
Roughage	$	562,96	93,19	146,14	195,57
	%	4.06	0.40	0.91	1.23
Clover	$	778,34	511,88	837,60	680,43
	%	5.62	2.17	5.20	3.91
Silage corn	$	1.887,58	3.029,01	2.282,00	2.549,22
	%	13.63	12.85	14.17	13.48
Non-permanent workers	$	0,00	64,64	0,00	29,12
	%	0.00	0.27	0.00	0.12
Veterinary and drug costs	$	333,85	304,50	337,66	322,05
	%	2.41	1.29	2.10	1.79
Artificial insemination	$	256,21	796,24	0,00	403,80
	%	1.85	3.38	0.00	1.85
Total of variable costs	$	13.853,63	23.577,99	16.101,75	19.074,89
	%	100.00	100.00	100.00	100.00
Share in total cost	%	74.62	73.32	64.71	71.57
Interest of cow capital	$	265,59	650,53	317,87	458,56
	%	5.64	7.58	3.62	6.05
Cow capital amortization	$	589,42	1.094,17	153,53	433,34
	%	12.51	12.75	1.75	5.72
Instrument machinery amortization	$	902,86	1.602,08	951,65	1.168,81
	%	19.16	18.67	10.84	15.43
Interest of instrument machinery	$	284,78	666,76	1.379,21	865,78
	%	6.04	7.77	15.71	11.43
Building amortization	$	982,13	1.495,06	919,98	1.124,50
	%	20.84	17.43	10.48	14.84
Interest of building capital	$	1.032,61	2.074,05	4.444,34	2.776,54
	%	21.91	24.17	50.62	36.64
Building repair maintenance costs	$	654,76	996,71	613,32	749,67
	%	13.90	11.62	6.99	9.89
Total fixed costs	$	4.712,15	8.579,36	8.779,90	7.577,20
	%	100.00	100.00	100.00	100.00
Share in total cost	%	25.38	26.68	35.29	28.43
Total production cost	$	18.565,78	32.157,35	24.881,65	26.652,10
	%	100.00	100.00	100.00	100.00

analysis and production cost analysis carried out in urban sprawl agricultural enterprises.

The costs of the examined enterprises for animal production are given in *Table 5*. According to the average of enterprises, 71.57% of total production costs compose from changing costs and 28.43% compose from fixed costs. Among the changing cost the highest share is compound feed with 59.22% and the highest share of fixed cost is interest of building capital with 36.64%. The reason for the high rate for amortization, interest and maintenance costs of the building capital in fixed costs is that the construction capital is much more in agricultural enterprises in the urban sprawl. As a matter of fact, the houses in the urban sprawl are similar to the ones in the urban area in terms of quality, and the house values are different from the houses in rural areas because of the proximity to the city. Therefore, houses in the urban sprawl should not be considered as an agricultural investment status.

The livestock production activity is determined in the examined urban sprawl agricultural enterprises and 84.88% of the animal production value is from milk sales, 10.30% is from the PSVI (Productive Stock Value Increase) and 4.82% is from the fertilizer *(Table 6)*. In Turkey sales of milk continues by milk marketing channels from producers to consumer known as the open sale of milk. This situation is more advantageous for urban sprawl agricultural enterprises. As a matter of fact, the province of Konya is the province where milk production is the most realized and therefore milk factories are concentrated. In addition, intensive population provides open milk marketing opportunities. This situation provides high market flexibility in terms of dairy cattle breeding in urban sprawl and positively affects producer income. Also, it has significant advantages in input procurement and in terms of finding, running and providing labour welfare.

In the case of where more than one product is produced at the end of production and the proportional share of the product in gross production value is close to each other, proportional cost calculation method is used to calculate the unit product costs. This method is based on the principle that the products obtained at the end of the production activity should take a share from the production cost as well as the share of the gross production value [18]. Proportional cost calculation method is used to calculate animal production costs in the examined enterprises. In order to find the total production cost of each product, the ratio of the animal production value is taken into consideration. In this case, the cost of milk production is calculated as $22.904,81 according to the average of enterprises. And it is seen that 85.94% of the production costs belong to milk, 9.44% to PSVI and 4.62% to fertilizer *(Table 6)*. In the study conducted by Aktürk et al.[8] in the rural area of Çanakkale province, production costs are distributed as 76.31% milk, 20.99% PSVI and 2.7% fertilizer.

Table 6. *Animal production value and production costs*

Enterprise Groups (da)	Milk		PSVI		Fertilizer		Total	
	$	%	$	%	$	%	$	%
0-30	12.291,02	84.88	1.491,37	10.30	697,97	4.82	14.480,36	100.00
31-120	24.701,18	86.37	2.588,97	9.05	1.309,82	4.58	28.599,96	100.00
121-+	16.702,20	85.54	1.918,16	9.83	903,67	4.63	19.512,85	100.00
Average of Enterprises	19.526,37	85.94	2.145,35	9.44	1.050,49	4.62	22.722,21	100.00
Distribution of Animal Production Costs According to Products								
0-30	15.758,63	84.88	1.912,28	10.3	894,87	4.82	18.565,78	100.00
31-120	27.774,31	86.37	2.910,24	9.05	1.472,81	4.58	32.157,35	100.00
121-+	21.283,77	85.54	2.445,87	9.83	1.152,02	4.63	24.881,65	100.00
Average of Enterprises	22.904,81	85.94	2.515,96	9.44	1.231,33	4.62	26.652,10	100.00

Table 7. *Unit milk cost*

Enterprise Groups (da)	Milk Production Costs ($)		Milk Yield (kg)	Unit Milk Costs ($/kg)	
0-30	15.758,63	14.207,59*	37.400,00	0.42	0.38*
31-120	27.774,31	25.448,39*	86.797,62	0.32	0.29*
121-+	21.283,77	18.395,58*	69.500,00	0.31	0.27*
Average of Enterprises	22.904,81	20.582,37*	70.398,94	0.33	0.29*

** Housing capital fixed costs, unprocessed production costs and unit milk cost*

While the cost of unit milk is determined as $0.33/kg in the examined enterprises, the net profit per unit is determined as $0.02/kg as the milk sales price is $0.35/kg *(Table 7)*. The cost of one kg of milk varies according to the enterprise groups and the cost decreases as the size of the enterprise increases. As a matter of fact, as the enterprise grows, the amount of input used and the amount of product obtained increase and the fixed cost per unit decreases as the amount of production increases. This situation causes the unit milk cost to be lower in large agricultural enterprises.

The cost of milk production includes building amortization, interest of building capital and repair-maintenance costs as a fixed cost. Due to the expectation of non-agricultural future, it is possible to qualify land and building capital as non-agricultural investment in agricultural enterprises. Therefore, it would be a more rational approach not to include the value of housing in the building capital in the calculation of unit milk cost. By subtracting the housing capital from the active capital the production costs are calculated again and as a result unit milk cost is determined. By subtracting the housing capital the average milk production cost decreased to $22.904,81 and the unit milk cost is calculated as $0.29/kg in the enterprise average. In this case, the unit cost is less than $0.04/kg and net profit per unit is increased to $0.06/kg. If housing capital costs are included in the production costs in the urban sprawl, unit milk costs are determined as higher.

DISCUSSION

In this study, the structural characteristics and location rent of the agricultural enterprises which carry out their activities in the urban sprawl and deal with dairy cattle breeding are examined. The capital structure of enterprises in this area shows discrepancy due to the evaluation of land and building capital as non-agricultural investment. Therefore, it is stated that these two capital elements should not be taken in the determination of the rantability of the enterprises in this field.

In the field of research, it is determined that feed production is realized in order to support animal husbandry activities and it is determined that feed needs, which are the biggest cost factor, are supplied. The average production costs of enterprises determined as $26.652,10 and animal production value is $22.722,21. Production cost for milk is calculated as $22.904,81 by applying the proportional cost method in total production cost of milk. The unit milk cost is determined as $0.33/kg, and Housing Capital is subtracted due to the assessment of non-agricultural investment in urban sprawl and milk cost is determined as $0.29/kg for urban sprawl. Within the scope of this study, the housing capital in the urban sprawl is considered as the location rent for enterprises deal with dairy cattle breeding.

Long-term investment planning is not possible in agricultural activities, as urban rents are expected in the future for urban sprawl [20,21]. Macroeconomic variables in the national and international context and microeconomic variables closely related to local supply and demand are effective in the evolution of land rents in urban sprawl [22]. With the effect of the agricultural policies, agricultural lands are shifting to non-agricultural uses which are able to obtain rent in the short term [23]. Because of the high cost of urban infrastructure, agriculture is neglected availability factor [24].

Because the agricultural lands in this area are expected to turn into land and the sustainability of agricultural production is at risk. However, dairy cattle breeding is a long-term activity and it is very difficult to convert this production activity into another investment. Although the input prices are high or the milk price is low, it is observed that the activity continues. Temel [25] examined the structure of enterprises producing ornamental plants and found that it was established close to the city centers and this provides a location rent.

In livestock enterprises, it has been seen some problems such as odor, waste into the water, disease outbreak, etc., and these are problems for urban sprawl. In the research area, some enterprises have a system in which solid wastes are converted into fertilizers by liquefied for agricultural land. This system should be used in all enterprises. Besides, it is recommended to establish organized livestock areas close to the cities for the sustainability of urban sprawl livestock activities in return for the conversion of agricultural land in urban sprawl into non-agricultural activities. Arrangements should be applied for these areas to continue their activities which do not harm the environment.

On the other hand, because milk is a product that can be quickly spoilt, it is a matter that needs to be close to the market, the urban sprawl is suitable for this. It is also important in terms of reducing urban sprawl transportation costs. The presence of potential consumers in these areas is seen as an advantage in milk marketing. This is an advantage for the consumer in terms of the availability of fresh milk. A cold chain system must be established in order to deliver the milk to the consumers in a healthy way.

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Short Communication

First Molecular Evidence for *Mycoplasma haemocanis* and *Candidatus Mycoplasma haematoparvum* in Asymptomatic Shelter Dogs in Kyrgyzstan

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Abstract

Mycoplasma haemocanis (Mhc) and *Candidatus Mycoplasma haematoparvum* (CMhp) have been investigated using species specific PCR and sequencing in 170 dogs from Kyrgyzstan. Maximum likelihood estimation (MLE) of the infection rates with 95% confidence intervals (CI) was calculated. The molecular prevalence of hemoplasma infection was 5.29% (CI 2.57-9.34). It was found that, five (2.94%, CI 1.06-6.22) samples were found to be infected with Mhc, one (0.59%, CI 0.03-2.57) sample with CMhp and three (1.76%, CI 0.44-4.52) samples with both species. These results demonstrate that dogs can be exposed to each haemoplasma species and provide first molecular evidence for these species in Kyrgyzstan.

Keywords: *Canine, Haemoplasmas, PCR, Kyrgyzstan*

Kırgızistan'da Asemptomatik Barınak Köpeklerinde *Mycoplasma haemocanis* ve *Candidatus Mycoplasma haematoparvum* İçin İlk Moleküler Kanıt

Öz

Kırgızistan'dan 170 köpekde tür spesifik PZR ve sekanslama ile *Mycoplasma haemocanis* (Mhc) ve *Candidatus Mycoplasma haematoparvum* (CMhp) araştırılmıştır. Enfeksiyon oranlarının en büyük olabilirlik tahmini (MLE) %95 güven aralığında (CI) hesaplandı. Hemoplasma enfeksiyonunun moleküler prevalansı %5.29 idi (CI 2.57-9.34). Beş (%2.94, CI 1.06-6.22) numunenin Mhc ile, bir (%0.59, CI 0.03-2.57) numunenin CMhp ile ve üç (%1.76, CI 0.44-4.52) numunenin de her iki tür ile enfekte olduğu bulundu. Bu sonuçlar, Kırgızistan'daki köpeklerin hemoplasma türlerinin her biri ile maruz kalabileceğini göstermekte ve bu türler için Kırgızistan'daki ilk moleküler kanıtı sağlamaktadır.

Anahtar sözcükler: *Köpek, Hemoplasma, PZR, Kırgızistan*

INTRODUCTION

Vectors and vector-borne diseases have a considerable impact for domestic and wild animals in tropical and subtropical climatic regions worldwide. Haemotropic mycoplasmas or hemoplasmas are bacteria infect in a wide range of vertebrate erythrocytes and recently renamed from *Haemobartonella* and *Eperythrozoon* [1]. Well known canine haemoplasma species, *Mycoplasma haemocanis* (Mhc) and *Candidatus Mycoplasma haematoparvum* (CMhp), cause subclinical or chronic disease in immunocompetent dogs and acute disease with hemolytic anemia in susceptible animals related

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to splenectomy, immunosuppression and concurrent infections. Lethargy, weight loss, fever and anorexia are the other symptoms for acute disease [2,3].

In several studies worldwide, *Mycoplasma* infections have been found in stray, wild and pet dogs in Turkey [4], Nigeria [5], United States [3], Brazil [6], Thailand [7], Iran [8,9], Italy [10], Spain, Portugal, Switzerland and France [2].

Recently, *Hepatozoon canis* infections with high prevalence in dogs from Kyrgyzstan were disclosured [11]. We aimed to investigate frequency of infection with Mhc and CMhp in dogs from Kyrgyzstan using polymerase chain reaction (PCR) and sequence analysis.

MATERIAL and METHODS

Ethic Statement

The Ethic statement was obtained from the Animal Experimentation Ethics Committee of Kyrgyz-Turkish Manas University (Document No: 29.06.2017/2017-06/01).

Study Area and Samples

Bishkek, largest city and capital of Kyrgyzstan, is located at 42.87 latitude and 74.59 longitude, 800 meters above sea level and has a surface area of 169.9 km^2 for city center. Bishkek can show both temperate and continental climate characteristics. Province has an average annual rainfall of 427 mm *(Fig. 1)*. The study was conducted on 170 apparently asymptomatic dogs from May 2016-October 2017. Five mL of blood sample were taken from the *vena cephalica antebrachii* into tubes containing K3EDTA-anticoagulant from shelter dogs with cooperation Kyrgyz-Turkish Manas University Veterinary Teaching Hospital.

Nucleic Acid Extractions and PCR Assay

For genomic DNA isolation, 200 µL blood was processed with a commercial kit [PureLink Genomic DNA mini kit (Invitrogen, Carlsbad, USA)]. Target DNA's were kept at -20°C until analysis. To determine each species, a single PCR analysis were made in a final reaction volume of 25 µL containing PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 5 mM $MgCl_2$, 125 µM deoxynucleotide triphosphates, 1.25 U Taq DNA polymerase (Promega, Madison, WI, USA), primers (20 pmol/µL) and template DNA. Sequence, specificity, target gene and product sizes for primers were demonstrated in *Table 1*. PCR was performed with an initial denaturation step of 94°C for 5 min was followed by 32 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. A final extension step at 72°C for 5 min was also applied [9]. Positive control DNA for Mhc (GenBank accession no: MG594502) and CMhp (GenBank accession no: MG594500) species and negative controls (nuclease-free water) were also used in the PCR

Fig 1. Map of Kyrgyzstan and location of Bishkek

Table 1. *Primers used in this study*

Primer	Sequence (5'-3')	Specificity	Target Gene	Product Length (bp)	Reference
Forward	GAAACTAAGGCCATAAATGACGC	Mhc	16S rRNA	309	[9]
Reverse	ACCTGTCACCTCGATAACCTCTAC				
Forward	ACGAAAGTCTGATGGAGCAATAC	CMhp	16S rRNA	328	[9]
Reverse	TATCTACGCATTCCACCGCTAC				

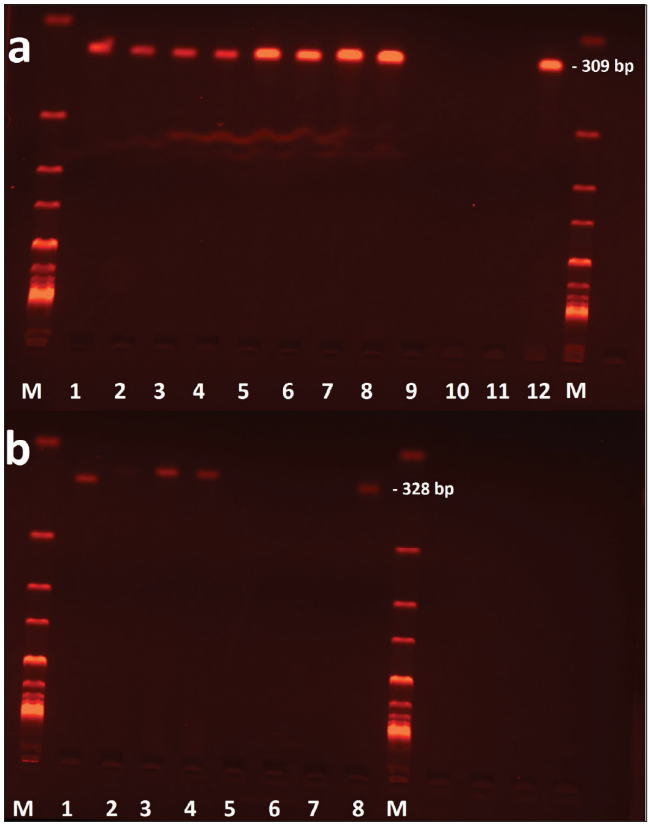

Fig 2. Agarose-gel electrophoresis of *Mycoplasma haemocanis* (a) and *Candidatus Mycoplasma haematoparvum* (b) specific polymerase chain reaction. M: 1 kb DNA ladder-marker, lane a1-a8: *Mycoplasma haemocanis* positive dog blood samples; lane a9, a10: negative dog blood samples, lane a11: negative control distilled water; lane a12: positive control DNA from dog, lane b1-b4: *Candidatus Mycoplasma haematoparvum* positive dog blood samples; lane b5, b6: negative dog blood samples, lane b7: negative control distilled water; lane b8: positive control DNA from dog

Table 2. *Molecular prevalence of canine hemoplasma species detected by PCR in Kyrgyzstan (n=170)*

Overall Prevalence (n = 9)		*Mycoplasma haemocanis*	*Candidatus Mycoplasma haematoparvum*	*Mhc+CMhp*
5		5	-	-
3		-	-	3
1		-	1	-
Total	9 (5.29%) (CI; 2.57-9.34)	5 (2.94%) (CI; 1.06-6.22)	1 (0.59%) (CI; 0.03-2.57)	3 (1.76%) (CI; 0.44-4.52)

reaction. Five microliters of PCR product was separated using electrophoresis (100 V, 60 min) in a 1.5% agarose gel stained with ethidium bromide and visualized using Gel Doc (Bio-Rad, Hercules, CA, USA) *(Fig. 2)*.

Sequencing and Molecular Classification

One positive sample for each species were selected to validate PCR results. After purification of PCR products by QIAquick PCR purification kit (Qiagen, Hilden, Germany) sequencing were performed by a commercial company (Macrogen, South Korea). Sequences were edited by Chromas versiyon 2.6.5 (http://technelysium.com.au/wp/) and compared with the other sequences available in Genbank *(https://blast.ncbi.nlm.nih.gov/Blast.cgi)*. The sequences of the partial 16S ribosomal RNA gene of Mhc and CMhp have been deposited in GenBank databases under accession no: MK015018 and MK026012 respectively.

RESULTS

Distribution and frequency of hemoplasma species in 170 dogs were determined. The overall prevalence for hemoplasma infection was 5.29% (CI 2.57-9.34). Nine animals were found to be infected by one or more species *(Table 2)*. Mixed infections were determined in 3 of 170 samples with a rate of 1.76% (CI 0.44-4.52) and single infections were in 6 with a rate of 3.53% (CI 1.41-7.03). While five (2.94%, CI 1.06-6.22) samples were found to be infected with Mhc, CMhp was detected in one (0.59%; CI 0.03-2.57) sample.

Obtained sequences comparisons exhibited that while Mhc sequence identified in this study (MK015018) showed 99-100% similarity with the previously reported sequences for the 16S ribosomal RNA gene of Mhc (KY117656, KP715858, EF416567, AB848714) and *Mycoplasma haemofelis* (KM275238, KR905462), CMhp sequence (MK026012) shared 99-100% identity with sequences for the 16S ribosomal RNA gene of CMhp (MG594500, KF366443, HQ918288) and 97-98% identity with *Candidatus Mycoplasma haemominutum* (JQ689947, AY150974, JQ044683).

DISCUSSION

This study exhibits, for the first time, molecular evidence and prevalence of hemoplasma in dogs in Kyrgyzstan. The overall molecular prevalence of canine hemoplasma species was 5.29% (CI 2.57-9.34). Mhc, CMhp and Mhc + CMhp prevalences were 2.94% (CI 1.06-6.22), 0.59% (CI 0.03-2.57) and 1.76% (CI 0.44-4.52) respectively with this study. Low prevalence of hemoplasmas determined with this study was similar to data from Italy 4.5% [10] and Nigeria with 7.7% [5]. While higher prevalences were reported in Portugal 40% [2], Iran 23% [9] and Turkey 15.3% [4]; low prevalence was from USA 1.3% [3]. Low prevalence in the studied area may be correlated to climate conditions and/or lack of vector diversity.

In the present study Mhc has higher molecular prevalence than CMhp and this result is in accordance with previous publications [5,9]. Three dogs (1.76%) were found to be infected for both Mhc and CMhp. Similar to our results, mix infections were determined in several studies [2,4,9].

Diagnosis of hemotropic *Mycoplasma* species is based on microscopic examination of thin blood smears [8] or PCR analysis targeting 16S rRNA gene fragment [1,12]. Microscopic examination of smears may be useful and cheap in acute cases but it is not possible to discriminate species

and also determine carrier animals with this method. Molecular methods have always been found superior to microscopic examination for detection and differentiation of hemotropic *Mycoplasma* spp. and other tick-borne agents [13,14]. In this study a species-specific PCR assay with high sensitivity and specificity were applied to determine carrier animals for hemotropic *Mycoplasma* spp. in dogs. It was determined that overall prevalence of canine hemoplasma species was 5.29% in Kyrgyzstan and Mhc and CMhp circulate there. We recommend species-specific PCR for Mhc and CMhp in routine screening of blood donors.

Transmission of canine hemoplasma species is associated with haematophagous arthropods like fleas and ticks [2]. Also, blood transfusion from carrier dogs to splenectomized dogs induces transmission the organism [12]. Furthermore, CMhp is accepted as a zoonotic microorganism [15]. Since it was determined with this study dog population in Kyrgyzstan is carrier for both Mhc and CMhp, veterinarians and medical doctors should take these species into consideration in suspected cases.

In conclusion canine hemoplasma species were determined in Kyrgyzstan dog population for the first time and the molecular prevalence for hemotropic *Mycoplasma* spp. in Kyrgyzstan dog population is 5.29%. It was also determined that dog population in Kyrgyzstan exposure to either or both hemoplasma species of Mhc and CMhp and Mhc has higher molecular prevalence than CMhp. It is suggested that further studies aimed to determine molecular prevalence of canine hemoplasmas and potential arthropod vectors should be conducted in other provinces of Kyrgyzstan. We also suggest to veterinarians to be conscious for canine hemoplasma among anemic dogs and routine screening of blood donors may be useful to prevent spread of disease.

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Short Communication

Osmanlı Dönemi Veteriner Hekimliği Eğitiminde İlk Etik Dersi Girişimi [1]

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Öz

II. Meşrutiyet'in (1908) ilanından sonra edebiyat, bilim ve felsefe konularında süreli yayınlar ile çeviri ve telif eserlerin yayımındaki artış dikkat çekicidir. Bu dönemde Askeri Baytar Suphi Ethem'in "botanik" ve "jeoloji" dersleri verdiği ve zooloji, botanik, biyoloji, kimya, antropoloji, sosyoloji, filoloji, tarih, felsefe, bilim tarihi, edebiyat tarihi gibi bilim alanlarında çalışmalar yaparak eserler ortaya koyduğu saptanmıştır. Bu çalışmada, Askerî Veteriner Okulu öğretmenlerinden Suphi Ethem'in, Ulum Tabi'iye ve İçtimaiye Kütüphanesi tarafından 1917 yılında yayımlanan, "Musahabelerim" adlı eserinin incelenmesi amaçlandı. İstanbul Büyükşehir Belediyesi Atatürk Kitaplığının "Belediye Osmanlıca Kitaplar Koleksiyonu"ndan elde edilen, 32 sayfalık kitapçığın transkripsiyonu yapıldı ve günümüz Türkçesine özetlenerek çevrildi. Eserin içeriği dönemin ve günümüzün bilgileri çerçevesinde ele alınarak değerlendirildi. Sonuç olarak, "etik" (ahlak felsefesi) konusunun, Osmanlı dönemi veteriner hekimliği eğitiminde ilk kez 1917 tarihinde yer aldığı söylenebilir.

Anahtar sözcükler: *Suphi Ethem, Veteriner hekimliği eğitimi, Etik dersi, Veteriner hekimliği tarihi*

First Ethic Course Attempt of Veterinary Medicine Education in Ottoman Period

Abstract

After the proclamation of the II. Constitutional Monarchy (1908), periodicals on literature, science and philosophy and the increase in the publication of translation and copyright works are noteworthy. In this period, Suphi Ethem lectured "Natural History" lessons "Botany" and "Geology" courses and worked on many branches such as zoology, botany, biology, chemistry, anthropology, sociology, philology, history, philosophy, history of science, history of literature. In this study, it was aimed to examine Suphi Ethem's booklet named "Musahabelerim" which was published by the Ulum Tabi'iye and İçtimaiye Library in 1917. A copy of the 32 - pages booklet obtained from the Istanbul Metropolitan Municipality, Atatürk Library's "Municipal Ottoman Books Collection" made transcription and translated by summarizing into today's Turkish language. The content of the booklet was evaluated within the framework of the information of the era and today. As a result, it can be said that the subject of ethics was first included in military veterinary medical education in 1917 in Ottoman era.

Keywords: *Suphi Ethem, Veterinary medical education, Ethics course, History of veterinary medicine*

GİRİŞ

Osmanlı Devleti'nde, 19. yüzyılın başlarında yaşanan siyasî, iktisadî, hukukî ve sosyal sorunlar nedeniyle, önce askerî eğitim alanında başlayan batılılaşma çalışmaları kısa sürede diğer alanlarda da etkisini göstermiştir. Özellikle II. Meşrutiyet'in (1908) ilanından sonra edebiyat, bilim ve felsefe konularında süreli yayınlar ile çeviri ve telif eserlerin yayımındaki artış dikkat çekicidir. Bu ortamda Askerî Baytâr Mektebi'nden mezun olan Suphi Ethem'in çeşitli askerî birliklerde görev almasının yanı sıra, Manastır Askerî İdadisi ve İstanbul'daki Rehber-i İttihad Lisesi'nde "doğa tarihi"; Askerî Baytâr Mektebi'nde de "botanik" ve "jeoloji" dersleri verdiği bilinmektedir [1,2]. Aynı zamanda zooloji, botanik,

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biyoloji, kimya, antropoloji, sosyoloji, filoloji, tarih, felsefe, bilim tarihi, edebiyat tarihi alanlarında çeşitli çalışmalar yaptığı ve eserler ortaya koyduğu saptanmıştır [1-3]. Bu çalışmada, Suphi Ethem'in Ulum Tabi'iye ve İçtimaiye Kütüphanesi tarafından 1917 yılında yayımlanan, "Musahabelerim"[1] [4] adlı eserinin incelenmesi amaçlandı. Eserin, askeri veteriner öğrencilerine anlatılan etik konulu bir dersin metni olduğu anlaşılmaktadır. Veteriner hekimliği eğitim-öğretiminde "ilm-i ahlak" (genel ahlak) dersinin sivil veteriner okulu müfredatına 1895 yılından itibaren konulduğu bilinmektedir [5]. Batılılaşma sürecinde, ihtiyaç duyulan kadroların şekillendirilmesinde eğitime büyük önem verilmiş; düşünsel, bilimsel ve toplumsal değişmelerin öğrencilere aktarılması hedeflenmiştir [6]. Bu çalışma ile Askeri Veteriner Okulunda "etik" (ahlak felsefesi) konusunun ilk kez Suphi Ethem tarafından işlendiği, basılı ders materyali ile ortaya konmaya çalışılacaktır.

MATERYAL ve METOT

Çalışmada, İstanbul Büyükşehir Belediyesi Atatürk Kitaplığının "Belediye Osmanlıca Kitaplar Koleksiyonu"nunda yer alan ve 32 sayfadan oluşan 1917 yılına ait nüsha esas alındı. Dersaadet Kader Matbaası'nda basılan, Arap alfabesi kullanılarak yazılmış olan kitabın transkripsiyonu yapıldı ve günümüz Türkçesine özetlenerek çevrildi. Kitabın içeriği, dönemin ve günümüzün bilgileri çerçevesinde ele alınarak değerlendirildi.

BULGULAR

Suphi Ethem'in ders notunun ilk sayfasında "Musahabelerim" başlığı mevcut olup; eserin kapağında çerçeve içerisinde konu başlıkları şu şekilde verilmiştir: Etiğin anlamı- Etik kuramları, Etik ekolleri, Eski-Günümüz medeniyetlerinde etik, Hukuk biliminin metafizik ve tedrici kuramları, Hukuk kuramları, kanun, yorum ve hukuk uygulamalarının gelişimi, Hukuk ve etiğin geleceği, Sonuç ve son söz.

Suphi Ethem, "*Efendiler,*" hitabı sonrasında, "*Bu ve izleyen haftalardaki sohbetlerimiz konusu genel edebiyat ile ilgili sorunlarla sınırlı olacaktır. Siz bütün bu sohbetlerin sonucunda meslek hayatınızın sosyal hayatınıza desteğini ve bağlılığını anlayacak ve birçok yorucu uğraşılar arasında haftada bir defacık olsun hoşça bir vakit geçirmiş olacaksınız.*" ifadesiyle amacını belirtmiştir. Sonrasında ise "*Bizde İlm-i ahlak Fransızca'da "Morale"nin mukabili olarak kabul edilmiş. "Ethique" kelimesi ise "ahlakiyat" veya "hasail-i ahlakiye[2]" tarzında yazılmıştır. Bu tamamiyle yanlıştır.* İlm-*i ahlak her halde "Ethique" kelimesinin mukabili olmalıdır.*" demiştir. "Etik" ve "Moral" kavramlarını etimolojik açıdan anlattıktan sonra, bu iki kavramın birbirinin yerine kullanılabildiğini söylemiştir. Öğrencilerin etiğin anlamını bu sohbetler sürecinin en sonunda anlayacağını çünkü etik konusunun ancak bir dizi *"tasvirat veya mefhumat-ı felsefe[3]"* ile açıklanabileceğini özellikle belirtmiştir. Yazar, etiğin insan eylemleri ile ilgili olduğunu ve kısmen hangi hareket tarzının, kişiyi veya toplumu refah ve mutluluğa ulaştıracağını gösterdiğini; hayır ve şer, fena ve iyi, haklı ve haksız, mübah ve gayri mübah ile ilgili bütün fikirlerin etiğin kapsamına girdiğini belirtmiştir.

İnsanlarda veya toplumlarda ilk felsefi fikrin gelişmesinden başlayarak teori ve gözlem yoluyla doğa olaylarının ve toplumsal hadiselerin anlaşılmaya ve açıklanmaya çalışıldığını ifade etmiş; bu çabaların tümdengelim ve tümevarım süreçlerinin oluşumunu sağladığını belirtmiştir. Gözlem yoluyla ulaşılabilecek bilgiler hakkında örnekler vermiş (suyun donması, erimesi, buharlaşması) ve eşyanın veya var olan her şeyin tamamını gözleme imkânı olmadığı için mevcut verilerden çıkarım yoluyla bazı bilgilere ulaşılabileceğini ancak tümdengelim yoluyla elde edilen bilginin her zaman doğru olmayacağını vurgulamıştır. Bilinen şeylerden bilinmeyenlere doğru gitmek (tümevarım) gerekir demiştir.

Auguste Comte'un insanlığın değişim ve ilerlemesini "Teolojik, Metafizik ve Pozitif" evreler şeklinde açıkladığı üç hal yasasına paralel olarak üç türlü felsefe olduğunu iddia etmiştir. Her döneme ilişkin düşünüş şekilleri ve yaygın inanışlara örnekler vermiş ve etiğin zaman ve mekâna bağlı olarak daima değiştiğini vurgulamıştır. Ondokuzuncu yüzyılda ulaşılan bilimsel bilgi seviyesinin "pozitivizm"in niteliğini ortaya koyduğunu vurgulayarak halkın hatta bazı yazar ve bilim insanlarının pozitivizmi yanlış anladığını belirtmiştir. Platon, Aristo ve Thomas Aquinas'ın yöntemlerini kısaca belirtmiş; Kant'ın "*vazife kaidesini*" (ödev ahlakı) öne çıkaran bir yeni felsefe geliştirdiğini iddia etmiştir.

Suphi Ethem, her toplumun kendine özgü bir ahlak anlayışı olduğunu, dolayısıyla geçmişten günümüze çok sayıda ahlak kuramı geliştirildiğini ve terimlerin anlamlarının da zamanla değiştiğini belirtmiştir. Friedrich Nietzsche'nin görüşlerini kısaca özetledikten sonra onun "Amoralist" olduğunu vurgulamış; Hobbes ile Bentham'ın sosyal faydacılık kuramını amoralizme karşı güçlendirmeye çalıştıklarını belirtmiştir.

"Bütün bu izahattan şunu anlatmak istiyorum ki, hükema[4] daima meşruiyet-i ahlakiyenin[5] şüpheli aksamını[6] *aramağa hiç de cesaret edemedikleridir. Onlar ahlakı bildirmemişler, yalnız muhakemat-ı ahlakiyenin[7] istinad ettiğ[8]i esaslardan bahs etmişlerdir. Gelecek haftaki musahabemde medeniyet-i kadimenin[9] ilm-i ahlakını mevzuu bahs edeceğimi va'd ederek sözüme hitam[10] veriyorum."* diyerek dersi bitirmiştir.

[1] söyleşi, bir konu üzerinde değerlendirme yapma
[2] huylar, tabiatlar
[3] felsefi tanım ve açıklamalar
[4] bilginler, alimler
[5] ahlaki geçerlilik
[6] kısımlar
[7] ahlaki değerlendirme
[8] dayanmak
[9] eski uygarlıklar
[10] son

Metnin sonunda *"Selimiye: Tarihi takrir*[11]*, 27 Şaban 1917"* şeklinde dersin verildiği yer ve tarih vurgulanmıştır.

TARTIŞMA ve SONUÇ

Suphi Ethem'in çeşitli bilim dallarında yaptığı çalışmalar bilim tarihi açısından incelenmiş ve değerlendirilmiş [1-3] olmakla birlikte, "ahlak kuramları" ve "etik" ile ilgili olduğu anlaşılan "Musahabelerim" adlı eseri üzerinde herhangi bir çalışma saptanamamıştır. Eserin içeriği göz önüne alındığında etik ve etik kuramları hakkında sosyolojik ve tarihsel açıdan bilgi verilmesinin amaçlandığı görülmektedir.

Sivil Veteriner Okulunda 1895 yılında müfredata eklenen "İlm-i Ahlak" dersinin içeriğine ilişkin ayrıntılı veri bulunmamakla birlikte, dönemin ahlak eğitimi anlayışı çerçevesinde toplumsal ilişkilerin düzenlenmesi, birlikte yaşama kuralları, ahlaki hikayeler, hoşgörü, fedakârlık, yardımlaşma, iş birliği, çalışkanlık, büyüklere saygı vb. konuları [6] kapsadığı söylenebilir. Çalışmanın konusu olan eserde ise felsefi anlamda ahlak, etik terimlerinin ele alındığı belirlenmiştir. Dolayısıyla, bu dersin "İlm-i Ahlak" dersinden farklı olduğu söylenebilir.

İncelenen eserin, Askeri Baytar Mektebinde, 27 Mayıs 1917 tarihinde verilen bir öğrenci dersinin metni olduğu ileri sürülebilir. Metinde geçen, "*Bu ve müteakib haftalardaki musahebelerimin mevzuu …*" ile "*Gelecek haftaki musahabemde …*" ifadelerinden, dersin birden fazla kez verilmesinin planlandığı kanısı çıkarılabilirse de konuyla ilgili başka bir kaynak bulunamamıştır. Aynı şekilde bu dersin müfredatta hangi adla yer aldığı da henüz belirlenememiştir.

Yazar'ın, ders konusunun "genel edebiyatla ilgili" olacağını belirtmesi ve *"birçok yorucu meseleler arasında haftada bir defacık olsun hoşça bir vakit geçirmiş olacaksınız."* ifadesi de konuya günümüzün "mesleki etik" içeriğinden farklı yaklaştığını düşündürmektedir. Demir [3], Odabaşı [1] Odabaşı [2]'nın da vurguladığı gibi, birçok bilim alanında kısa sürede yaptığı çalışmalar göz önüne alınacak olursa, Suphi Ethem'in, bu dersi sadece genel kültür yönüyle vermeyi amaçlamış olabileceği akla gelmektedir. Söz konusu dersin ilk ders olması nedeni ile tanımlar ve felsefi akımların verilerek konuya giriş yapıldığı ve sonraki haftalarda konunun detaylandırılmasının planlandığı iddia edilebilir. Eserde yer alan *"Siz onun içerdiği anlamı bu sohbetler dizisinin en sonunda anlayacak ve amacımın "hak ve kuvvet" fikri üzerinde durduğunu göreceksiniz."* ifadesinin de bu düşünceyi destekler nitelikte olduğu öne sürülebilir.

Bilimsel veteriner hekimliği eğitim öğretiminin 1762 yılında Fransa-Lyon'da başlamasından sonra mesleki davranış kuralları ile ilgili saptanabilen ilk eser, 1876 yılında Emile Thierry tarafından yayımlanan "Déontologie Vétérinaire-Devoirs et Droits des Vétérinaires (Veteriner Deontoloji-Veteriner Hekimlerin Görev ve Hakları)" başlıklı kitaptır [7]. İncelenen eserinde Suphi Ethem; Sokrates, Aristo, Platon, Nietzsche gibi felsefeciler ile Auguste Comte gibi sosyal bilimcilerin çalışmalarından ve görüşlerinden söz etmiş; ancak Thierry'nin anılan kitabı hakkında bilgi vermemiştir. Bu durum, daha önce araştırma ve yayım yaptığı alanların sosyoloji, felsefe ve doğa bilimleri olması nedeniyle, etik konusunu veteriner hekimlik açısından ele almadığını düşündürmektedir.

1849-1920 yılları arası ders müfredatı incelendiğinde Osmanlı Döneminde "Veteriner Hekimliği Etiği" veya "Etik" başlıklı hiçbir derse rastlanmamıştır [5]. Oysaki, aynı dönemde Mayo, "Veterinary Ethics" başlıklı makalesinde, öğrenciliğinde Amerika Birleşik Devletleri'nde "*Ethics"* veya "*The Science of Duty"* isimli dersi aldıklarını açıkça vurgulamıştır [8]. Ancak ulaşılabilen ders müfredatlarında 1910-1921 yılları arasında "Veteriner Hekimliği Etiği" veya "Etik" başlıklı bir ders olmadığı tespit edilmiştir [9-11]. Ayrıca Liautard'ın [12] Massachusetts Veteriner Hekimler Birliği'nin toplantısında yaptığı konuşma, "Veteriner Hekimliği Mesleğini Değerlendirme Anlamında Etik" başlığıyla American Veterinary Review'de 1892 yılında yayımlanmıştır. Bütün bu kaynaklardan hareketle, Amerika Birleşik Devletleri'nde etik konusunun veteriner hekimlerin görev ve hakları çerçevesinde ele alındığı söylenebilir. Ancak Suphi Ethem'in Amerika'daki veteriner hekimliği etiği çalışmalarını takip ettiğine dair de bir bulgu yoktur.

Metinde geçen, "Bu ve müteakib haftalardaki musahebelerimin mevzuu …" ile "Gelecek haftaki musahabemde …" ifadelerinden, dersin birden fazla kez verileceği kanısı çıkarılabilirse de konuyla ilgili başka bir kaynak bulunamamıştır. Beşer ve Tabiat (1919) dergisinin 4 ve 5inci sayılarında *"Ahlâk Nazariyyeleri*[12]*"* başlıklı iki yazıda konunun özetlenerek tekrar yayımlandığı saptanmıştır [2]. Bu yazıların devam edeceği vurgulanmakla birlikte bu tarihten sonra herhangi bir çalışmaya rastlanamamıştır.

Sonuç olarak, incelenen eserin Türkiye'deki veteriner hekimliği eğitiminde etik konusunun, ilk kez 1917 yılında yer aldığını açıkça ortaya koyduğu söylenebilir.

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Short Communication

Polymorphism in *GHRH* Gene and Its Association with Growth Traits in Tibetan Sheep

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Abstract

The objectives of this study were to identify single nucleotide polymorphisms (SNPs) in the growth hormone-releasing hormone *(GHRH)* gene and to evaluate their associations with growth traits in two main Tibetan sheep breeds. Through sequencing technology, four SNPs were identified in the 5'UTR region of *GHRH* gene. Both g.794A>C and g.1497C>A exhibited significant influence on the growth-related traits in Tibetan sheep (P<0.05 or P<0.01). Hence, the biochemical and physiological functions, together with the results obtained in our investigation, suggest that the *GHRH* gene could serve as genetic marker for growth in Tibetan sheep breeding.

Keywords: *Tibetan sheep, Mutation, GHRH gene, Body measurement*

Tibet Koyunlarında Büyüme Hormonunu Salgılatıcı Hormon *(GHRH)* Genindeki Polimorfizmlerin Büyüme Özellikleri İle İlişkisi

Öz

Bu çalışmanın amacı, büyüme hormonu salgılatıcı hormon *(GHRH)* genindeki tek nükleotid polimorfizmlerini (SNPs) tanımlamak ve bunların iki Tibet koyun ırkının büyüme özellikleri ile olan ilişkilerini değerlendirmektir. Sıralama teknolojisi ile, *GHRH* geninin 5'UTR bölgesinde dört SNP tanımlandı. Hem g.794A> C hem de g.1497C> A, Tibet koyunlarında büyümeyle ilgili özellikler üzerinde önemli bir etki gösterdi (P<0.05 veya P<0.01). Bundan dolayı, araştırmamızda elde edilen sonuçlarla birlikte, biyokimyasal ve fizyolojik fonksiyonlar *GHRH* geninin Tibet koyun yetiştiriciliğinde büyüme için genetik belirteç görevi görebileceğini ortaya koydu.

Anahtar sözcükler: *Tibet koyunu, Mutasyon, GHRH geni, Vücut ölçüsü*

INTRODUCTION

Tibetan sheep (ovine) were the first artificially bred sheep in the Qinghai-Tibetan plateau and hold enormous potential for animal production [1], which showed high tolerance to the extreme environments, such as extreme cold, low oxygen concentrations, and low air pressure [2]. As an anabolic hormone, growth hormone *(GH)* was synthesized and secreted by the anterior pituitary eosinophil cells in mammals [3]. It could perform crucial effect on tissue growth, reproduction, muscle accretion and fat catabolism by binding to various hormones of the somatotropic axis [4]. In the anterior pituitary gland and tissues, the synthesis and secretion of *GH* was highly affected by *GHRH* [5,6]. Because of the specific role of the *GHRH* gene in metabolism, we hypothesized that the variations in *GHRH* gene would be a candidate for heritable differences in growth traits of Tibetan sheep.

MATERIAL and METHODS

Blood samples were obtained from 565 female Tibetan sheep (aged 6 to 8 months) belonging to two different breeds: Black Tibetan sheep (BT, N=210) and Oula Tibetan

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sheep (OT, N=355). The sheep were reared in Henan County and Haiyan County respectively of Qinghai Province. In this study, all individuals were raised in five different farms. Meanwhile, the growth traits including body weight, withers height, body length and heart girth were measured on one day. Genomic DNA were isolated from whole blood samples using the OMGAM Blood DNA Kit (OMGAM Bio-Tek, Doraville, USA) and were stored at -20°C. On the basis of the *GHRH* gene sequence (Accession no NC_019470), four pairs of PCR primers *(Table 1)*. were designed by Primer Premier Software (Version 5.0). PCR amplification was performed following the method of Sun et al.[7] Amplification were then sequenced using an ABI 3730 sequencer (ABI, Foster City, CA, USA).

Gene frequencies, Hardy-Weinberg equilibrium and polymorphism information content were calculated by POPGENE software package (Version 3.2). Linkage disequilibrium (LD) was estimated by the web-based tool *(http://analysis.bio-x.cn/myAnalysis.php)*. SNPs interaction was assessed based on likelihood ratio statistic test from logistic regression [8]. Statistical analysis was performed using the general linear model (GLM) procedure implemented in SPSS 16.0 (IBM Company, NY, USA) software package. The basic linear model was: $Y_{ijk} = \mu + G_i + A_j + F_k + e_{ijk}$, G_i is the fixed effect of genotype, A_j is the fixed effect of age, F_k is the fixed effect of farm and e_{ijk} is the random error.

RESULTS

Four SNPs were identified in the 5'UTR of ovine *GHRH* gene including g.794A>C, g.987T>C, g.1480G>A and g.1497C>A *(Fig. 1)*. Summary statistics for each of those SNPs were presented in *Table 2*. The frequencies of allele A (g.794A>C), T (g.987T>C), G (g.1480G>A) and C (g.1497C>A) were found to be predominant in the studied samples. Except for g.987T>C in OT breed and BT breed, and g.1497C>A in OT breed, the genotypic frequencies conformed to Hardy-Weinberg equilibrium (P>0.05). According to the classification of PIC value [9], four SNPs were within the range of moderate genetic diversity (0.25<P<0.50). The values of r^2 between the four SNPs in the studied samples were from 0.000 to 0.043, indicating that those SNPs had weak LD.

The results of association study between BT breed and growth traits were presented in *Table 3*. For the g.794A>C and g.1497C>A, the influence of AA genotype resulted in the highest mean for body weight compared to animals with genotype CC (P<0.05). As shown in *Table 4*, the g.1497C>A polymorphism affected the body weight, and heart girth in animals with the AA genotype to a much greater extent than in animals with the CC genotypes (P<0.01 or P<0.05) in OT breed.

The prediction indicated that substituting A with C at g.794A>C locus produced a putative loss of binding sites myf3, bHLH transcription factor 1, TGFB-induced factor homeobox 2-like and myf4. In addition, the prediction suggested that g. 1497 C and g. 1497 A could in sequence binding in six and four cis-acting elements *(Table 5)*.

As it is presented in *Table 6*, an examination of the epistatic effect of the three-gene SNP genotypes on the growth traits was attempted, with significant interactions detected for four growth traits (body weight, withers height, body length and heart girth).

DISCUSSION

Our results were consistent with the previous study. Piorkowska et al.[10] reported that the *GHRH/AluI* SNP had significant effects on water-holding capacity and meat colour in pigs of three breeds reared in Poland. One novel SNP *(GHRH*/HealII) was demonstrated to improve body

Table 1. *Primers used in these experiments*

Name	Primer Sequence (5' to 3')	Tm (°C)	Product Length	Amplified Region
L1	CGTCAGTGCTTTAGGGTTC	58.8	695 bp	Part of 5'UTR
	GATTGGCAGATTTGGGAG			
L2	CTGGCTTTACTGCGACTT	60.0	550 bp	Part of 5'UTR
	TGGCATTCTACTCCCTCC			
L3	GTGACTGGCAGAGGCAGA	61.5	760 bp	exon 1 and exon 2
	GAAGTGACAGCTGCTGTG			
L4	AAAGGGCAGTTCTTCATA	63.5	771 bp	exon 3
	TCTTCTGGTTCTTGATGTAT			

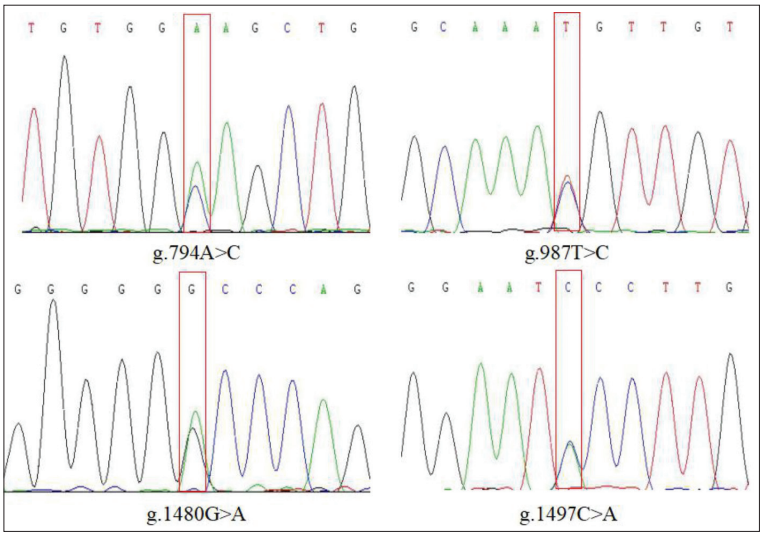

Fig 1. The sequencing map of the GHRH gene SNPs

Table 2. *Genotype frequencies (%) of the GHRH gene for the single nucleotide polymorphisms (SNPs)*

Locus	Breeds	Genotypic Frequency (%)			PIC	Maximum Allele Frequency	HWE
g.794A>C	BT	AA (48.57)	AC (37.14)	CC (14.29)	0.34	67.14% (A)	5.26
	OT	AA (56.62)	AC (36.06)	CC (7.32)	0.31	74.65% (A)	0.80
g.987T>C	BT	TT (61.90)	TC (27.14)	CC (10.95)	0.30	75.48% (T)	14.95
	OT	TT (72.11)	TC (19.15)	CC (8.73)	0.26	81.69% (T)	45.92
g.1480G>A	BT	GG (59.52)	GA (34.29)	AA (6.19)	0.29	76.67% (G)	0.37
	OT	GG (46.48)	GA (41.97)	AA (11.55)	0.34	67.46% (G)	0.68
g.1497C>A	BT	CC (52.86)	CA (37.62)	AA (9.52)	0.32	71.67% (C)	1.13
	OT	CC (67.04)	CA (24.51)	AA (8.45)	0.31	79.30% (C)	22.83

Polymorphism information content (PIC), heterozygosity (He), Hardy-Weinberg equilibrium (HWE), $\chi^2_{0.05(1)} = 3.840$, $\chi^2_{0.01(1)} = 6.630$

Table 3. *Association of different genotypes of SNPs in GHRH gene with growth traits in BT breed*

Locus	Genotypes (N)	Body Weight (kg)	Withers Height (cm)	Body Length (cm)	Heart Girth (cm)
g.794A>C	AA (102)	47.60 ± 0.26^{a}	67.90±0.24	71.00±0.24	90.78±0.41
	AC (78)	44.56 ± 0.30^{ab}	67.05±0.28	70.13±0.27	91.47±0.47
	CC (30)	43.08 ± 0.47^{b}	66.37±0.45	69.82±0.43	90.02±0.76
g.987T>C	TT (130)	45.29±0.27	67.40±0.22	70.57±0.21	90.49±0.37
	TC (57)	46.21±0.41	67.65±0.33	70.40±0.32	91.71±0.55
	CC (23)	47.87±0.65	66.38±0.53	70.43±0.51	91.48±0.68
g.1480G>A	GG (126)	45.86±0.28	65.54±0.22	70.22±0.21	90.98±0.31
	GA (72)	45.78±0.37	67.21±0.29	71.07±0.28	90.84±0.49
	AA (12)	45.74±0.63	66.74±0.52	70.16±0.59	90.85±0.69
g.1497C>A	CC (111)	44.64 ± 0.28^{b}	67.00±0.23	69.97±0.21	90.54±0.39
	CA (79)	46.80 ± 0.32^{ab}	67.57±0.27	70.68±0.26	91.45±0.47
	AA (20)	48.83 ± 0.55^{a}	68.74±0.46	72.51±0.45	90.99±0.84

^{a,b} Means with different superscripts are significantly different (P<0.05); ^{A,B} Means with different superscripts are significantly different (P<0.01)

Table 4. *Association of different genotypes of SNPs in GHRH gene with growth traits in OT breed*

Locus	Genotypes (N)	Body Weight (kg)	Withers Height (cm)	Body Length (cm)	Heart Girth (cm)
g.794A>C	AA (201)	58.87 ± 0.35^{a}	71.60±0.26	75.29 ± 0.26^{a}	96.34 ± 0.33^{a}
	AC (128)	58.49 ± 0.44^{a}	71.20±0.31	74.80 ± 0.32^{ab}	95.82 ± 0.42^{a}
	CC (26)	52.40 ± 0.73^{b}	70.00±0.53	71.95 ± 0.47^{b}	92.12 ± 0.64^{b}
g.987T>C	TT (256)	57.69±0.32	71.16±0.24	74.58±0.23	95.77±0.30
	TC (68)	58.78±0.62	71.35±0.45	75.12±0.45	95.84±0.58
	CC (31)	61.83±0.75	72.78±0.67	76.69±0.66	96.40±0.70
g.1480G>A	GG (165)	58.71 ± 0.39^{a}	71.32±0.29	74.93±0.28	95.91±0.37
	GA (149)	58.97 ± 0.43^{a}	71.68±0.31	75.59±0.29	96.37±0.39
	AA (41)	53.89 ± 0.71^{b}	70.19±0.58	72.03±0.56	93.62±0.74
g.1497C>A	CC (238)	57.99 ± 0.31^{B}	70.99±0.24	74.71 ± 0.28^{b}	95.94±0.31
	CA (87)	56.55 ± 0.51^{B}	71.12±0.39	73.94 ± 0.38^{b}	94.92±0.51
	AA (30)	65.29 ± 0.82^{A}	74.77±0.66	78.89 ± 0.64^{a}	97.76±0.80

measurement in limousine cattle [11]. *Cheong* et al.[12] showed that one SNP (c.-4241A>T) in promoter region of *GHRH* gene had a strong effect on cold carcass weight and longissimus muscle area in Korean Hanwoo cattle. The research of Zhang et al.[13] showed that a novel marker (g.4251C>T) on *GHRH* gene was associated with body weight for different growth periods (6, 12, 18, and 24 months old) in Nanyang cattle. Based on the outcomes, it is our belief that the *GHRH* gene could be an excellent candidate gene for growth-related traits in livestock.

Table 5. *Transcription factor binding sites identified at SNP's identified within 5'UTR of ovine GHRH gene*

Locus	Genotype	Transcription Factors	Core Similarity	Cis-acting Elements (Recognition sequence)	Target Strand
g.794A>C	A/C	Transcription factor AP-2, alpha	0.952	tggcCCTGtgga/cagc	(+)
	A	CCAAT/enhancer binding protein beta	0.928	ggcccTGTGgaagct	(+)
	C	TGFB-induced factor homeobox 2-like, X-linked, dimeric binding site	0.925	cagacagctGCCAcagg	(-)
	C	Myogenic regulatory factor MyoD (myf3)	0.977	ctgtggCAGCtgtctgc	(+)
	C	Achaete-scute family bHLH transcription factor 1	0.978	cagacaGCTGccaca	(-)
	C	Myogenic bHLH protein myogenin (myf4)	0.992	ggcagaCAGCtgccaca	(-)
	C	TALE homeobox protein Meis 2, dimeric binding site	0.779	gtggCAGCtgtctgcca	(+)
g.1497C>A	C/A	PAX6 paired domain and homeodomain are required for binding to this site	0.880	Atc/accttgtgCCAGccctg	(+)
	C/A	Stimulating protein 1, ubiquitous zinc finger transcription factor	0.885	CccagGGGCtgggaatc/a	(+)
	C	Ikaros 1, potential regulator of lymphocyte differentiation	0.947	ggctGGGAatccc	(+)
	C	NF-kappaB (p50)	0.851	gctGGGAatcccttg	(+)
	C	SRY (sex-determining region Y) box 9	0.968	ctggcACAAgggattcccagccc	(-)
	C	Transcription factor with 8 central zinc fingers and an N-terminal KRAB domain	0.772	agggggggCCAGgggctgggaatcc	(+)
	A	Ikaros 3, potential regulator of lymphocyte differentiation	0.997	ggctgGGAAtacc	(+)
	A	Brn-5, POU-VI protein class (also known as emb and CNS-1)	0.754	ggctggCACAaggtattcccagc	(-)

Table 6. *Multi-marker interaction analysis of GHRH with GH and Myogenin*

Traits	Gene	df	Sum of Square	Mean of Square	F Value	P Value
Body length	GHRH	2	309.1	154.54	15.371	6.39E-07
	GH	2	170.3	85.16	8.47	0.000298
	MY	2	259.7	129.83	12.914	5.45E-06
	GHRH:GH	4	27.9	6.97	0.694	0.59711
	GHRH:MY	3	22.9	7.62	0.758	0.519159
	GH:MY	4	19.9	4.97	0.494	0.740071
	GHRH:GH:MY	4	43.7	10.92	1.086	0.364838
Body weight	GHRH	2	910.8	455.4	40.089	2.75E-15
	GH	2	308.9	154.5	13.597	2.99E-06
	MY	2	485.4	242.7	21.362	4.16E-09
	GHRH:GH	4	11.4	2.8	0.251	0.909
	GHRH:MY	3	49.6	16.5	1.454	0.228
	GH:MY	4	4.1	1	0.089	0.986
	GHRH:GH:MY	4	26.2	6.6	0.577	0.680
Heart girth	GHRH	1	2405	2405	0.581	0.447
	GH	2	8532	4266	1.03	0.359
	MY	2	3275	1637	0.395	0.674
	GHRH:GH	2	5169	2585	0.624	0.537
	GHRH:MY	2	2110	1055	0.255	0.775
	GH:MY	4	8878	2220	0.536	0.709
	GHRH:GH:MY	4	4506	1126	0.272	0.896
Withers height	GHRH	2	502.2	251.1	17.596	9.62E-08
	GH	2	60.9	30.46	2.134	0.12114
	MY	2	168.9	84.44	5.917	0.00321
	GHRH:GH	4	79.5	19.88	1.393	0.23794
	GHRH:MY	3	45.5	15.18	1.064	0.36561
	GH:MY	4	56.4	14.09	0.988	0.41543
	GHRH:GH:MY	5	136.3	27.25	1.91	0.09446

Silico analysis was used to predict the effects of the alternative alleles in the 5′ UTR of the *GHRH* gene on the transcription factor binding sites by online analysis website *(http://www.genomatix.de/)* [14]. The predictable results showed that differences existed for the transcription factors of the different genotypes at these two mutations. Thus, it can be reasonably inferred that the identified SNPs within the *GHRH* gene 5′UTR regions would modify transcription factor binding affinity, thereby affecting phenotypes in Tibetan sheep.

Our previous work revealed that SNPs of candidate (i.e., *GH* and *Myogenin*) significantly influenced growth traits in Tibetan sheep [7,15]. Multi-marker interaction analysis suggested the influence of the mutations in three different genes were additive effects, which is not consistent with the the function of *GHRH* in regulating the *GH* secretion. This discordance may be due to relatively small sample size used. Further studies are required to address other SNPs of the three genes and their associated gene network.

In summary, four polymorphisms in the *GHRH* gene were identified in Tibetan sheep. The association analysis of single markers revealed that g.794A>C and g.1497C>A exhibited prominent effects on growth traits. Our investigation provides evidence that *GHRH* gene could be used as molecular markers and could contribute to the expanding panel of functional variation.

Conflicts of Interest

The authors declare no conflict of interest.

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