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RESEARCH ARTICLE

Textural, Chemical and Sensory Properties of Döners Produced from Beef, Chicken and Ostrich Meat

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Summary

In this study, the chemical, textural and sensory properties of beef, chicken, or ostrich meat döners were compared. Ostrich döner samples had lower (P<0.0001) cholesterol content than beef or chicken döners but higher calorific values. It is observed that beef döners had higher (P<0.0001) Warner-Bratzler shear force (WBSF) than chicken and ostrich döners. According to the texture analyses results it is concluded that hardness measured by Texture Profile Analyzer was found to be a better predictor of sensory tenderness than WBSF. Panelists rated ostrich meat with highest point in terms of sensory properties. Ostrich döners had better overall acceptance than beef or chicken döners. Therefore it is concluded that ostrich döner can be taken into consideration as an alternative protein source to beef or chicken döners.

Keywords: *Ostrich meat, Döner, Texture*

Sığır, Tavuk ve Devekuşu Etinden Üretilmiş Dönerlerin Tekstürel, Kimyasal ve Duyusal Özellikleri

Özet

Bu çalışmada deneysel olarak üretilmiş sığır, tavuk ve devekuşu etinden üretilmiş dönerlerin kimyasal, tekstürel ve duyusal özellikleri karşılaştırılmıştır. Sığır ve tavuk dönerlerine göre devekuşu etinden üretilmiş döner örneklerinin kolesterol içeriği daha düşük, kalori değerleri ise daha yüksek bulunmuştur. Sığır etinden üretilmiş döner örneklerinin daha yüksek Warner Bratzler kesme kuvveti (WBSF) değerlerine sahip olduğu gözlenmiştir (P<0.0001). Yine elde edilen doku analizi bulgularına göre Tekstür Profil Analizi ile ölçülen sertlik değerinin WBSF değerine göre duyusal gevrekliğin daha iyi bir belirleyicisi olduğu sonucuna varılmıştır. Panelistler duyusal özellikler bakımından en yüksek puanla devekuşu etini tercih etmişlerdir. Devekuşu döneri sığır eti ve tavuk etinden üretilmiş döner örneklerinden daha yüksek beğeni almıştır. Sonuç olarak devekuşu dönerinin sığır ve tavuk dönerlere alternatif bir protein kaynağı olarak değerlendirilebileceği kanısına varılmıştır.

Anahtar sözcükler: *Devekuşu eti, Döner, Tekstür*

INTRODUCTION

Döner is a traditional Middle Eastern meat product, which is consumed widely in Turkey and many parts of the world [1-3]. Although döner appeals people who are accustomed to the Anatolian culture, it has become a competitor in the fast-food market [4]. Döner is traditionally made from lamb, veal, beef, or poultry meat or by mixing them at certain proportions in the presence of onion, pepper, tomatoes, and other spices [5]. As an extra flavor, several other spices (white pepper, black pepper, cumin, allspice, curry, and thyme), tomato paste or juice, milk powder, lemon juice, yoghurt, chicken egg and sugar may be added [6-8]. Sauce composition may vary [9]. Based on the production style, döners are classified as leaves, leaves and ground, and ground [10]. After the raw döner on a vertical stick is slowly rotated to roast in an open gas or electric oven, cooked portions are removed as thin slices and consumed in bread with sliced tomato, onions and lettuce [1,10-12].

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Altough döner made from beef, veal, or lamb meat are the most common, the use of chicken meat in döner production has become very popular because it is cheap and digested easily. However some quality problems like non-uniform shaped pieces occured while shaving off, juiciness and texture are not desirable to the consumer [7,10]. Beef döner is a tasty meal but its comparatively high fat content makes it not a very healthy choice. It is suggested that, the higher fat intake increases risk of certain types of cancer, and coronary heart disease. Several health-related organizations, including American Cancer Society and World Health Organization have recommended that fat intake be reduced to less than 30% of total calories received daily [13]. Therefore other meat source like ostrich meat may also be used in place of beef or chicken meat. Ostrich meat has a darker color than beef due to its higher pH and higher myoglobin content. Ostrich meat is considered a healthier choice because it has lower saturated fatty acids and higher polyunsaturated fatty acids than red meats. Moreover, ostrich meat has lower overall lipid content [14-17]. Compared to beef, lower lipid content, lower saturated fatty acid content and higher polyunsaturated fatty acid content of ostrich meat might make it a possible candidate for döners provided that it yields at least similar or better quality döner than beef. Therefore, the goals of this study were to determine and compare chemical, textural, and sensory properties of döners made from beef, chicken, or ostrich meat to develop better quality döner.

MATERIAL and METHODS

Sample Preparation

The beef, chicken, and ostrich döner samples provided by a local company in Manisa were formulated for this research and prepared complying with retailer's recipe and cooking conditions. The experiment was repeated 3 times and 9 samples for each treatment were analyzed. Deboned meats were trimmed of skin, connective tissue and visible fat in a processing hall at 4°C. Beef döners had beef, tallow, onion, milk, sunflower oil, black pepper, thyme and salt. Ostrich döners were produced for our research and had the same formula except for that ostrich meat was used instead of beef. Chicken döners had chicken breast meat, skin, sweet pepper paste, curry, paprika, and salt. Prepared döners were spitted on a döner kebap stick and slowly rotated for 2 min in front of a gas oven. Cooked surface was shaved off using automatic knives in the form of thin slices (0.25 cm). About 250 g of sample was packed in polyethylene bags and transferred with use of this retailer's cold chain cars to the laboratory. All analyses were performed at Celal Bayar University Food Engineering Department Laboratories.

Chemical Analysis

Chemical properties of döner samples including moisture, protein, fat, and ash content were determined using official methods [18] and expressed as %. Cholesterol content was determined using the procedure by Naeemi, Ahmad, Al-Sharrah, and Behbahani [19]. The pH was measured in a homogenate prepared by blending 10 g döner samples with 100 ml of distilled water for 30 s. Readings were taken with a WTW model pH 521, digital pH-meter and a WTW, type E56, combination electrode (WTW-Wissensehaftlich-TechnischeWerkstaetten GmbH, Weilheim, Germany). Water activity was measured using a portable hygrometer (AM/Wert-Messer, Germany).

Color Measurement

L*, a*, b* values were determined *with* a digital *Minolta CR300 chromometer* (*Minolta* Co., *Osaka*, *Japan)* The samples were homogenized and transferred to petri dishes before taking the readings. It was ensured that there was no gap between the sample and the petri lid and the lenses of colorimeter touched to the lid of the petri dish. Six readings per sample were taken and mean values were calculated.

Warner-Bratzler Shear Force and Texture Profile Analysis

Samples were sheared using a WSBF head with a load cell of 50 kg with crosshead spead and chart speed of 10 cm/min attached to a Texture Analyzer (Stable 80 Micro Systems, England). The texture profile analysis (TPA) parameters, including hardness (peak force on first compression, N), cohesiveness (ratio of the work done under the second fore-displacement curve to that one during the first compression) and gumminess (force to disintigrate a semisolid sample for swallowing, N) were determined according to the procedure suggested by Bourne [20].

Energy Values

Total calories were calculated taking a 100-g cooked sample as a reference using values for protein (4.02 cal/g), fat (9.3 cal/g) and for carbonhyrate (3.87 kcal/g) and were given as kcal.

Sensory Properties

Panelists from the faculty and research assistants at Food Engineering Department, evaluated overall acceptability, apperance, flavor, juiciness, tenderness, and ease of swallowing of samples by following AMSA quidelines [21]. Unsalted crackers and water (at room temperature) were served to panelists to clean the palate between the samples. No more than two sessions per day was scheduled to prevent fatigue. Panelists evaluated samples using an 8-point hedonic scale.

Statistical Analysis

The design was completely randomized. The treatments

were beef, chicken, and ostrich meat döners. Three replications were done. The analysis of variance was done using the PROC GLM procedure of SAS (version 8.2, SAS Institute Cary, NC 2001). LSMEANS for teatments were generated and separated when significant (P<0.05) using the pdiff statement. Correlation coefficients were calculated using PROC CORR procedure of SAS [22].

RESULTS

Chemical Analysis

Chicken and ostrich döners had statistically similar protein contents, but had higher protein contents than beef döner *(Table 1)*. This finding is in agreement with Kayisoglu et al.[23]. Beef döners had the highest ash content, followed by ostrich and chicken döners. Moisture content was not affected by type of döner (P>0.05), or was water activity (P>0.05). pH was not different (P>0.05) among chicken ostrich, and beef döners. Chicken and beef döners were reported to have similar pH values [23]. Ostrich döners had the highest fat content followed by beef and chicken döners. This observation could be attributed to the fact that ostrich döners cooked fastest and there was not enough time for the added fat to be removed from the ostrich meat. On the other hand, beef döners cooked the slowest resulting in removal of fat, thus having the lesser fat than ostrich döners. As for chicken döners, chicken skin rather than fat was used in the formulation, probably leading to the least amount of fat among the type of döners studied. Chicken döners were also reported to have less amount of fat than beef döners in another study [23].

Cholesterol content was the highest in chicken döners because chicken skin was in the chicken döner formulation and it was lowest in the ostrich döner. Similary, other authors [15,17] pointed out that ostrich meat was a healthy red meat due to its low cholesterol content [15,24]. However, other study by Hoffman [25] did not agree and suggested that ostrich meat had similar cholesterol values to other lean type meats. Beef döners had lower cholesterol content than chicken döners, which could be attributed to the fact that on the average beef (muscles) had 60 mg/100 g whereas chicken (muscles) had 80 mg/100 g cholesterol [26].

Color Values

Beef and ostrich döners had similar L* values (P>0.05), whereas they had lower (P<0.001) L* values than chicken döners. The a* (redness) value was the highest for chicken döners (P<0.0001). This could be explained by the chicken döner formulation because chicken döners contained considerable amount of red pepper paste, which probably resulted in redder color. Similarly, chicken döners had higher (P<0.0001) b*(yellowness) than beef or chicken döners. Inclusion of skin and the color of chicken fat in chicken döner formula could have caused this.

Warner-Bratzler Shear Force and Texture Profile Analysis

Beef döners had higher (P<0.0001) Warner-Bratzler shear force (WBSF) than chicken or ostrich döners. Ostrich döners had significantly higher (P<0.01) WBSF (less tender) than chicken döners *(Table 3)*. Hardness followed the same trend with beef döners having higher hardness (P<0.0001) values than ostrich or chicken döners. Chicken döners were found to have the least hardness value. Hardness values for chicken döners were comparable to those reported by Kilic [27]. The type of döner (beef, chicken, or

Table 1. *Chemical properties and color values of beef, chicken, or ostrich döners*
Tablo 1. *Sığır, tavuk ve devekuşu dönerlerin kimyasal özellikleri ve renk değerleri*

Sample	Protein (%)	Ash (%)	Moisture (%)	Fat (%)	pH	aw	Cholesterol	L*	a*	b*
Beef	34.02^{a}	5.15^{a}	51.40^{b}	9.75^{b}	6.37	0.93	49.16^{b}	43.96^{b}	3.65^{b}	5.05^{b}
Chicken	33.73^{a}	2.31^{c}	54.35^{a}	9.25^{b}	6.33	0.95	68.72^{a}	60.23^{a}	8.67^{a}	23.6^{a}
Ostrich	31.56^{b}	3.45^{b}	51.69^{b}	14.68^{a}	6.42	0.93	22.77^{c}	43.14^{b}	3.43^{b}	3.48^{c}
P-value	0.0002	0.001	0.073	0.28	0.37	0.0003	<0.0001	<0.0001	<0.0001	<0.0001

^{a,b,c} *Means in the same row with different letters differ significantly (P<0.05)*

Table 2. *Sensory properties of beef, chicken, and ostrich döners*
Tablo 2. *Sığır,tavuk ve devekuşu dönerlerin duyusal özellikleri*

Sample	Taste	Ease of Swallowing	Appearance	Juiciness	Overall Tenderness	Overall Acceptability
Beef	6.52^{b}	6.05^{c}	6.26^{b}	5.75^{c}	5.73^{c}	5.93^{c}
Chicken	6.28^{c}	6.55^{b}	6.51^{b}	6.30^{b}	6.70^{b}	6.33^{b}
Ostrich	6.80^{a}	7.22^{a}	6.97^{a}	6.98^{a}	7.47^{a}	7.40^{a}
P-value	0.003	<0.0001	0.008	0.0007	<0.0001	<0.0001

^{a,b,c} *Means in the same row with different letters differ significantly (P<0.05)*

Table 3. *WBSF (kg) values of beef, chicken, and ostrich döners*
Tablo 3. *Sığır,tavuk ve devekuşu dönerlerin WBSF (kg) kesme kuvveti değerleri*

Sample	WBSF (kg)
Beef	6.05[a]
Chicken	1.58[c]
Ostrich	3.16[b]
P-value	0.0002

[a,b,c] *Means in the same row with different letters differ significantly (P<0.05)*

significantly correlated (P>0.05, r=-0.63) to cohesiveness. Safari et al.[28] also found significant correlation between WBSF and sensory tendemess [28]. Correlation coefficient between hardness and sensory tendernes was higher (P<0.05) than that between WBSF and sensory tendernes. Huidobro et al.[29] similarly reported texture profile analysis parameters as better predictors of sensory tendemess as compared to WBSF, agreeing with our findings. Ease of swallowing was significantly correlated to hardness (P<0.01, r=-0.89), cohesiveness (P<0.05, r=-0.67), and gumminess (P<0.01, r=-0.83), but was not significantly

Table 4. *Texture profile analysis parameters of beef, chicken, and ostrich döners*
Tablo 4. *Sığır,tavuk ve devekuşu dönerlerin tekstür profil analiz parametreleri*

Sample	Hardness	Chewiness	Cohesiveness	Gumminess
Beef	5.74[b]	1.54[b]	0.75	2.52[b]
Chicken	4.52[b]	1.23[b]	0,80	2.74[b]
Ostrich	5.12[a]	2.18[a]	0.82	4.21[a]
P-value	0.0002	<0.0001	<0.21	0.0005

[a,b,c] *Means in the same row with different letters differ significantly (P<0.05)*

ostrich) did not affect (P>0.05) cohesiveness value. Beef döners had higher (P<0.0001) gumminess values than chicken or ostrich döners. However, chicken and ostrich döners yielded similar (P>0.05) gumminess values *(Table 4)*.

Energy Values

Beef and chicken döners had about 232 kcal, and 226 kcal (100-g cooked), respectively, whereas ostrich döners had approximately 262 kcal. The higher caloric content of ostrich döners might poise a problem from nutritional point of view.

Sensory Properties

Ostrich döners had better taste (P<0.01), ease of swallowing (P<0.01), overall appearance (P<0.01) higher juiciness (P<0.01), higher overall tendemess (P<0.01), and better overall acceptability (P<0.01)than beef or chicken döners *(Table 2)*. Beef döners had higher (P<0.05) taste, but lower (P<0.05) ease of swallowing, lower overall tendemess (P<0.0001), lower juiciness (P<0.05) and lower (P<0.05) overall acceptability than chicken döners. Beef and chicken döners had similar (P>0.05) appearance. Overall, panelists clearly chose ostrich döners over beef or chicken döners *(Table 2)*.

Correlation Coefficients

Flavor was not correlated (P>0.05) to any of the textural parameters. Appearance was not highly (P>0.05, r=-0.46) correlated to WBSF, but was highly negatively correlated to hardness (P<0.05, r=-0.72), cohesiveness (P<0.01, r=-0.82), or gumminess (P<0.05, r=-0.79). Overall tenderness was negatively correlated (P<0.05, r=-.710) to WBSF, hardness (P<0.01, r=-0.89), gumminess (P<0.01, r=-0.9), but was not correlated (P>0.05, r=-0.6) WBSF. Similarly, juiciness was negatively correlated to hardness (P<0.01, r=-0.82), cohesiveness (P<0.05, r=-0.74), and gumminess (P<0.01), but not to WBSF (P>0.05, r=-0.56). Overall acceptability followed the same trend. Overall acceptability was negatively correlated to hardness (P<0.05, r=-0.72), cohesiveness (P<0.05, r=-0.71), and gumminess (P<0.05, r=-0.76), but was not significantly correlated to WBSF (P>0.05, r=-0.44).

Ostrich meat in place of beef or chicken meat can successfully be used in the production of a traditional product (döner) without significantly affecting its eating quality. Ostrich meat was found to produce better döners in terms of sensory properties, and textural characteristics, which further justifies its use in processed meat products although ostrich meat döners were found to have the highest fat content. Moreover, cholesterol content of the ostrich meat döners was the lowest making it as an alternative fast food for the people who suffer from cholesterol related diseases. Our results suggest that ostrich meat döners will be a novel product and will have a promising future in the fast food sectors provided that use of ostrich meat in processed meat products become a common practice in the meat industry.

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RESEARCH ARTICLE

Effect of Width and Depth of Bell Drinker and Sex on Fattening Performance and Carcass Characteristics of Pekin Ducks

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Summary

The aim of this study was to determine the effects of width and depth of bell drinker and sex on fattening performance and carcass characteristics of Pekin ducks. A total of sixty male and sixty female ducklings (Star 53 H.Y., Grimaud Freres) were used. Each sex group was randomly allocated into two bell drinker groups (narrow bell drinker with 47 mm water depth and 48 mm water width and wide bell drinker with 78 mm water depth and 90 mm water width) each of 30 ducklings. Groups were located in floor pens of 170 x 94 cm width x length, each of 6 chicks, in a naturally ventilated house. Sex affected the hatching weight (P<0.01), total body weight gain (P<0.001), feed intake (d 22 to 42 and d 1 to 42, P<0.05), feed conversion ratio (d 1 to 21, P<0.05), slaughter weight (P<0.001) and percentages of hot carcass (P<0.05). Wide bell drinker increased the body weight gain (d 22 to 42 and d 1 to 42, P<0.001), feed efficiency (d 22 to 42 and d 1 to 42, P<0.01), slaughter weight (P<0.001) and percentages of breast and wings (P<0.01), however decreased the percentages of skin subcutaneous fat (P<0.01). More width and depth bell drinker was positively contributed to the fattening performance in rearing of Pekin ducks.

Keywords: *Pekin duck, Bell drinker, Sex, Fattening performance, Carcass characteristics*

Pekin Ördeklerinde Farklı Genişlik ve Derinlikteki Askılı Sulukların ve Cinsiyetin Besi Performansı ve Karkas Özelliklerine Etkisi

Özet

Bu çalışmanın amacı Pekin ördeklerinde farklı genişlik ve derinlikteki askılı sulukların ve cinsiyetin besi performansı ve karkas özelliklerine etkisini belirlemektir. Çalışmada toplam 60 erkek ve 60 dişi palaz (Star 53 H.Y., Grimaud Freres) kullanılmıştır. Her cinsiyet grubu her birinde 30 palaz olacak şekilde rastgele iki farklı askılı suluk grubuna (47 mm su derinliği ve 48 mm su genişliğine sahip dar askılı suluk ve 78 mm su derinliği and 90 mm su genişliğine sahip geniş askılı suluk) ayrılmıştır. Çalışma 170 x 94 cm (genişlik x uzunluk) boyutlarındaki yer bölmelerinde 6 palaz bulunacak şekilde doğal havalandırmalı kümeste yapılmıştır. Cinsiyetin çıkım ağırlığı (P<0.01), toplam canlı ağırlık artışı (P<0.001), yem tüketimi (22 ile 42. günler arasında ve 1 ile 42. günler arasında, P<0.05), yemden yararlanma oranı (1 ile 21. günler arasında, P<0.05), kesim ağırlığı (P<0.001) ve sıcak karkas randımanı (P<0.05) üzerine etkisi önemli bulunmuştur. Geniş askılı suluklar canlı ağırlık artışını (22 ile 42. günler arasında ve 1 ile 42. günler arasında, P<0.001), yemden yararlanmayı (22 ile 42. günler arasında ve 1 ile 42. günler arasında, P<0.01), kesim ağırlığını (P<0.001) ve göğüs ile kanat yüzdelerini (P<0.01) artırırken, deri ve deri altı yağ yüzdelerini azaltmıştır (P<0.01). Pekin ördeği yetiştiriciliğinde derinlik ve çapı daha fazla olan askılı sulukların kullanılması besi performansını olumlu yönde etkilemiştir.

Anahtar sözcükler: *Pekin ördeği, Askılı suluk, Cinsiyet, Besi performansı, Karkas özellikleri*

INTRODUCTION

Genetic selection and improvements in nutrition and husbandry techniques have made the shorter growing periods in Pekin ducks [1]. Within the years the final market weight has increased from 2400 g at 56 d of age [2] to nearly 4000 g within a 43 d growing period [3]. Also the proportion of breast muscle in the carcass increased from 9% [2] to 16.6% [4].

In recent years increasing attention has been focused on animal welfare. The Council of EUROPE [5] recommends

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that ducks should also be able to dip their heads in water and spread water over their feathers. Ducks are waterfowl, but they grow under intensive system without water for swimming. They do not do their natural behavior in intensive system. In general intensive system offer advantages such as controlled conditions and lower environmental impact, but they may also have disadvantages for the animals' welfare, the main concern being the lack of access to bathing water [6]. Studies have shown that easily accessible open water (in the form of troughs, baths or showers) seems to be beneficial for the welfare, including the health, of the birds and that it encourages the display of natural bathing behaviours [3,7]. Erişir et al.[8] determined that the Pekin ducks reared with swimming pool reached higher live weights than those with nonswimming pool and effects of swimming pool on carcass characteristics were not statistically significant in their study. However in an intensive system access to pool had a negative effect on duck body weight. The authors claimed that open water in intensive system resulted in negative environmental consequences such as increased ammonia concentration and poor litter quality. This problem should be solved by constructed drainage area to minimize contamination of bedding with excess water [3] or by used different width and depth of drinkers. For duck production, nipple or narrow bell drinkers are used generally in intensive system. But nipple and narrow bell drinkers do not provide an opportunity for ducks to immerse any parts of their body [3]. Therefore width and depth of bell drinker is important for the natural behaviour. Wide bell drinkers may positively affect the basic duck behaviour due to providing an opportunity immerse any parts of their body. However, available literature on the topic provides no information regarding the effects of bell drinker systems according to the different water depth and width on growth and carcass characteristics in male and female Pekin ducks. Therefore the aim of this study was to determine the effects of width and depth of bell drinker systems on growth and carcass characteristics in male and female Pekin ducks.

MATERIAL and METHODS

This study was approved by Ankara University Animal Care and Use Committee (2011/112/423). Sixty male and sixty female ducklings (Star 53 H.Y., Grimaud Freres) were obtained from the local hatchery (Köy-Tav, Turkey). Each sex group was randomly allocated into two bell drinker groups (narrow bell drinker with 47 mm water depth and 48 mm water width and wide bell drinker with 78 mm water depth and 90 mm water width) each of 30 ducklings. Treatments were subdivided into 5 replicates of 6 chicks each, located in floor pens of 170 x 94 cm width x length in a naturally ventilated house. Each pen had wood shavings litter. During the first week each pen was equipped with one bell drinker and one chick feeder and the other weeks each pen was equipped with one bell drinker and one hanging suspended feeder. Birds were fed with a starter diet from 1 to 21 day of age (2830 kcal/kg metabolizable energy and 18.2% crude protein) and grower diet from 22 to 42 day of age (2720 kcal/kg metabolizable energy and 17.3% crude protein). Food and water were offered *ad libitum* during the experiment. Birds were provided with continuous light. Fattening duration was 42 days.

Ducklings were weighed at the beginning of the experimental period and weekly to determine body weight and body weight gain by pen. Feed intake was recorded weekly and expressed as g per duck per week and the feed conversion ratio was calculated as g feed per g body weight gain. After final weighing at 42 day of age, feed was removed 6 h prior to slaughter. Slaughtering was conducted by a commercial slaughterhouse. Slaughter weight was determined before slaughtering. Hot carcass, gizzard, liver and heart were weighed. These weights were also expressed as percentages of slaughter weight. The carcasses were stored at 4°C for 24 h by hanging. Cold carcass weights were recorded and were expressed as percentage of slaughter weight as cold carcass yield. Each carcass was cut into the neck and wings with skin and legs and breast without skin and subcutaneous fat and weighed. Abdominal fat and total of skin with subcutaneous fat were separately weighted. They were expressed as a percentage of cold carcass weight.

Statistical analyses were performed using the software package SPSS for Windows (SPSS Inc., Chicago, IL). Data were tested for distribution normality and homogeneity of variance. A two-way ANOVA was used to determine the differences between sex and bell drinker groups as well as their interactions with respect to the studied parameters. When a significant difference was found among groups for post-hoc multiple comparisons, Duncan test was used [9]. A value of $P<0.05$ was considered statistically significant.

RESULTS

Duckling weight at hatch and body weight gain in Pekin ducks according to the drinker system and sex are summarized in the *Table 1*. In this study hatching weights were 50.3 g and 46.7 g in male and female ducklings, respectively. Type of bell drinkers affected the body weight gain from d 22 to 42 and from d 1 to 42 ($P<0.001$), however sex affected the body weight in all examined period ($P<0.01$). The feed intake and feed conversion ratio of groups are shown in *Table 2*. Sex also affected the feed intake (d 22 to 42 and d 1 to 42, $P<0.05$) and feed conversion ratio (d 1 to 21, $P<0.05$). Wide bell drinker decreased the feed conversion ratio (d 22 to 42 and d 1 to 42, $P<0.01$). As shown in *Table 3* and *4*, slaughter weight, hot carcass yield, relative organ weights and carcass characteristics of Pekin ducks were summarized. Sex affected the slaughter weight ($P<0.001$) and percentages of hot carcass ($P<0.05$).

Table 1. *Effects of drinker system and sex on duckling weight at hatch (DW) and body weight gain (BWG) of Pekin ducks (n=5)*
Tablo 1. *Pekin ördeklerinde suluk sistemi ve cinsiyetin çıkım ağırlığına ve canlı ağırlık artışına etkisi (n=5)*

Type of Bell Drinkers	Sex	DW (g)	BWG (g/duck) d 1 to 21	BWG (g/duck) d 22 to 42	BWG (g/duck) d 1 to 42
Narrow bell drinker	Female	46.5	1143	1790	2932
	Male	50.2	1344	1860	3203
Wide bell drinker	Female	47.0	1255	1893	3148
	Male	50.4	1312	2093	3405
Main effect mean					
Narrow bell drinker		48.3	1243	1825	3068
Wide bell drinker		48.7	1284	1993	3277
Female		46.7	1199	1841	3040
Male		50.3	1328	1976	3304
Pool SEM		0.59	12.53	17.89	15.97
Significance		P[1]			
Type of bell drinkers		NS	NS	***	***
Sex		**	***	**	***
Type of bell drinkers X Sex		NS	*	NS	NS

[1] *Statistical significance, *** P<0.001, ** P<0.01, * P<0.05,* ***NS:*** *Non-significant*

Table 2. *Effects of drinker system and sex on feed intake (FI) and feed conversion ratio (FCR) of Pekin ducks (n=5)*
Tablo 2. *Pekin ördeklerinde suluk sistemi ve cinsiyetin yem tüketimi ve yemden yararlanma oranına etkisi (n=5)*

Type of Bell Drinkers	Sex	FI (g/duck) d 1 to 21	FI (g/duck) d 22 to 42	FI (g/duck) d 1 to 42	FCR (g/g) d 1 to 21	FCR (g/g) d 22 to 42	FCR (g/g) d 1 to 42
Narrow bell drinker	Female	1845	4713	6558	1.62	2.63	2.24
	Male	1891	4901	6791	1.41	2.64	2.12
Wide bell drinker	Female	1809	4573	6382	1.44	2.43	2.03
	Male	1898	4763	6662	1.45	2.28	1.96
Main effect mean							
Narrow bell drinker		1868	4807	6674	1.51	2.64	2.18
Wide bell drinker		1854	4668	6522	1.45	2.35	2.00
Female		1827	4643	6470	1.53	2.53	2.13
Male		1894	4832	6726	1.43	2.46	2.04
Pool SEM		24.62	42.04	60.10	0.02	0.04	0.03
Significance		P[1]					
Type of bell drinkers		NS	NS	NS	NS	**	**
Sex		NS	*	*	*	NS	NS
Type of bell drinkers X Sex		NS	NS	NS	*	NS	NS

[1] *Statistical significance, ** P<0.01, * P<0.05,* ***NS:*** *Non-significant*

Wide bell drinker increased the slaughter weight (P<0.001) and percentages of breast and wings (P<0.01), however decreased the percentages of skin subcutaneous fat (P<0.01).

DISCUSSION

Hatching weight of 1-d-old ducklings was statistically different between sex group (P<0.01). Male ducklings were heavier than female ducklings at hatch and under all the examined weeks body weight gain of male ducklings were higher than female ducklings (P<0.01). This data demonstrate that the effects of hatching weights of different sex are noticeable when ducks reached to market weights. Also male ducklings consumed totally more food than females (P<0.05). This result is consistent with previous reports for body weight of male and female ducks [10-12]. Feed conversion ratio was only statistically significant (P<0.05) in the three weeks of rearing. This statistically difference might be explained that body weight gain in the first three weeks was higher in males than females.

Table 3. *Effects of drinker system and sex on slaughter weight, hot carcass yield and relative organ weights of Pekin ducks (n=30)*
Tablo 3. *Pekin ördeklerinde suluk sistemi ve cinsiyetin kesim ağırlığı ile sıcak karkas ve organ yüzdelerine etkisi (n=30)*

Type of Bell Drinkers	Sex	Slaughter Weight (g)	Hot Carcass Yield (%)	Liver (%)	Heart (%)	Gizzard (%)
Narrow bell drinker	Female	2768	70.9	1.71	0.72	3.05
	Male	3035	72.1	1,62	0.70	3.00
Wide bell drinker	Female	3004	72.0	1,69	0.69	2.95
	Male	3182	72.4	1,68	0.64	2,94
Main effect mean						
Narrow bell drinker		2901	71.5	1,66	0.71	3.00
Wide bell drinker		3093	72.2	1,68	0.66	2.94
Female		2886	71.5	1,70	0.70	3.00
Male		3109	72.3	1,65	0.67	2.94
Pool SEM		15.97	0.171	0.024	0.012	0.041
Significance		P[1]				
Type of bell drinkers		***	NS	NS	NS	NS
Sex		***	*	NS	NS	NS
Type of bell drinkers X Sex		NS	NS	NS	NS	NS

*[1] Statistical significance, *** P<0.001, * P<0.05,* ***NS:*** *Non-significant*

Table 4. *Effects of drinker system and sex on carcass characteristics of Pekin ducks (n=5)*
Tablo 4. *Pekin ördeklerinde suluk sistemi ve cinsiyetin karkas özelliklerine etkisi (n=5)*

Type of Bell Drinkers	Sex	Neck[1] (%)	Breast[1] (%)	Legs[1] (%)	Wings[2] (%)	Skin with Subcutaneous Fat (%)	Abdominal Fat (%)
Narrow bell drinker	Female	6.69	28.37	18.61	12.71	21.58	1.36
	Male	7.08	29.08	16.81	12.45	21.30	1.30
Wide bell drinker	Female	6.89	30.13	16.83	12.86	19.95	1.26
	Male	6.67	30.04	16.98	13.20	19.78	1.38
Main effect mean							
Narrow bell drinker		6.89	28.73	17.71	12.58	21.44	1.33
Wide bell drinker		6.78	30.09	16.90	13.03	19.86	1.32
Female		6.79	29.25	17.72	12.78	20.76	1.31
Male		6.88	29.56	16.90	12.82	20.54	1.34
Pool SEM		0.093	0.206	0.213	0.075	0.222	0.032
Significance		P[3]					
Type of bell drinkers		NS	**	NS	**	**	NS
Sex		NS	NS	NS	NS	NS	NS
Type of bell drinkers X Sex		NS	NS	*	NS	NS	NS

*[1] Without skin and subcutaneous fat, [2] With skin, [3] Statistical significance, ** P<0.01, * P<0.05,* ***NS:*** *Non-significant*

In the first three week, the average body weight gains were statistically similar in different bell drinker systems; however, after three weeks of rearing ducklings used wide bell drinkers were higher weight gain than used narrow bell drinkers (P<0.001). This result showed that after the three weeks of rearing, wide bell drinker system contributed to higher body weight gain of ducklings. Similarly İpek et al.[13] reported that broilers in the groups on round type drinkers reached higher body weight gain compared with those in the groups in which nipple type drinkers after the age of 3 weeks. O'Driscoll and Broom [3] examined the effects of different access to water (narrow bell drinker, trough and bath) on body weight. They showed that body weight of Pekin ducks at 43 d in the trough treatment was significantly higher (P<0.05) than in the narrow bell drinker treatment. In our study feed intakes in all examined weeks were statistically similar. Drinking system affected the feed conversion ratio from d 22 to 42 and from d 1 to 42 (P<0.01). And wide bell drinker positively affected the feed efficiency in these weeks. These differences may be due to the less food consumption but higher weight gain in reared with wide bell drinkers than the other group. Food is partitioned between body functions, including maintenance, growth and health. In healthy animals, 10%

of food ingredients consumed are used to maintain health. In negative conditions, most of the consumed food is used to cope with unpleasant conditions [14]. In the present study, this condition may be explained that wide drinker system positively affects the weight gain and feed efficiency. Mortality was not seen in examined groups during the experiment.

Slaughter weight and hot carcass yield were affected by the sex. These characteristics were higher in male ducklings. Wide bell drinkers increased the slaughter weight. Percentages of liver, heart and gizzard were not affected from the examined parameters.

Sex did not affect the examined percentages of carcass parts including neck, breast, legs, wings, abdominal fat and skin with subcutaneous fat. Similarly Sarı et al.[15] concluded that breast percentages were not different in sex groups. Slaughter age may be important in these parts. Bochno et al.[11] reported that the deposition of fat and skin increased significantly up to 10 weeks of age (from 98 g in 2-week-old birds to 843 g in 10-weeks-old ones). However types of bell drinkers only affected the percentages of breast, wings and skin with subcutaneous fat. Percentages of breast and wings were increased, but skin with subcutaneous fat was decreased in ducklings with rearing wide bell drinkers. Leg muscles of hatching duckling are more developed than breast muscles. Leg muscles grow very quickly to the age of 2 weeks, and breast muscles to about 7 weeks [11,16,17]. A rapid growth rate of wing muscles in Pekin ducks to about 5 weeks is worth noting [11]. These knowledge explained our results. Results of carcass parts were similar with results in body weight gain and feed efficiency. Wide bell drinker was important after the three weeks of rearing according to our results of body weight gain and feed efficiency therefore the percentages of legs were not statistically different in bell drinking groups because the first growing part is legs. The second growing parts are breast and wings, therefore these parts were different bell drinker groups. However, these growing parts may be affected by slaughter age. The interactions of sex and type of bell drinker were found for body weight gain and feed conversion ratio in the first three week and for the percentage of legs ($P<0.05$).

As a conclusion, we said that sex and bell drinker system are important for some examined parameters. Sex affected the hatching weight, body weight gain, feed intake (d 22 to 42 and d 1 to 42), feed conversion ratio (d 1 to 21), slaughter weight and the percentages of hot carcass. Wide bell drinker increased the body weight gain, feed efficiency (d 22 to 42 and d 1 to 42), slaughter weight and the percentages of breast and wings and decreased the percentages of skin with subcutaneous fat. Wide bell drinker system was positively contributed to the fattening performance in both sex. If pool were not used in duck production, wide bell drinkers should be used. Further studies should be carried out to investigate the different access to water on production, behaviour, welfare and economics.

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RESEARCH ARTICLE

Distribution of Different Alleles of Aromatase Cytochrome P450 *(CYP19)* and Melatonin Receptor 1A *(MTRN1A)* Genes among Native Turkish Sheep Breeds [1]

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Summary

In this study, 257 animals from eight native Turkish sheep breeds (Akkaraman, Dağlıç, Gökçeada, İvesi, Karacabey Merino, Karayaka, Kıvırcık, Sakız) were investigated for three SNPs located at *CYP19* and *MTRN1A* genes which are important for reproductive traits. The A→G transition within *CYP19*, the C→T and G→A transitions within *MTRN1A* were investigated by using PCR-RFLP method. To analyze *CYP19* locus *DraI*, for *MTRN1A* locus *MnlI* and *RsaI* restriction enzymes were used. Two alleles were found for all the loci investigated. These alleles named as A and G for *CYP19*, M and m for *MTRN1A-MnlI,* and R and r for *MTRN1A-RsaI*. A and m alleles were predominant at *CYP19* and *MTRN1A-MnlI* loci, respectively. While R allel was prevalent in majority of breeds investigated, r allele was predominant in Sakız, Karacabey Merino, and Karayaka breeds for *MTRN1A-RsaI.* UPGMA test results show that the allele frequencies distributions of these loci can be used for calculating genetic distance between breeds.

Keywords: *Sheep, Reproduction, Polymorphism, CYP19, MTRN1A, Genetic distance*

Aromatase Cytochrome P450 *(CYP19)* ve Melatonin Receptor 1A *(MTRN1A)* Genlerinin Farklı Allelerinin Türkiye Yerli Koyun Irkları Arasındaki Dağılımı

Özet

Bu çalışmada Türkiye yerli koyun ırklarından (Akkaraman, Dağlıç, Gökçeada, İvesi, Karacabey Merinosu, Karayaka, Kıvırcık, Sakız) 257 hayvan üreme özellikleri açısından önem taşıyan *CYP19* ve *MTRN1A* genlerinde bulunan üç adet TNP bakımdan incelenmiştir. *CYP19* lokusunda bulunan A→G transisyonu ile *MTRN1A* lokusunda bulunan C→T ve G→A transisyonları PCR-RFLP metodu kullanılarak incelenmiştir. *CYP19* lokusunu analiz etmek için *DraI, MTRN1A* lokusunu analiz etmek için *MnlI* ve *RsaI* restriksiyon enzimleri kullanılmıştır. İncelenen tüm lokuslar için iki allel bulunmuştur. Bu alleler *CYP19* lokusu için A ve G *MTRN1A-MnlI* lokusu için M ve m, *MTRN1A-RsaI* lokusu için R ve r olarak isimlendirilmiştir. *CYP19* ve *MTRN1A-MnlI* lokusları için sırasıyla A ve m alleleri predominanttır. *MTRN1A-RsaI* lokusu bakımdan incelenen ırkların çoğunda R alleli yaygın iken, Sakız, Karacabey Merinosu ve Karayaka ırklarında r alleli predominanttır. UPGMA test sonuçları bu lokusların allel frekans dağılımlarının ırklar arasındaki genetik mesafenin hesaplanmasında kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Koyun, Üreme, Polimorfizm, CYP19, MTRN1A, Genetik mesafe*

INTRODUCTION

Reproduction is an economically important complex trait influenced by many environmental and genetic components. Classical selection methods for complex composite traits such as reproduction do not result in conclusive genetic improvement. In this sense a gene has major affect can be used for selection criteria for this kind of complex traits to obtain more rapid genetic improvement. Reproductive traits are affected by many genes but a limited

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number of major genes associated with reproductive traits have been reported in sheep [1-4]. Aromatase cytochrome p450 (*CYP19*) and melatonin receptor 1a *(MTRN1A)* genes are two of the candidates genes for estrogen biosynthesis and seasonality, respectively. While aromatase cytochrome p450 (*CYP19*) is located in chromosome 7, melatonin receptor 1a *(MTRN1A)* is located chromosome 26 [5,6].

Understanding of genetic mechanism of seasonal reproduction in sheep is very important because this seasonal variation in fertility is an important factor limiting efficiency of sheep production. The photoperiod drives their reproductive cycle, which comprises a season of high sexual activity during short days and an anestrous season that occurs during long days. Melatonin is called the "hormone of darkness," because it is released by the pineal gland during the night. The pattern of secretion of melatonin provides information that is apparently 'read' by cells within the brain that have the appropriate receptors and controls reproductive function [7,8]. In mammals, two high-affinity melatonin receptor subtypes have been cloned and characterized, namely the MTNR1A and MTNR1B receptors, also known as the MT1 and MT2 receptors, respectively [9,10]. The MT1 receptor seems to be the only one involved in the regulation of reproductive activity [11,12]. Association studies carried out in the MTNR1A reveal different interesting results for estrus and fertility in the spring [13-15].

During late maturity, the ovaries are in a state of true anestrum. One of the predominant causes of true anestrum is a low level of ovarian estrogens. The aromatase cytochrome P450 enzyme plays an important role in estrogen biosynthesis by conversion of androgens to estrogens, and due to its critical function it is an important hormone both in the controlling of reproduction for male and female [16-18] and of the other traits like fat deposition and growth [19,20]. It maps to bands q24-q31 of the ovine chromosome 7 [5]. In sheep *Cyp19*, there are four different promoter regions (P1.1, P1.4, P1.5 and P2) that show organ-specific activities. As P2 is mainly active in ovarian granulosa cells [21] mutations occurred in these mentioned regions may be significant for obtaining valuable knowledge on reproductive traits. In a previous study some important relationships were determined between the C242T transition and some reproductive traits as litter weight, lambing interval, lambing age, reproductive performance, and maternal ability in Brazilian sheep breeds [22].

There are few studies carried out to light up reproductive characteristic of Turkish native sheep breeds up to present. Due to limited studies the native sheep breeds remain unperceived for reproductive characteristics.

In this study presence and the allelic frequency of A→G transition within P2 at position 113 of *CYP19*, C→T and G→A transitions within exon 2 at position 606 and 612 of *MTRN1A*, respectively were investigated in eight sheep breeds from Turkey. Mutations occurred such loci may be useful tolls as molecular markers in the selection programs. It must be put in mind that to reveal function of these kinds of mutations and to use them in animal production systems it should be understood genetic structure of populations for these loci. Hence understanding of genetic structure of Turkish native sheep breeds for these kinds of loci will contribute to establish organized selection, breeding and conservation programs.

MATERIAL and METHODS

Blood samples used for DNA isolation were collected from 257 animals from both sexes belonging to eight native Turkish sheep breeds and one crossbred population well adapted to local conditions. Total eight sheep breeds; Akkaraman (akk, n=28), Dağlıç (dgl, n=28), Gökçeada (gda, n=28), İvesi (ivs, n= 33), Karacabey Merino (mrns, n=37), Karayaka (kry, n=25), Kıvırcık (kvrck, n=39), Sakız (skz, n=39) were sampled from private farms, conservation farms and research institutes of government at different regions of Turkey.

Total DNA was extracted using a genomic DNA purification kit (K0512, Fermentas, Lithuania) according to the manual instructions. Spectrophotometric methods were used to determine DNA quality and quantity. PCR conditions were carried out according to Zsolnai et al.[23] and Martinez-Royo [4]. Primers and restriction enzymes used for PCR amplifications are given in *Table 1*. The restriction fragments were directly analyzed by electrophoresis in 2% and 2.5% agarose gels in 1× TAE buffer, stained with ethidium bromide, and visualized under UV light.

Direct counting was used to estimate phenotype and allele frequencies of the genetic variants for all loci. The chi-square test ($\chi 2$) was used to check whether the populations were in Hardy-Weinberg equilibrium. All calculations and the $\chi 2$ analyses were carried out using PopGene32 software [24]. Genetic relationships among populations were visualized in a dendrogram constructed by unweighted paired group cluster analysis (UPGMA), from a modified NEIGHBOR procedure implemented in PHYLIP version 3.5 software also using PopGene32. The UPGMA dendrogram of population was constructed based on Nei's genetic distance [25].

Table 1. *Primers sequences and restriction enzymes used in the study*
Tablo 1. *Çalışmada kullanılan primer dizileri ve restriksiyon enzimleri*

Locus	Primers (5′ ⟶ 3′)	R.E	Reference
CYP19-P2	ACAATGGGAGGCTCTGAGAATG GAAAAATTAGAAAATCCCCAAAA	*DraI*	[23]
MNRTA1	TCCCTCTGCTACGTGTTCCT GTTTGTTGTCCGGTTTCACC	*RsaI* *MnlI*	[4]

RESULTS

Two alleles and three genotypes were detected in overall breeds investigated for three loci. In order to genoyping *MTRN1A-Mnl*I and *MTRN1A-Rsa*I the nomenclature of Martinez-Royo was used [4]. To determine SNP 612 in *MTRN1A*, 237 bp PCR products were digested with *Mnl*I restriction endonuclease. M homozygote individuals have 237 and 13 bp fragments, the mm homozygotes have 170 bp, 67 bp, and 13 bp and the heterozygotes have four bands as 237 bp, 170 bp, 67 bp, and 13 bp (the 13 bp could not seen in the gels). The *RsaI* restriction enzyme was used to determinate SNP606 in the same PCR products of *MTRN1A*. RR genotype has 148 bp and 79 bp, and 23 bp (the 23 bp could not seen in the gels), rr genotype has 148 bp and 102 bp, and the heterozygote has all three bands 148 bp, 79 bp, and 102 bp. while the restriction enzyme site absent the allele is named as "r".

To 517 bp PCR fragment was digested by using *Dra*I to analyze CYP 19 P2 region. The G homozygote individuals have only 517 bp uncut fragment. Homozygotes A have 401 bp and 116 bp fragments and the heterozygotes have 517 bp, 401 bp and 116 bp fragments. The fragments obtained from agarose gel electrophoresis are given in the *Fig. 1*.

While in the *CYP19* and *MTRN1A-Mnl*I loci A and m alleles were predominant, respectively, allelic frequencies vary among breeds for *MTRN1A-Rsa*I alleles as given in *Table 2*.

Samples from eight sheep breeds were found to be in Hardy-Weinberg equilibrium at three loci investigated, except Dağlıç and Karayaka sheep breeds. Deviations from the HWE were significant at *CYP19* in Dağlıç breed ($P<0.01$) and *MTRN1A-Mnl*I in Karayaka breed ($P<0.05$).

UPGMA dendrogram obtained revealed two main genetic groups of breeds investigated *(Fig. 2)*.

a)CYP19-*Dra*I b) MNTR1A-*Mn*II c) MNTR1-*Rsa*I

Fig 1. Diagnostic PCR-RFLP fragments of loci investigated (a, b, c)

Şekil 1. İncelenen lokuslara ait PCR-RFLP parçalarının tanımlanması (a, b, c)

Table 2. *Distribution of allelic frequencies among the breeds investigated*
Tablo 2. *Allelik frekansların incelenen ırklar arasındaki dağılımı*

LOCUS	Alleles	BREEDS															
		Fat-tailed						Semi-fat tailed		Thin-tailed							
		ivs	χ^2	dgl	χ^2	akk	χ^2	skz	χ^2	mrns	χ^2	kvrck	χ^2	kry	χ^2	gda	χ^2
Cyp19	A	1	--	0.9286	8.14**	1		0.8462	1.17	0.8378	1.26	0.6795	2.00	0.9600	0.02	0.9286	0.12
	G	-		0.0714		-		0.1538		0.1622		0.3205		0.0400		0.0714	
MT1-*MnlI*	M	0.2576	0.67	0.1071	2.35	0.3214	0.46	0.2308	3.28	0.3108	1.17	0.2564	1.68	0.3200	5.13*	0.4821	0.08
	m	0.7424		0.8929		0.6786		0.7692		0.6892		0.7436		0.6800		0.5179	
MT1-*RsaI*	R	0.7273	2.12	0.6071	0.03	0.6429	1.63	0.4103	0.14	0.4189	0.06	0.5256	0.17	0.2400	3.40	0.7857	1.88
	r	0.2727		0.3929		0.3571		0.5897		0.5811		0.4744		0.7600		0.2143	

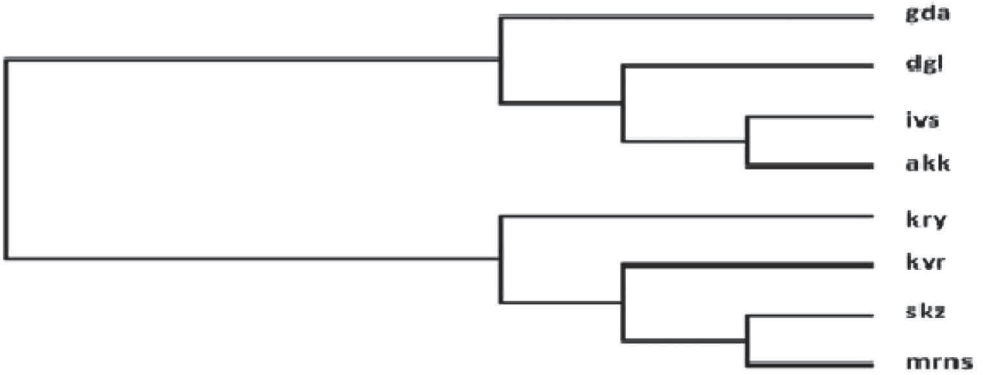

Fig 2. Genetic distance between populations investigated. Dendrogram based on Nei's [25] genetic distance (PopGene program UPGMA PHYLIP Version 3.5, modified NEIGHBOR method)

Şekil 2. İncelenen populasyonlar arasındaki genetik mesafe. Nei'nin [25] genetic mesafesine dayanan UPGMA dendrogramı (PopGene programı UPGMA PHYLIP Version 3.5, adapte edilmiş NEIGHBOR metodu)

Table 3. *Observed and Expected heterozygosity values for all loci in investigated breeds*
Tablo 3. *İncelenen ırklarda tüm lokuslar için gözlenen ve beklenen heterozigotluk değerleri*

LOCUS	BREEDS															
	Fat-tailed						Semi-fat tailed		Thin-tailed							
	ivs		dgl		akk		skz		mrns		kvrck		kry		gda	
	Obs-Het	Exp-Het	Obs-Het	Exp-Het	Obs-Het	Exp-Het	Obs-Het	Exp-Het	Obs-Het	Exp-Het	Obs-Het	Exp-Het	Obs-Het	Exp-Het	Obs-Het	Exp-Het
Cyp19	-	-	0.0714	0.1351	-	-	0.3077	0.2637	0.3243	0.2755	0.5385	0.4412	0.0800	0.0784	0.1429	0.1351
MT1-*MnlI*	0.3333	0.3883	0.1429	0.1948	0.5000	0.4442	0.4615	0.3596	0.4054	0.4343	0.3077	0.3863	0.6400	0.4441	0.5357	0.5084
MT1-*RsaI*	0.3030	0.4028	0.5000	0.4857	0.3571	0.4675	0.4615	0.4902	0.5135	0.4935	0.5385	0.5052	0.2400	0.3722	0.4286	0.3429

Observed heterozygosity for *CYP19* was the highest in Kıvırcık sheep breed and the lowest in Dağlıç sheep breed. Observed heterozygosity for *MTRN1A-Mnl*I was the highest in Karayaka sheep breed and the lowest in Dağlıç sheep breed. For the *MTRN1A-Rsa*I locus the higher value was observed in Kıvırcık breed and the lowest value was observed in Karayaka sheep breed *(Table 3)*.

DISCUSSION

In the present study while Native Turkish sheep breeds were investigated for *CYP19* P2 polymorphism for the first time, Sakız, Akkaraman and İvesi sheep breeds investigated for *MTRN1A Mnl*I and *Rsa*I polymorphisms by Seker et al.[26]. The rest of the breeds were analyzed for the first time for *MTRN1A Mnl*I and *Rsa*I polymorphisms, as well.

The A→G transition within P2 of *CYP19* has been reported in for the first time and the authors analyzed total seven breeds from Spain and Hungaria [23]. For all the breeds investigated, the A allele was found as predominant in the present study. While in the present study the frequencies of A allele were found between 1 and 0.3205, Zsolnai et al.[23] found the frequencies of A allele between 1 and 0.750. In the other study for determinate *CYP19* P2 polymorphism in Tiberian sheep population the *CYP19* P2 locus was found to be monomorphic [27]. The frequencies found for *CYP19* P2 locus were concordance with these previous studies [23,27].

Both *MTRN1A Mnl*I and *Rsa*I loci were found as polymorphic and frequencies for the alleles found were given in *Table 2*. While in the *MTRN1A-Mnl*I locus m alleles were predominat, allelic frequencies vary among breeds for *MTRN1A-Rsa*I alleles. The frequencies of *MTRN1A Mnl*I alleles obtained in the present study were similar to the frequencies reported by Notter et al.[13] and Kaczor et al.[28], but were disagreement with many reports for *MTRN1A Mnl*I polymorphism [4,14,26,29]. In the study carried out on Native Turkish sheep breed the M allele was also found predominant in the Sakız, Akkaraman and İvesi sheep breeds, inconsistency with the present study. On the other hand there is also some discordance between the allele's frequencies for *MTRN1A Rsa*I locus. In the present study frequency of r allele vary from 0.2143 to 0.7600. The results obtained by the other authors also vary breed to breed. For example while in the Columbia and Dorset sheep breeds the r allele frequencies were found 0.97 and 0.65, respectively [13,30] in the Sarda and Tisdale sheep breeds it found as 0.34 and 0.24, respectively [15,29]. These discordances can be explained in the differences in sampling strategies or nomenclature.

Interestingly the dendrogram obtained from analysis of the three loci is quite logical. While all of the majority of thin tail breeds are grouped together except Gökçeada sheep breed, the fat tail breeds are grouped together *(Fig. 2)*. This finding can be accepted as an evidence for utilities of these loci to breed separation.

Majority of breeds investigated in this study have been examined for *MTRN1A- MnlI* and *Rsa*I loci for the first time and for the *CYP19* P2 all of the breeds have been examined for the first time. Results of this study demonstrate that native Turkish sheep breeds have a high level of genetic variation in these three loci investigated. To provide better understanding of function of these mutations it should be needed further analysis to determinate their affects on productive traits by using phenotypic data. Further analysis at expression level it should be also carried out. Number of studies should be increased by using more animals from each breed to be clear the discordance between the allele distributions in *MTRN1A Rsa*I locus to reveal genetic structure of Turkish native sheep breeds for this locus.

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RESEARCH ARTICLE

Köpeklerin Ön Çapraz Bağ Lezyonlarının Tedavisinde "Tibial Plato Düzeltici Osteotomi" Yönteminin Kullanılması ve Sonuçlarının Değerlendirilmesi [1] [2]

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Özet

Bu klinik çalışmada, köpeklerde ön çapraz bağ kopuklarının (ÖÇB) tedavisinde kullanılan ekstrakapsüler tedavi yöntemlerinden Tibial Plato Düzeltici Osteotomi (TPDO) operasyon tekniğinde bir implant sistemi olan kilitli TPDO plakların klinik olgularda kullanılması ve yöntemin klinik ve radyolojik sonuçlarının değerlendirilmesi amaçlanmıştır. Çalışma materyalini, Ankara Üniversitesi Veteriner Fakültesi küçük hayvan kliniğine topallık şikayeti ile getirilen ve klinik, radyografik ve artroskopik muayeneyle ÖÇB kopuğu tanısı konan, değişik yaş ve cinsiyette 15 büyük ırk köpek oluşturdu. Tüm olgular postoperatif 0, 10, 25, 60, 90 ve 120. günlerde ilgili ekstremite yönünden klinik ve radyografik muayeneleri yapılarak TPDO plağı gevşemesi, implant yetersizliği ve gelişebilecek diğer komplikasyonlar yönünden izlendi. Ayrıca postoperatif muayene periyotlarında olguların ilgili ekstremitedeki topallık ve ağrı duyumları skorlanarak değerlendirildi. Çalışma materyalini oluşturan olguların TPDO operasyonu ve tibia proksimalindeki osteotomi hattının kilitli TPDO plakları ile yapılan fikzasyonunu izleyen sürelerde özellikle implant gevşemesi dahil olmak üzere herhangi bir komplikasyon gelişimi gözlenmedi.

Anahtar sözcükler: *Köpek, Kilitli TPDO plağı, Ön çapraz bağ, TPDO*

The Use and Interpretation of Tibial Plateau Leveling Osteotomy (TPLO) in Dogs in Cranial Cruciate Ligament Lesions

Summary

In this clinical study, utilisation of a newly described implant system which is called locking TPLO plates in TPLO surgery technique, one of the extracapsular treatment methods, for treatment of anterior cruciate ligament rupture in dogs in clinical cases and evaluation of clinical and radiological results of the method is aimed. The materials of this study are, 15 large breed dogs with different age and sex which were brought to Ankara University Faculty of Veterinary Medicine Small Animal Clinic with clinical evidence of rear limb lameness, in these dogs the anterior cruciate ligament rupture was diagnosed after clinical, radiological and arthroscopic examination The dogs which were included in the study, were examined clinically and radiographically on 0, 10th, 25th, 60th, 90th, and 120th days. All cases were examined for TPLO plate loosening, implant failure and other complications that may develop postoperatively. Also in the postoperative examination period, the limb lameness and pain sensations of the patients were scored. After TPLO surgery and fixation of the osteotomy line proximal to tibia with locking TPLO plates, patients were examinated in post operative period for possible complications, especially implant loosening, but no complication was observed.

Keywords: *Cranial cruciate ligament, Dogs, locking TPLO plate, TPLO*

GİRİŞ

TPDO, bütün ırklarda kullanılması avantajı ile gündeme gelmiş bir ekstrakapsüler tedavi tekniği olup köpeklerin 6 ayın üzerinde (proximal tibial büyüme plağı açısından) olması gereklidir. Ayrıca bilateral şekillenen ÖÇB kopuk-

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larında 3 hafta arayla her iki tarafa da uygulanabilir. Köpeklerde TPDO tekniği, ÖÇB'nin kısmi yırtıklarında aşırı stresi engellemek için, bazen de kranial tibial itme kuvvetinin (KTİK) büyüklüğünü azaltmak için uygulanır [1].

TPDO operasyonu için tibial plato açısı (TPA) çok önemlidir. TPA, tibianın kondilus medialisinin eğimi ile tibianın fonksiyonel aksisinden geçen açıdır [2]. TPA ölçümü, TPDO tekniğine başlamadan önce yapılması gerekli bir prosedürdür. Operasyon sırasında tibianın proksimal rotasyonuna izin verecek açının belirlenmesi, operasyon öncesi ölçüm ile hesaplanarak kararlaştırılmalıdır. ÖÇB yırtığı olan köpeklerde yapılan çalışmalarda, olmayanlara oranla daha büyük TPA tespit edilmiştir [3]. ÖÇB yırtığı olan ve olmayan Labrador Retriever'lar üzerinde yapılan benzer bir çalışmada ise TPA'larda bir fark tespit edilmediği bildirilmiştir [4]. Wilke ve ark.[5] Labrador Retriever ile Greyhounds cinsi köpeklerde geleneksel TPA ölçümü (lateral radyografi) ile ayakta TPA ölçümünü (ayakta duruş sırasında alınan radyografi) yapmışlardır. Çalışmanın sonucunda klinik yönden normal olan Greyhound'lar ve ÖÇB yırtığı olmayan Labrador Retriever'larda ayakta duruş ölçümlerinde bir fark tespit edilmemiştir. Geleneksel ölçümde ise ÖÇB yırtığı olan Labrador Retriever'larda olmayanlara göre daha küçük TPA tespit edilmiştir. Halen TPA'nın ÖÇB ile bağlantısı tam olarak açıklanamasa da, KTİK üzerine etkisi olduğu bilinmektedir [5].

TPDO operasyonun amacı; ayakta duruş sırasında oluşan KTİK'in eklem üzerindeki zarar verici etkisini nötralize etmek ve eklemde fonksiyonel stabilizasyon sağlamaktır. Operasyonda, KTİK'i kapsayan pasif kuvvetler onarılmaz (ÖÇB ve medial menisküsün kaudal kısmı), sadece tibial platonun düzeltilmesiyle ekstremitenin fleksor kaslarının aktif güçlerinin etkisi arttırılır (hamstring ve biceps femoris kasları) ve KTİK kontrol altına alınır.

Bu çalışmada, özel şekilli kilitli TPDO plakları kullanılarak yapılan operasyon prosedürünün ve postoperatif belirli periyotlarda klinik-radyografik sonuçların değerlendirilmesi amaçlanmıştır.

MATERYAL ve METOT

Çalışma materyalini; Ankara Üniversitesi Veteriner Fakültesi Cerrahi Anabilim Dalı Kliniği'ne getirilen ve yapılan muayenelerin ardından ÖÇB kopuğu tanısı konulan değişik yaş ve cinsiyette 15 büyük ırk köpek oluşturdu.

Çalışmada, rutin yumuşak doku ve ortopedik cerrahi setlerine ek olarak 3.5 mm kilitli TPDO plak seti, power drive matkap ve testere ünitesi (Synthes USA, Paoli, PA) kullanıldı. Çalışmaya dahil edilen olguların hasta sahiplerinden alınan anamnez bilgileri ve klinik olarak hastanın duruş, oturuş ve yürüyüş şekillerinin izlenmesinden sonra hastalar klinik muayeneye alındı. Klinik muayeneleri sırasında çapraz bağ kopuğunu belirlemek için öne çekmece hareketi ve tibial kompresyon testleri uygulandı. Olgularda radyografiler kraniokaudal (CrCd) pozisyonda ve TPA açıları ölçümü için lateral yatar pozisyonda ve sedasyon uygulandıktan sonra mediolateral (ML) pozisyonda elde edildi. Köpeklerde sedasyon sağlamak için IM yolla medetomidin HCl (Domitor 100 µg/kg canlı ağırlık) uygulandı. Radyografi alımı sırasında sedasyonun yetersiz kaldığı durumlarda anestezi protokolüne ketamine HCl (Ketalar 10 mg/kg) ile devam edildi.

Tüm hastalara artroskopi uygulaması yapıldı *(Şekil 1)*. Hasta sırtüstü pozisyonda yatırıldı, ilgili ekstremitenin ve genu ekleminin etrafı serviyetlerle sınırlandırıldı. Artroskopiden 10-15 dakika önce eklem içine 2.5 mg/kg dozunda bupivacain hidroklorid (Marcaine®, Astra Zeneca) epinefrinle (Adrenalin®, Galen İlaç) birlikte uygulandı. Olgularda artroskopik giriş yapılmadan önce eklem içi 10-15 ml Steril Ringer Laktat solüsyonu verilerek eklemin gerginliği sağlandı. Daha sonra eklem içine tekniğine uygun olarak girildi. Lateral femur kondilusu, lateral menisküs, medial tibia platosu interkondiler boşluk, ÖÇB, arka çapraz bağ (AÇB) ve sinovial membran değerlendirildi. Medial yaklaşımla medial femur kondilusu, medial menisküs, menisküslere ait ligament, medial tibia platosu, sinovial membranın muayenesi yapıldı. Artroskopik muayene sırasında eklem içi ringer laktat solüsyonu ile devamlı yıkandı. Femoro-patellar eklemde medial trohlear sınır, lateral trohlear sınır ve suprapatellar poş muayene edilerek inceleme tamamlandı. Tüm artroskopik ekipman eklemden çıkarıldıktan sonra deriye yapılan ensizyon 2/0 ipek iplikle basit ayrı dikiş uygulaması ile kapatıldı. Artroskopiden sonra hastayı uyandırmak amacıyla atipemazol hidroklorür (Antisedan 100 µ/kg canlı ağırlık) uygulandı.

Artroskopik muayeneyle ÖÇB kopuğu bulunduğu teyit edilen olguların sağaltımı için randevu verildi. Analjezi amacıyla tüm hastalara operasyondan yaklaşık 30 dk önce 0.2 mg/kg (0.4 ml/10 kg) dozda, tek doz olarak SC meloksikam (Maxicam®, 5 mg/50 mL, Sanovel) ve antibiyoterapi amacıyla 22 mg/kg dozda IV sefazolin sodyum (Sefazol®, 500mg IV, Mustafa Nevzat) uygulandı. Anestezinin indüksiyonu için 0.5 mg/kg diazepam (Diazem Ampul® IM/IV, 10 mg/2 mL, Deva) uygulamasını takiben entübasyona yetecek miktarda propofol (Propofol Enj. Emülsiyon® IV, 200 mg/20 mL, Abbott) uygulandıktan sonra olgular uygun boyuttaki kaflı endotrakeal tüple entübe edildi. Spontan ventilasyonda %2-3'lük izofloran ile (Isoflurane®, 100 mL Abbott) anestezinin devamı sağlandı. Hasta uygun pozisyonda masaya yatırıldı. Proksimal tibianın radial kesisinden önce medial artrotomi yapılarak eklem içinin muayenesi yapıldı ve ÖÇB kalıntıları uzaklaştırıldı ayrıca menisküsler tekrar kontrol edildi. Kemik kesisini yapabilmek için proksimal tibianın medial yüzeyine ulaşıldı. Kemiğin medial yüzeyine jig uygulaması yapıldı. Sonra proksimal tibia radial osteotomileri gerçekleştirildi. Operasyonlarda 3.5 mm kilitli TPDO plak ve vidaları kullanıldı *(Şekil 2)*. Hastalara postoperatif antibiyoterapi

amacıyla 7 gün süreyle SC enrofloksasin (Baytril K ®, 20 mL enj, flakon, Bayer) ve analjezik olarak da 3 gün süreyle SC meloksikam uygulandı.

Operasyon sonrası olgularda 10 gün süre ile operasyon yarasının korunması ve ilgili ekstremitenin hareketlerinin sınırlandırılması amacıyla Robert-Johns koruyucu bandaj uygulaması yapıldı. Bütün olgularda 10, 25, 60, 90, 120. günlerde klinik ve radyolojik muayeneleri tekrarlandı *(Şekil 3)*. Postoperatif 10. gün yapılan klinik muayenede bandaj açılarak operasyon yarasının kontrolü yapıldı, hareket kısıtlaması önerildi. Postoperatif muayene periyotlarında ilgili ekstremitedeki ağrı ve topallık değerlendirildi. Ağrı değerlendirmesi 4 değişkenli bir skorlama sistemiyle yapıldı: 1 (Bacağın manüplasyonunda ağrı yanıtı yok), 2 (Hafif, normal eklem hareketiyle manüplasyona izin verir, fakat başını çevirerek veya bacağını çekerek ağrı hissettiğini belli eder), 3 (Orta, normal eklem hareketiyle manüplasyona izin vermez, Skor 2'deki gibi ağrısını belli eder), 4 (Şiddetli, bacağın manüplasyonuna izin vermez) [6]. Hastaların topallık değerlendirmeleri ise Mükemmel (Çok iyi, topallık olmadan basabilme), İyi (Egzersiz sonrası hafif topallık), Orta (Hafif veya orta şiddetli topallık fakat ekstremite üzerine tutarlı yükleniş), Zayıf (Kötü, aralıklı veya sürekli olarak yüklenmenin olmadığı bir topallık) olmak üzere 4 değişken kullanılarak yapıldı [7].

Hastalar postoperatif kontrol günlerinde; tibia kemiği proksimalinde oluşturulan radial kesi hattındaki kemiksel kaynama, kortikal devamlılık, kallusun varlığı, primer ve sekonder redüksiyon kaybı, implant yetersizliği bulunup bulunmadığı, gecikmeli kaynama, kaynama yokluğu ve osteomyelitis yönünden klinik ve radyografik olarak değerlendirildi.

BULGULAR

Çalışma materyalini; 4 kangal çoban köpeği, 3 melez ırk, 2 golden retriever, 1 sibiryan husky, 1 alman çoban köpeği,1 boxer, 1 pitbull, 1 cani corsa, 1 rotweiller ırkı olmak üzere toplam 15 köpek oluşturdu. Olguların 5'i dişi ve 10'u erkek olup yaşları 7 ay ile 7 yıl arasında idi (ortalama 36.5 ay). Vücut ağırlıklarının, 24 ile 45 kg (ort. 32.5 kg) arasında olduğu belirlendi. Hasta sahiplerinden alınan anemnez sonrası oluşum nedenlerinin çoğunlukla direkt travmatik etkiler (yoğun egzersiz ve sportif aktivite, trafik kazası) olduğu belirlendi. Olguların 3'ünde (olgu 1, 2, 15) ise neden belirlenemedi.

Muayene edilen tüm olgularda öne çekmece hareketi pozitif olarak bulundu. Tibial kompresyon testi radyografi alımı sırasında uygulandı ve sonuçlar açısından daha şüpheli bulundu. Testlerde şüpheye düşülen olgularda testler sağlam genu eklemi ile karşılaştırmalı olarak yapıldı ve ayrıca anestezi uygulaması sonrasında tekrarlandı. Bir olguda (olgu 10) ÖÇB kopuğu ile birlikte lateral kollateral bağ kopuğu tespit edildi. Olguya TPDO operasyonu ile birlikte lateral kollateral bağ rekonstrüksiyonu operasyonu uygulandı.

Değerlendirilen her olgu için elde edilen TPA açıları ve tibia proksimal kesisinde uygulanan rotasyon oranları *Tablo 1*'de sunulmuştur. Hastaların postoperatif dönem TPA açısı ölçümleri *Tablo 2*'de verilmiştir.

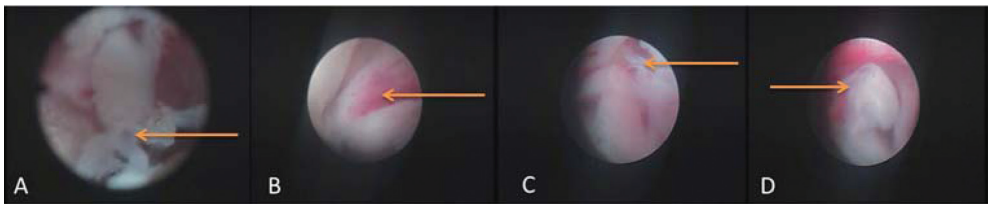
Olguların hiçbirinde anestezi komplikasyonu ile karşılaşılmadı. Ayrıca operasyon yarası komplikasyonu ve postoperatif enfeksiyon gelişimine de rastlanmadı. Olgularda implant yetersizliği, implant gevşemesi, vida migrasyonu, kemik enfeksiyonu, kaynama gecikmesi, kaynama yokluğu ve kesi hattında redüksiyon kaybına ilişkin bir komplikasyona da rastlanmadı.

TARTIŞMA ve SONUÇ

Köpeklerde, ÖÇB lezyonlarına oldukça sık rastlanmaktadır [8-13]. ÖÇB kopmaları sonrasında gelişen instabilite ve kronik dönemde oluşan osteoartritis lezyonları, köpeklerde arka ekstremite topallıklarının en yaygın nedenlerinden biridir [10].

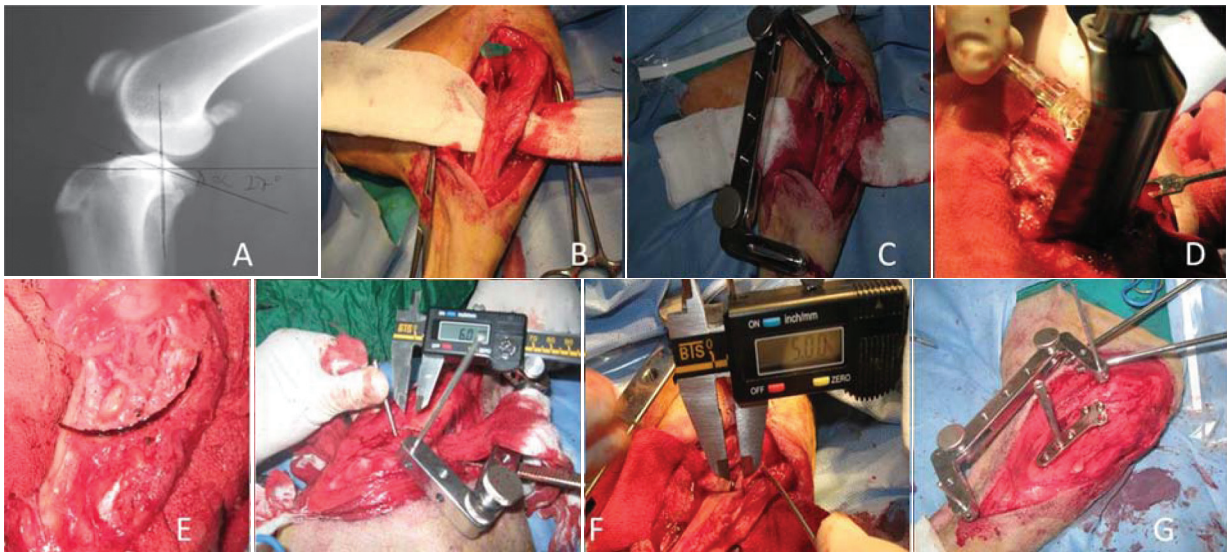
Çapraz bağ travmalarının tanısında klinik, radyolojik ve artroskopik bulgulardan yararlanılabilir. ÖÇB kopuklarının klinik tanısında "öne çekmece hareketi testi" önemli bir klinik tanı yöntemidir [9-11,13-15]. Ayrıca "tibial kompresyon" testi de çapraz bağ lezyonlarının tanısında klinik muayene yöntemlerinden biri olarak bildirilmiştir [14-17].

Çalışma materyalini oluşturan 15 büyük ırk köpekte literatür bilgileri doğrular yönde klinik, radyolojik ve artroskopik muayeneler sonrasında ÖÇB lezyonu belirlendi.



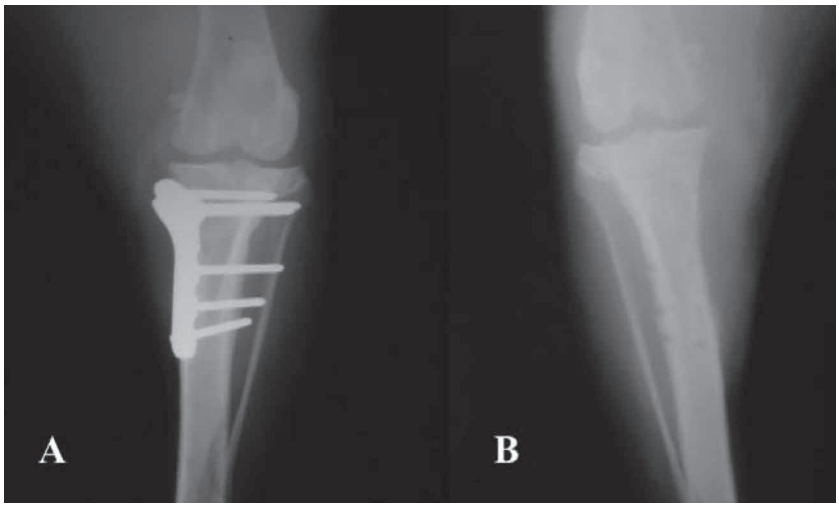
Şekil 1. Çeşitli olgulara ait artroskopik görünümler. **A-** Olgu no. 5. ÖÇB'de tam ruptur ve sinovitis, **B-** Olgu no. 6. Osteokondritis, **C-** Olgu no. 8 ÖÇB'de tam ruptur ve belirgin sinovitis, **D-** Olgu no. 15 ÖÇB'de tam ruptur

Fig 1. Arthroscopic images of different cases. **A-** Case no. 5. Complete rupture of anterior cruciate ligament and synovitis, **B-** Case no. 6. Osteochondritis, **C-** Case no 8. Complete rupture of anterior cruciate ligament and pronounced synovitis, **D-** Case no 15. Complete rupture of anterior cruciate ligament



Şekil 2. Operasyon aşamaları. **A-** Referans noktaların belirlenmesi ve çizgilerle birleştirilmesinden sonra TPA açısının ölçülmesi, **B-** Tibianın lateral yüzeyindeki dokuları korumak için tampon uygulaması, **C-** Tibianın medial yüzeyine jig uygulaması, **D-** Kesi sırasında oluşan ısının yumuşak dokularda ve kemikte olası yıkılmayıcı etkisini azaltmak için bölgenin serum fizyolojikle yıkanması, **E-** Döndürme oranının belirlenmesi için kesi hattının her iki tarafına Kirschner pinlerinin yerleştirilmesi, **F-** Rotasyon pininin yerleştirilmesi sonrasında rotasyon yapılması ve rotasyon derecesinin ölçülmesi, **G-** Özel şekillendirilmiş kilitli TPDO plağında tibianın distal fragmanında kilitli vida uygulanma yeri

Fig 2. Stages of the operation. **A-** Measuring the TPA angle after determination of reference points and combining with lines, **B-** Pad application to protect tissues of the lateral surface of the tibia, **C-** Jig application to the medial surface of the tibia, **D-** Washing the area with saline to reduce the potential destructive effects to the soft tissues and bone from incision heat, **E-** Placing Kirschner wires on both sides of the incision line to determine the rate of rotation, **F-** After placement of the rotation pin, rotating and determining the degree of rotation, **G-** Locked screw application location with specially shaped TPLO plate in distal fragment of the tibia



Şekil 3. Olgu 8. **A-**Postoperatif 140. gün CrCd radyo-grafik görünümü, **B-** Postoperatif 140. günde radyo-grafik görünüm

Fig 3. Case 8. **A-** CrCd radiographic appearance on the postoperative 140th day, **B-** Radiographic appearance on postoperative 140th day

Çalışmada, klinik muayeneler sırasında "öne çekmece hareketi" ve "tibial kompresyon" testi uygulandı. Uygulanan her iki testin, özellikle "tibial kompresyon" testinin ML yönde radyografi ile birleştirilmesi eklem stabilitesinin kaybını açıkça göstermekte idi. Muayene edilen tüm olgularda öne çekmece hareketi pozitif olarak bulundu. Bu çalışmada, öne çekmece hareketi testinin klinik sonuçlarının daha değerlendirilebilir bulunduğu belirlendi. Tibial kompresyon testi ise sonuçlar açısından daha şüpheli bulundu. Klinik değerlendirmelerimize göre, öne çekmece hareketi testinin, uygulanabilirliğinin pratik ve sonuçlarının daha kabul edilebilir bir test olduğu gözlemlendi.

Tibial plato açısının hesaplanması ile köpeklerin çapraz bağ kopuklarına predispoze olup olmadıkları belirlenerek gerekli önlemlerin alınmasıyla morbidite azaltılabilir [3,5,18]. TPA hesaplaması, TPDO ve Üçlü Tibia Osteotomisi (ÜTO) gibi diz eklemi cerrahisinde hem preoperatif ölçülerek operasyon için istenen en uygun eksenin açısının bulunmasında, hem de operasyon sonrası ölçülerek kontrol

Tablo 1. *Olgularda TPA açısı ölçümleri ve rotasyon oranlarına ait veri*
Table 1. *TPA angle measurements and rotation rates data of patients*

Olgu No	Sol Arka Ekstremite TPA	Sağ Arka Ekstremite TPA	Tibial Rotasyon Oranı (mm)
1		13º	4.00
2	19º		6.00
3		22º	7.00
4		23º	7.50
5		27º	9.00
6	30º		10.25
7	24º		8.00
8		21º	6.75
9	23º		7.50
10		21º	6.75
11	20º		6.25
12	24º		8.00
13	23º		7.50
14	18º		5.50
15		29º	10.00

Radyografi alımında çok çeşitli metotlar kullanılmaktadır. Birinci radyografi alma tekniğinde hayvan sedasyona alınıp ardından yan yatırılarak tibianın tümünün radyografisinin alınmasına dayanmaktadır. Başka bir yöntemde radyografi alımı için benzer hazırlıklar yapıldıktan sonra tibianın boydan görüntüsü yerine röntgen ışınları önce genu eklemine odaklanıp radyografisi alındıktan sonra hayvan ya da masa hareket ettirilmeden tarsal ekleme odaklanıp ikinci bir görüntü alımı şeklinde yapılmaktadır [18]. Bu çalışmada, tanımlanan birinci radyografi elde etme yöntemi tercih edilerek, TPA ölçümü içi alınan ML radyografiler bu yöntemle alınmıştır. İkinci yöntemin tercih edilmemesinin nedeni ise bu yöntemde bacağı kaydırmadan görüntü elde etmenin oldukça zor olmasıdır.

Artroskopik olarak parsiyel ve tam çapraz bağ kopmaları belirlenebilir, aynı zamanda bu yöntemle dejeneratif değişiklikler, menisküs lezyonları ve sinovit bulguları da daha kolay ve şüpheye yer vermeyecek şekilde gözlenebilir [12,14,26]. Çalışmamızda, artroskopik bulgular ve operasyon sırasındaki gözlemler klinik muayene yöntemlerinde kullanılan testleri doğrulamıştır. Artroskopik mua-

Tablo 2. *Postoperatif radyografik muayene periyotlarında TPA açısı ölçüm sonuçları*
Table 2. *TPA angle measurement results on postoperative radiographic examination periods*

Olgu No	Gün					
	0*	7-10**	25	60	90	120
1	13°	5°	5°	5°	-	-
2	19°	6°	-	-	-	-
3	22°	5°	-	-	-	-
4	23°	5.5°	5.5°	5.5°	5.5°	5.5°
5	27°	6°	-	-	-	-
6	30°	6°	6°	6°	6°	6°
7	24°	5°	5°	5°	-	-
8	21°	6°	6°	6°	6°	6°
9	23°	6.5°	6.5°	-	-	-
10	21°	6°	6°	6°	6°	6°
11	20°	5°	5°	5°	5°	5°
12	24°	6°	6°	6°	-	-
13	23°	5°	5°	5°	5°	5°
14	18°	6°	6°	6°	6°	6°
15	39°	5°	5°	5°	5°	5°

amaçlı olarak kullanılır [19-23]. Ayrıca Fetting ve ark.'larına göre bu tür operasyonlarda başarılı bir prosedür için TPA'nın ölçülmesi şarttır [2]. Köpeklerde TPA'yı saptamak amacıyla tibianın proksimal bölümünün lateral radyografik görüntüsünden yararlanılır [24]. TPA ölçümü yaparken radyografik muayene sırasında pozisyon verilmesi oldukça önemlidir [25]. Bu çalışmada Reif ve ark.'larının [25] önerileri doğrultusunda, TPA ölçümleri, merkez X-ray ışın demetinin diz eklemi merkezine gelmesine dikkat edilerek yapıldı. Bu sayede doğru TPA ölçümleri elde edildi.

yenelerde olgular çapraz bağ lezyonları, eklem içi dejeneratif değişiklikler ve menisküs lezyonları yönünden değerlendirildi. Tüm olgularda tam çapraz bağ kopuğu belirlendi. Menisküs yırtılması ve lezyonuna ise rastlanmadı.

ÖÇB lezyonlarının operatif sağaltımında, eklem stabilitesinin yeniden oluşturulmasına yönelik çok sayıda yöntem tanımlanmıştır [12,15]. Operatif sağaltım yöntemleri intrakapsüler ve ekstrakapsüler girişim teknikleri olarak iki ana başlık altında toplanabilir [13,15,26]. Ekstrakapsüler

tekniklerde, uygulamalar eklem içine ulaşmadan eklem stabilizasyonunu sağlama prensibine dayanmaktadır. Burada lateral retinaküler imbrikasyon; ethylene tetrafluoroethylene, absorbe olmayan dikiş materyalleri (supramid, prolone), balıkçı misinası veya serklaj telleri ile "U" şeklinde eklem dışından dikiş uygulamaları [13,15,26-31] ya da kapitilum fibula'nın kraniale çekilmesi (fibula başı transpozisyonu) ve bununla oluşturulan kollateral lateral ligamentin gerginlik gücünden yararlanılmasına yönelik uygulamalar tanımlanmıştır [12,26,27,32-34]. Ekstrakapsüler stabilizasyon yöntemlerinin, eklem hareketlerini değiştirdiğinin ve genu ekleminin aksiyel rotasyonunda değişiklikler oluşturabildiğinin bilinmesine rağmen, 15 kg'a kadar olan köpekler tarafından iyi şekilde tolere edilebildiği ve yeterli eklem stabilitesinin sağlandığı bildirilmektedir [11,13,28,35].

Klasik ekstrakapsüler yöntemler genelde 20 kg'a kadar olan ırklarda kullanıldığı için 20 kg'ın üzerindeki olgularda yetersiz kalmıştır. Bu ağırlığın üzerinde kullanılan klasik ekstrakapsüler yöntemlerinde (sentetik dikiş materyaller ve serklaj teli ile yapılan stabilizasyon teknikleri) kullanılan materyalin kopma riski, periartiküler dokulara uygulanan aşırı gerilme kuvvetine bağlı tibia'nın içe rotasyon hareketini yapamaması, tibial rotasyon hareketlerinin sınırlandırılması, tibio-femoral eklem yüzüne, menisküslere, eklem kıkırdağına uygulanan kompresyonun artmış olmasına bağlı eklem kıkırdağının zarar görmesi ve osteoartritis şekillenmesi gibi gelişimler söz konusu olmaktadır [36].

Son yıllarda, tibial plato düzeyinden yapılan osteotomi ile kalçadan genu eklemine gelen fleksor güçlerin arttırılması ve eklemin stabilizasyonuna yönelik bir teknik tanımlanmıştır [36]. ÖÇB'de parsiyel veya tam rupturu olan köpeklerin sağaltımında TPDO etkili bir yöntemdir. TPDO'nun amacı, tibial platoda 5°'lik bir eğim sağlayarak AÇB ve dizin aktif bileşenleri (uyluk bölgesinde bulunan, genu ekleminde fleksiyona neden olan kaslar) ile KTİK'i etkili bir şekilde kontrol etmektir [37]. Köpek tibiasının proksimal ucunun medial kenarına uygulanmak üzere geliştirilmiş TPDO sabitlenmesini sağlayan yeni bir kilitleme plağı geliştirilmiştir. Bu plak daha önce kullanılan TPDO plaklarındaki bazı sınırlamaların üstesinden gelmek amacıyla tasarlanmıştır. Plak, 316L paslanmaz çelikten imal edilmiştir, sağ ve sol çeşitleri tibianın medial kenarına uygun şekilde tasarlanmıştır. Bunlara ek olarak, plağın başında kilitleyici vida başları kullanılarak tibial plato sabitlenmiştir. Bu nedenle, plağın kemik şeklini tam olarak alması önemli değildir. Ayrıca, vidaları bu plağa kilitleyerek redüksiyonun azalmasını engeller. Plağın gövdesinde, iki dinamik kompresyon ve bir kombine olmak üzere üç delik bulunur. Bu nedenle, kilitleyici vidalarla metafizeal fragment sabitlendikten sonra, standart vidalarla kırığa kompresyon yapılabilir. Plağın alt yüzeyi sınırlı temaslı dinamik kompresyon plaklarıyla aynı yapıya sahiptir, içe doğru çukur yüzey plağın kemikle temasını ve kan akımının engellenmesini azaltmayı amaçlar [37].

Bu klinik çalışmada kullanılan kilitli TPDO plaklarına ilişkin herhangi bir komplikasyon gelişimi gözlenmemiştir. Plağın eğimli yüzeyi tibianın proksimaline tam uyum sağlamakta ve redüksiyon kayıpları minimal düzeye inmektedir. Böylece plak uygulaması sırasında klasik TPDO plaklarında yapılması gereken plağın proksimal tibianın medial yüzeyine göre şekillendirilmesi işlemi ortadan kalkmış olmaktadır. Hatalı plak bükülmesine bağlı olarak gelişebilecek redüksiyon kayıpları ve hatalı uygulamalar ve en önemlisi de postoperatif komplikasyon gelişimi ve revizyon operasyonları en aza indirilmekte hatta ortadan kalkmaktadır. Bu çalışmada hiçbir olguda plak gevşemesi, hatalı redüksiyon uygulaması gözlenmemiş ve hiçbir olguda revizyon operasyonuna ihtiyaç duyulmamıştır. Çalışmanın özellikle iri yapılı, atletik ve hareketli hayvanlarda gerçekleştirildiği düşünülürse implant gevşemesinin görülmemesi kilitli plağın biyomekanik özelliklerine atfedilebilir.

TPDO cerrahisinin potansiyel komplikasyonları arasında enfeksiyon, zayıf kemik iyileşmesi, patellar ligamentin gerginliği, tibial krestin kırılması, artritis, menisküs hasarı ve anestezi komplikasyonları sayılabilir [38]. Bu çalışmada herhangi bir enfeksiyon belirtisi görülmediği gibi, postoperatif kontroller sırasında da osteotomi hattının iyileşmesinde anormal bir bulguya rastlanmamıştır.

TPDO cerrahisi, ÖÇB yetersizliklerinde tercih edilen yeni bir tekniktir. Tekniğin yapısından ve komplike uygulama aşamalarından dolayı komplikasyon riski yüksektir [39]. Konuyla ilgili detaylı literatür veri analizinin yapıldığı bir çalışmada [40] TPDO cerrahisini takiben bacak fonksiyonlarının gelişiminin başarılı olduğu bildirilmektedir. Jandi ve Schulman, TPDO cerrahisinden 2 yıl sonrasına kadar topallığı değerlendirmişler ve ilk 6 aylık sürede olgularının %69'unun, 12 ay sonrasında ise %94'ünün normal veya normale yakın yürüyüşünün bulunduğunu bildirmişlerdir [41]. Bu çalışmada topallık ve ağrı değerlendirilmiştir. Olguların takip periyotları neticesinde Jandi ve Schulman'ın [41] sonuçlarına benzer olarak, topallığın başlangıçtan itibaren tedricen azaldığı ve izleme periyodu tamamlanan olguların tamamında yürüyüşün normale döndüğü gözlenmiştir.

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RESEARCH ARTICLE

Minimally Invasive Motor Nerve Conduction Study of the Rat Sciatic and Tail Nerves

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Summary

Human and veterinary scientific researchers widely use rats as experimental animals. The rat nerves conduction studies are used for investigations of neural tissue injury, neural regeneration, peripheral neuropathies etc. in experimental models. Nerve conduction studies on animals do not follow strict rules, as studies in humans. Various methods and lack of the single recognized technique of nerve conduction study on the animals often lead to difficulties in the interpretation and comparison of the results of similar studies. In the present study, we have described the minimally invasive motor nerve conduction study on the rat sciatic and tail nerves. Electrophysiological examination including nerve conduction velocity and compound muscle action potential amplitudes measurements were performed on six normal growing male adult Wistar Albino rats. The mean motor nerve conduction velocity of the sciatic nerves was 58.90±5.07 m/s and of the tail nerves was 40.23±2.39 m/s. The mean compound muscle action potential amplitudes of the sciatic and tail nerves were 17.91±6.75 mV and 1.89±0.49 mV, respectively. Similar results of previous *in vitro* and *in vivo* studies prove the objectivity and reliability of our nerve conduction technique by bipolar needle electrodes on the non-exposed nerves.

Keywords: *Nerve conduction study, Sciatic nerve, Tail nerves, Rat*

Rat Siyatik ve Kuyruk Motor Sinirlerinin Minimal İnvaziv İletim Çalışması

Özet

Beşeri ve veteriner bilimsel araştırmacılar, ratları deneysel hayvan olarak yaygınca kullanmaktadırlar. Rat sinir iletim çalışmaları sinir dokusu hasarı, sinir rejenerasyonu, periferik nöropati ve buna benzer deneysel araştırma modellerinde kullanılmaktadır. Hayvanlar üzerinde yapılan sinir iletim çalışmaları, insan çalışmalarından farklı olarak, katı kurallara uymaz. Hayvanlarda sinir iletim çalışmalarının çeşitli yöntemleri ve tek tanınan tekniğin olmayışı benzer çalışmaların sonuçlarının yorumlanmasında ve kıyaslanmasında zorluklara yol açar. Çalışmamızın amacı, rat siyatik ve kuyruk motor sinirlerinin minimal girişimsel iletim çalışma yöntemlerini tanımlamaktır. Altı erkek erişkin Wistar Albino ratın sinir iletim hızları ile bileşik kas aksiyon potansiyelleri ölçümlerini içeren elektrofizyolojik incelemesi yapıldı. Ortalama motor sinir iletim hızları siyatik sinirlerde 58.90±5.07 m/sn ve kuyruk sinirlerinde 40.23±2.39 m/sn idi. Siyatik ve kuyruk sinirlerin ortalama bileşik kas aksiyon potansiyellerinin genliği sırasıyla 17.91±6.75 mV ve 1.89±0.49 mV idi. Bipolar iğne elektrotlar ile ve sinir ekspozisyonu yapılmadan uyguladığımız bu sinir iletim tekniğimizin sonuçlarının daha önceki *in vitro* ve *in vivo* çalışmaların sonuçları ile benzerlik göstermesi bu tekniğinin objektiflik ve güvenilirliğini desteklemektedir.

Anahtar sözcükler: *Sinir iletim çalışması, Siyatik sinir, Kuyruk sinirleri, Rat*

INTRODUCTION

One of the methods for study peripheral nerves is electroneurography (ENG) or, in other words, nerve conduction velocity (NCV) study. This method is used for stimulating the various nerves and recording the potentials. It can be said that, the NCV is an indicator of the physiological or pathophysiological state of the nerves.

The motor nerve conduction velocity (MNCV) is tested by stimulating the motor nerve with a supramaximal stimulus at two points of a nerve trunk. Recording electrodes are placed on the muscles innervated by the nerve and then record the potential which is called a compound muscle action potential (CMAP). CMAP is a

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maximally evoked electrical activity of all active neural fibers. The CMAP recorded from indicator muscle reflects the three values: the size of the motor unit innervated by the axons, the size of motor nerve fibers responding to the stimulus and the synchronization of their response. The greater number of active fibers increases the CMAP amplitude [1,2]. The amplitude of the CMAP recorded by the proximal and distal stimulations conventionally is measured from the peak of the negative deflection to the peak of the positive deflection [1]. The time required for the onset of CMAP with distal stimulation is called the terminal distal latency. The MNCV can be calculated by dividing the distance between proximal and distal stimulation site with the difference of onset latencies of two CMAP. The MNCV reflects the degree of myelination of the fastest axons [3].

As conventional, surface or needle electrodes are use for CMAP recording in human and animal practice. When placed to the skin, surface electrodes allow a non-invasive assessment of the nerve. Surface electrodes are adhered to the skin overlying the selected muscle for indirect measure of muscle generated potentials. Invasive methods consist of surgically placing electrodes on exposed nerve or introducing needles near to nerve. Needle electrodes convert direct signals from selected region of the muscle. Needle electrodes which used in the electrophysiological studies have monopolar, concentric and bipolar properties.

Human and veterinary scientific researchers widely use rats as experimental animals. Viability, fast reproduction and simplicity of these animals make them indispensable in experimental researches. In neurology, rats are used in models of human neurological diseases, neurological drug researches and for development new diagnostic neurological approaches. Particularly, rats are used for investigation of models of peripheral axonal and demyelinating neuropathies, neural tissue injury and regeneration [1-6]. Usually, in these studies scientists have used rats' sciatic nerve, because easy for stimulation, has the simple anatomy and relatively thicker than other peripheral nerves [1-3,6]. As known, the rat sciatic nerve originates from spinal segments L4-L5. In the middle of thigh sciatic nerve is separated into two major branches: the tibial and peroneal branches. The peroneal nerve passes laterally and innerves anterior muscles. The tibial nerve takes a depth course, passes between the lateral and medial heads of gastrocnemius muscle and innervates the flexor muscles [7].

Also, the rat tail nerves conduction studies are used for non-invasive investigations of peripheral neuropathies in experimental models [5,6]. Caudal nerves of rat tail originate from sacrocaudal spinal roots and pass along the length of the tail where they are divided into two minor dorsal and two major ventral nerves [8].

Frequently, for selective stimulation of the nerve and recording the CMAP's in rats are used surgical exploration of the sciatic nerve or other nerves and muscles. These methods can result in sacrificing the animal, because this trauma leads to severe disability or death. As a result, researchers can not track changes in nerves in the same animal over time. Also, multiple animals are needed for the same study.

On the other hand, there are some issues such as ethics and humanistic use of animals in studies. The most appropriate research method and minimum number of animals, which may lead to scientific results, must be preferred in animal studies. Minimizing the pain of the animal during the experiment and less traumatic and appropriate methods of anesthesia and analgesia must be used [9-11].

For these reasons we tried to describe two useful and objective methods of motor nerve conduction study of the sciatic and tail nerves in rats without exploration of nerves and animal sacrification or disability.

MATERIAL and METHODS

This study was performed on six normal growing male adult Wistar Albino rats, weighting 200-225 g. These rats were used as controls in previous study with the approval of the Ethic Committee on Research Animal Care at Veterinary Faculty of Kafkas University of Kars, Turkey (No 2012/30) [1].

All procedures were performed aseptically. The rats were anesthetized by using intraperitoneal 10 mg/kg xylazin HCl and 80 mg/kg ketamin HCl. The animals were positioned prone with maximally straightened hindlimbs. The rat's body temperature was maintained by keeping the animal on an electric pad that was switched off during recording. Core temperatures were measured by placing the probe on the sacrum. Rat's core temperature was maintained at between 36-38°C. Electrophysiological measurements were taken using Neuropack M1 MEB-9200 (Nihon-Cohden Corp, Tokyo, Japan) device. No animal were killed or disabled during the present study.

Sciatic Nerve Conduction Study

In this study, the sciatic nerve was stimulated percutaneously from bipolar needle electrodes which were placed at the level of hip joint and popliteal fossa. Recording bipolar needle electrodes were placed in medial gastrocnemius muscle belly *(Fig. 1)*.

Accurate placement of the recording needle on the gastrocnemius muscle is very important, because the volume conduction from neighboring muscles could result in false positive signals, mostly from the biceps femoris muscle [3,12]. The common reference (ground electrode) was placed on the basis of tail. Electrical stimulation was square pulse with an frequency of 1 Hz and duration of 0.2 m/s. The stimulus current intensity was increased gradually up to

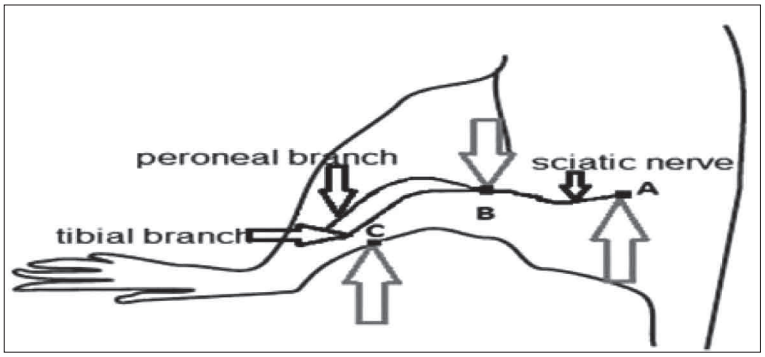

Fig 1. Stimulation and recording points of rat sciatic nerve motor conduction study; **A-** proximal stimulation point, **B-** distal stimulation point, **C-** recording point

Şekil 1. Rat siyatik sinir motor iletim çalışmasında uyarım ve kayıt noktaları; **A-** proksimal uyarım noktası, **B-** distal uyarım noktası, **C-** kayıt noktası

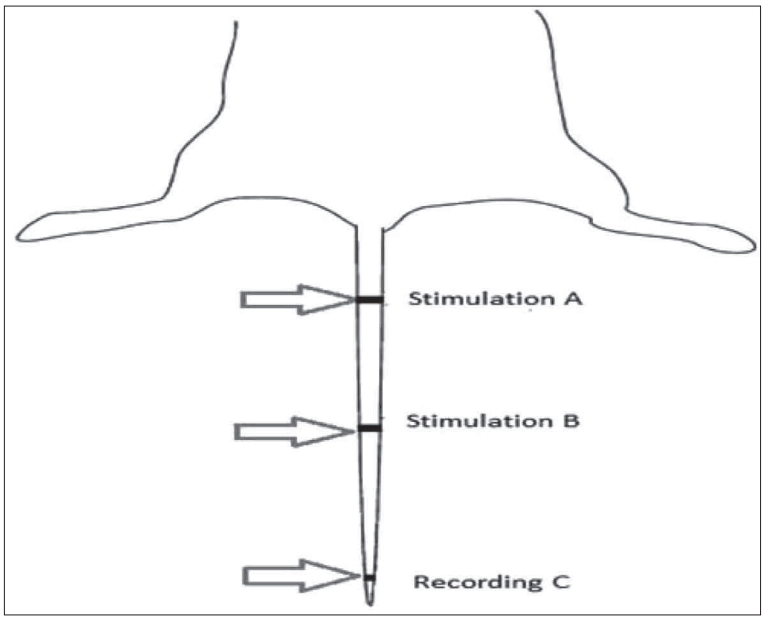

Fig 2. Stimulation and recording points in motor nerve conduction study of rat tail; **A-** proximal stimulation point, **B-** distal stimulation point, **C-** recording point

Şekiil 2. Rat kuyruk motor sinirlerinin iletim çalışmasında uyarım ve kayıt noktaları; **A-** proksimal uyarım noktası, **B-** distal uyarım noktası, **C-** kayıt noktası

supramaximal intensity (current intensity 30% above the value to evoke the maximal CMAP). The stimulation was repeated 5 times for all measurements and the average values were recorded. Then the rat's hindlimbs were straightened and the distance between the stimulating electrodes were ascertained with a tape measure to the nearest millimeter.

In each animal, both sciatic nerves on all 12 hindlimbs were investigated.

Tail Nerve Conduction Study

Fig. 2 shows schematic illustration of the bipolar needle electrode arrangement for the measurement of MNCV in the tail nerves. All electrodes were inserted intramuscularly. CMAPs were evoked by stimulation at point B on the middle part of the tail and point A in the proximal part of the tail. CMAP was recorded at point C, located about 2-3 cm from the end of tail. Frequency, duration and intensity of the electrical stimulation were performed as on the sciatic nerve. The distance between the stimulation and recording electrodes was measured by the tape measure in the straight tail position. MNCV was calculated by the conventional method: the distance was divided by the difference between proximal and distal latencies.

We performed all measurements as accurately as possible because the small error in distance may lead to incorrect results in MNCV. Also, we tried to minimize the artifacts and to record a response, when the electro-negativity from the isoelectric line was started.

Fig. 3 and *Fig. 4* show sample traces of sciatic and tail nerves motor conduction studies.

Statistical Analysis

The results were analyzed using Minitab-12 statistical software. For tests normality of distributions were used the Anderson-Darling test. The following variables were considered: MNCV and CMAP amplitude. All results were expressed as the mean ± standard deviation (SD).

RESULTS

The mean MNCV of the sciatic nerves were 58.90±5.07 m/s and of the tail nerves were 40.23±2.39 m/s *(Fig. 5)*. The mean MNCV were statistically slow in the tail nerves than in the sciatic nerves.

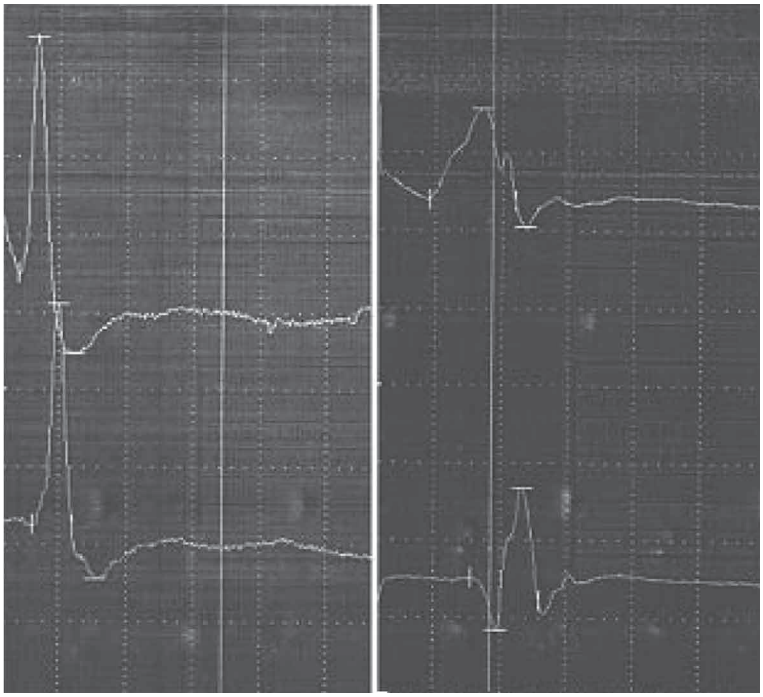

Fig 3. Sample traces of sciatic motor nerve conduction study

Şekil 3. Siyatik sinir motor iletim çalışmasının örnek traseleri

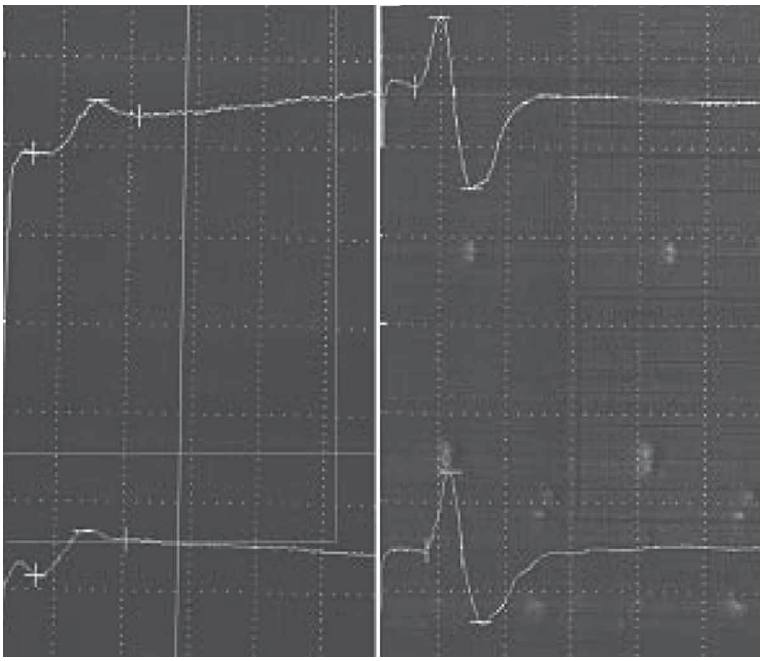

Fig 4. Sample traces of rat tail motor nerve conduction study

Şekil 4. Rat kuyruk motor sinirlerinin iletim çalışmasının örnek traseleri

The mean CMAP amplitudes of the sciatic and tail nerve were 17.91±6.75 mV and 1.89±0.49 mV, respectively *(Fig. 6)*. The CMAP amplitudes of the tail nerves were statistically lower compared with the amplitudes of the sciatic nerves.

DISCUSSION

Nerve conduction studies in rats are used in animal experimental models of chronic or acute axonal and demyelinating neuropathies, in nerve injury and regeneration studies and for better understanding effects and toxicity of several drugs [1-6,8,12]. In the human and animal medical literature, particularly in rats, numerous nerve conduction techniques have been described. Nerve conduction studies on animals do not follow strict rules, as studies in humans [13]. Various techniques of nerve conduction were described in each animal study. Most of these techniques were performed on the exposed nerves *in vivo* or on the nerve segments *in vitro* [1-6,14,15]. Sometimes, researchers preferred recording from the different muscles (gastrocnemius, tibialis anterior or interosseous muscles) and by different type of electrodes (surface or needle electrodes). Various methods and the lack of the single recognized technique of nerve conduction study often

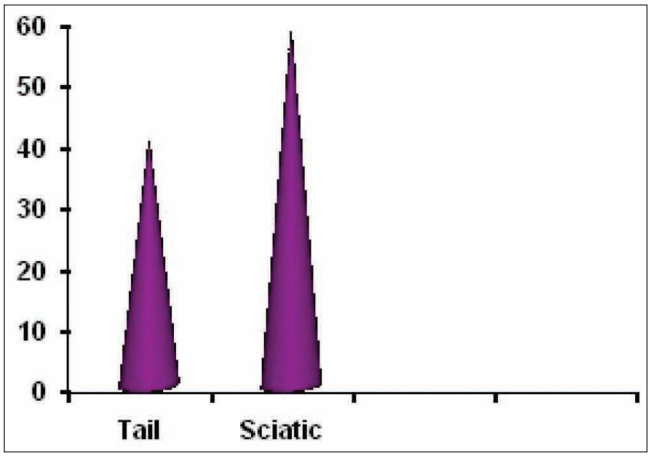

Fig 5. Sciatic and tail MNCV (m/s)
Şekil 5. Siyatik ve kuyruk motor sinirlerinin iletim hızları (m/sn)

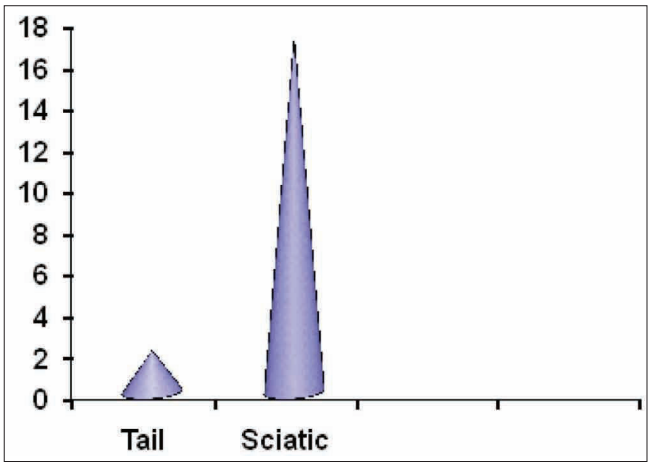

Fig 6. Sciatic and tail CMAP amplitudes (mV)
Şekil 6. Siyatik ve kuyruk bileşik kas aksiyon potansiyeli amplitüdleri (mV)

lead to difficulties in the interpretation and comparison of the results of similar studies.

In the present study we have described the motor nerve conduction study methods of the sciatic and tail nerves and attempted to verify the results of our study, comparing with the results of previous researches. Although the purpose of this study is not to compare the velocities and CMAP amplitudes between the sciatic and the tail nerves, we found that the sciatic MNCV investigated by needle electrodes from gastrocnemius muscle were faster than the tail MNCV. Also, the sciatic CMAP amplitudes were higher than the tail CMAP amplitudes. Those were the expected results and explained by the "size principle" of Henneman-larger muscles (gastrocnemius muscle) have bigger motor unites with thicker nerve fibers and thus, have faster conduction velocities than small muscles (tail muscles) [16].

Some electrophysiological studies on rats were performed using surface recording and stimulating electrodes [4,8,17] As known, surface electrodes non-invasive, painless, easy and quick to use [18,19]. Surface electrodes record the activity of a large area and as a result, give the information about electrophysiological activity from the large muscle groups. On the other hand, surface electrodes lack specificity because they sample a large volume of muscle and thus, are not be able to distinguish signals from the target muscle or neighboring muscles. Also, surface electrodes detect signals from the superficial muscles and not from the deep muscles.

Nevertheless, using of needle electrodes is an invasive procedure and can lead to pain, neural and muscular injury, we preferred to use the bipolar needle electrodes, because they can record and distinguish signal from selected muscle, especially from the thin muscles in the small laboratory animals, such as the gastrocnemius muscle in the present study [9]. According to our knowledge, this is the first study where were used bipolar needle electrodes for stimulation non-exposed nerves and recording from the gastrocnemius muscle.

The sciatic and tail nerves conduction studies by needle electrodes were performed in some studies on rats [3-6]. Relatively long distance between two stimulation pointes, thickness of these nerves, easy to stimulation, simple anatomy of these nerves are advantages to use in nerve conduction studies [20]. Study technique, sciatic and tail MNCV and CMAP amplitude values in previous studies were similar to our results. Oğuzhanoğlu et al.[4] found that sciatic nerve motor conduction velocities were 55.12±9.63 m/s and CMAP amplitudes of gastrocnemius muscle were 31.87±9.38 mV. Also, Kasselman et al.[6] reported the motor nerve conduction velocities in the tail and sciatic nerves were similar to our study results. Another author reported that the sciatic- tibial MNCV was 46.3±12.4 m/s and CMAP amplitudes were 10.2±3.9 mV; and the tail MNCV was 21.9±3.2 and CMAP amplitudes were 8.3±3.3 mV in rats at the 9th week of age [17]. Additionally, some studies, which were performed on isolated sciatic nerve segments and on the exposed nerve, support our results. Dalkılıc et al.[15] performed sciatic nerve conduction study *in vitro* on isolated nerve segment. Conduction velocities in their study were 55.7±2.6 m/s similar to our values. The study on exposed sciatic nerve was performed by Wang et al. and the results were close to ours [14]. Another study, performed on exposed sciatic nerve, reported that the sciatic nerve MNCV as 46.9±2.2, 53.0±2.1 and 48.9±3.8 m/s depending on dietary supplementation [21]. Similar results *in vitro* and *in vivo* studies prove the objectivity and reliability of our nerve conduction technique by bipolar needle electrodes on the non-exposed nerves.

However, Rupp et al.[22] considered that recording CMAPs from the gastrocnemius muscle in rats should be treated with caution, especially, if monopolar needle electrodes are used for recording. On the other hand, recordings from the gastrocnemius muscle have been applied in various studies. These studies do not contain

any inconsistencies about recording from gastrocnemius muscle [23-25].

In addition to the above, the minimally invasive measurements may be valuable tool to displaying peripheral nerve changes and it was confirmed by histomorphometric evaluation in the previous study [1].

In conclusion, the results of present study and other similar studies show that minimally invasive sciatic and tail nerves conduction studies with bipolar needle electrodes may be the suitable methods in rat models of peripheral nervous system diseases. These methods of nerve conduction may be preferable in cases where researchers need further observation of surviving animals after examination. On the other hand, from an ethical point of view, these methods are minimally invasive and not the cause of death or disability of animals, such as in the present study.

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RESEARCH ARTICLE

17β-Estradiol Inhibites Nitric Oxide-cGMP-Dependent Pathway but may Activate Independent Pathway in Small Intestinum of Ovariectomized Rat [1] [2]

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Summary

This study was designed to investigate the effect of 17β-estradiol on small intestinal motility of ovariectomized rats and the possible role of nitric oxide (NO) in this activity. A total of 24, 3 to 6 month-old female Sprague Dawley rats (270±20 g) were ovariectomized and divided into four groups as one control and three experimental groups. The control group received 0.2 mL, sesame oil once daily for three days, whereas the experimental groups were treated with 25, 50 and 100 µg/rat intramuscular 17β-estradiol, respectively. The rats were sacrificed by cervical dislocation under anaesthesia 18 h after the termination of last treatment. Immediately, duodenum, jejunum and ileum were isolated for organ bath contractility experiments to evaluate isometric smooth muscle motility *in vitro*. It was observed that application of 100 µg/rat 17β-estradiol showed a decreasing tension in duodenum, whereas none of the different doses of 17β-estradiol showed any significant difference in jejunum. The application of 50 and 100 µg/rat 17β-estradiol decreased the spontaneous contractile tension of ileum. However, L-arginine (10^{-5}M), 8-Br-cGMP (10^{-6}) and SNP (10^{-3}M) decreased the spontaneous contractions of smooth muscle of duodenum, jejunum and ileum. Moreover, it was demonstrated that 17β-estradiol decreased the relaxing activity of L-arginine and 8-Br-cGMP but increased the activity of SNP in dose dependent manner. In conclusion, it is suggested that 17β-estradiol has a relaxative effect in duodenum and ileum and particulary inhibits the activity of cGMP-PK. However, endogenous NO-NOS pathway mediated by 17β-estradiol may play a key role in secretory and/or ciliary activity of intestinum.

Keywords: *17β-estradiol, Small intestine, cGMP, Nitric oxide, Rat*

17β Östradiol Overioktomize Sıçanların İnce Bağırsağında Nitrik Oksit-cGMP'den Bağımsız Yolu Etkinleştiriyor Olabilirken Bağımlı Yolu Engelliyor

Özet

Bu çalışmada ovaryumları çıkarılmış sıçanlarda 17β-östradiolün ince bağırsak kasılımları üzerine etkisi ile bu etkinin ortaya çıkmasında nitrik oksitin rolü incelenmiştir. Bu amaçla çalışmada 3-6 aylık ve ortalama 270±20 g ağırlığında, 24 Sprague Dawley dişi sıçan her grupta 6 sıçan bulunacak şekilde, kontrol (Ov) ve 3 deneme grubu olmak üzere 4 gruba ayrılmıştır. Çalışmada kontrol grubuna günlük olarak kas içi susam yağı enjeksiyonu yapılmış (0,2 ml), deneme gruplarına sırasıyla 25, 50 ve 100 µg/sıçan 17β-östradiol, üç gün uygulanmıştır. Son uygulamadan 18 saat sonra genel anestezisi altında ötenazi yapılmıştır. Takibinde ince bağırsak, duodenum, jejenum ve ileum bölgelerine ayrılmış ve *in vitro* izometrik düz kas hareketleri kaydedilmiştir. 17β-östradiolün 50 ve 100 µg/sıçan dozları spontan ileum kasılımlarının gerimini azaltırken duodenumda aynı etkiyi 100 µg/sıçan dozunun oluşturdu gözlemlendi. Jejenumda ise gruplar arasında farklılık oluşmadığı görüldü. Buna karşın L-arjinine (10^{-5}M), 8-Br-cGMP (10^{-6}M) ve SNP (10^{-3}M) uygulamaları duodenum, jejenum ve ileumda spontan düz kas kasılımlarını azalttı. Ayrıca 17β-östradiol uygulamasının L-arjinin ve 8-Br-cGMP'nin gevşetici etkisini azalttığı tersine SNP'nin etkinliğini ise doza bağımlı olarak arttırdığı gösterildi. Sonuç olarak 17β-östradiolün duodenum ve ileumda özellikle cGMP-PK etkinliğini engellemesine rağmen gevşetici etkiye sahip olduğu buna karşın 17β-östradiol aracılığıyla endojen NO-NOS yolunun bağırsağın sekresyon ve/veya siliyar etkinliklerinde rol oynayabileceği önerilmektedir.

Anahtar sözcükler: *17β-östradiol, İnce bağırsak, cGMP, Nitrik oksit, Sıçan*

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INTRODUCTION

Estrogens are basic female sex hormones and exist in forms of estron, estrol and 17β-estradiol in the body. Estrogens increase the concentration of receptors for oxytocin [1] and adrenergic agents [2], which modulate membrane calcium channels. Estrogens are capable to increase the contraction of uterine smooth muscle [3] however, they produce relaxation in smooth muscles of bile duct [4], trachea [5] and blood vessels [6]. Intestinal motility in human [7] and animals [8] are closely associated with estrous cycle, pregnancy and menopause. It has been demonstrated that changes in concentration of estrogen and progesteron as well as local hormones of digestive tract affects electromechanical behaviour of smooth muscles of gastro-intestinal (GI) tract [9]. It has been reported that estrogens may hyperpolarize the cell membrane by stimulating extracellular discharge of potassium (K^+) in smooth muscle cells, thus the inhibition of spontaneous contraction is occurred [10]. However, the blocking of entrance of calcium (Ca^{+2}) into cells by estrogens inhibits voltage-dependent Ca^{+2} and K^+ channels and this further produces relaxation in smooth muscle [11]. It has been indicated that 17β-estradiol significantly inhibites the carbachol-induced contractions of ileum [12]. Moreover, in a previous study, it has been clearly demonstrated that estrogens increase plasma concentration of cholecystokinin and activates cholecystokinin receptors thus; the inhibitor effect of estradiol on motility of gastrointestinal system occurs [9]. Some molecules mediating 17β-estradiol activity, such as nitric oxide (NO), is also responsible for control of motility and passage speed in GI system. Nitric oxide released from neuronal nitric oxide synthase (nNOS) located in nonadrenergic and noncholinergic (NANC) nerves are very important factor for the control of GI system motility and emptying time [13]. Nonadrenergic and noncholinergic nerves that are dependent on NO release play a key role for regulating the secretion, motility and amplitude of smooth muscles in GI tract [13,14].

As a chemical mediator, NO enters into the cell swiftly, activates cGc in sitozol and stimulates the formation of cGMP, which is an inner cell secondary messanger in simple muscle cells [19]. GMP paves the way for relaxation in a simple muscle via two mechanisms. Firstly, NO_2 reduces levels of Ca^{++} within a cell and increases the permeability of K^+ channels. Thus, it hyperpolarizes the plasma membrane [15,16]. Secondly, cGMP blocks miyozin/aktin interaction by activating cGMP dependent protein kinaz (PKG) which leads to the dephosphorization of light miyozin chains and which is reported to play a key role in NO/cGMP signals [15].

Previous reports have been clearly demonstrated that the effect of estrogens on the GI system motility displayed various responses regarding to different anatomical region of intestinum [12,17]. To the authors' knowledge, the role of NO on 17β-estradiol mediated contractility of small intestine was still unclear. Therefore, this study was designed to evaluate the effect of different doses of 17β-estradiol, a femal sex steroid, on small intestine motility and the possible role of NO in ovariectomized rats.

MATERIAL and METHODS

Animals

A total of 24, 3 to 6 month-old female Sprague-Dawley rats (270±20 g) were used in the study. Rats were fed *ad libitum* with a commercial rat diet. (This research has been approved by Institutional Etchic Committee of Afyon Kocatepe University. Date and number is; 2008/203).

Experimental Groups and Study Design

The ovariectomy procedure was performed under general anesthesia by an intra-peritoneal ketamine (21.2 mg/kg) and xylazine (4.2 mg/kg) combination. Small bilateral incisions were made on the dorsum to expose the ovaries retroperitoneally. The ovarian vessels were then clamped and the ovaries were removed. Afterwards, the uterine tubes were ligated and the muscles and skin were sutured. Two weeks after operation, the ovariectomized rats were randomly assigned to four groups of 6 rats each. Rats in the control group received sesame oil once daily for three days, whereas rats in the experimental groups were treated with 25, 50 and 100 µg/rat 17β-estradiol, intramuscularly.

After 18 h of the last hormone treatment, the rats were killed by cervical dislocation under sodium penthotal (25 mg/kg, intraperitoenal) anaesthesia. Immediately after death, the abdominal cavity of the rats was opened and the mid-duodenum, mid-jejunum and mid-ileum were carefully removed avoiding physical damage. Each individual isolated tissues was placed in Krebs' solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1mM $MgSO_4$, 1 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose). Subsequently, the surrounding mesentery and fat tissues were carefully removed from the tissues, and a strip-shaped tissue samples with dimensions of 0.1x0.3x1.0 cm were obtained. One edge of each intestine tissue preparation was fixed to platinum ring electrodes. The opposite edge of the tissue was connected to a force-displacement transducer (model 10-A; MAY, Commat, Ankara, Turkey). Isolated intestine tissues were placed in organ baths filled with 20 mL Krebs' solution, which were continuously ventilated with a gas mixture (95% O_2 - 5% CO_2) at 37ºC. The isometric smooth muscle activity of the intestine samples were monitored and recorded by computer via the force transducer and an acquisition system (model MP30 WSW with Biopac Student Lab PRO Software, Biopac Systems). An electrical field stimulation (EFS) device (model ISO 150-

C, MAY, Commat, Ankara, Turkey) was used for maximal contractility.

Recording of Isometric Intestine Contractility

Samples in organ baths were kept in Krebs' solution for at least 1 h prior to the recordings to enable the tissues to adapt to the environment, and the solution was refreshed in 15-min intervals. The appropriate resting tension for the strips was determined in initial experiments. Optimal tension relationships were achieved with resting tensions of 2 g for the intestinum strips. Therefore, a resting tension of 2 g was applied to the tissues. Electrical field stimulation was then used to define submaximal contraction, carried out at various frequencies (2, 4, 8, 16, 32 and 64 Hz) and voltage (10, 20, 30 and 40 voltage). In order to determine endogenous NO activity, L-arginine (10^{-5} M) (Sigma, Cat # A5131) was added to the Krebs' solution. 8-Bromo guanosine 3',5'-cyclic monophosphate sodium salt (8-Br-cGMP, a membrane-permeable analog of cyclic guanosine monophosphate) (10^{-6} M) (Sigma, Cat # B13815131) was added in order to determine the activity of cyclic guanosine monophosphate-protein kinase (cGMP-PK) pathway and sodium nitroprusside (SNP) (10^{-3} M) (Sigma, Cat # S0501) was added in order to determine the exogeneous NO activity. All applications were performed on the same tissue. The mean tension of spontaneous contractions for each strip calculated for a 10-min period before administration of examined substances was set as 100% [18,19]. Thereafter, changes in small intestinal contractions caused by the examined substances were recorded and compared to the control period.

Statistical Analysis

All values are presented as mean ± S.D. For statistical evaluation of the data, one-way ANOVA was used. To compare the individual means of treatment groups, the Tukey test was performed (SPSS 16.0). Group differences were declared significant at P<0.05.

RESULTS

Amplititude of spontaneous contractions obtained in duodenum, jejunum and ileum are given in *Table 1*. It was observed that application of 25 and 50 µg/rat 17β-estradiol did not alter the spontaneous contractile tension of duodenum, whereas application of 100 µg/rat 17β-estradiol showed a decreasing tension as compared to control group (P<0.01). Moreover, none of the different doses of 17β-estradiol showed any significant difference in jejunum. Similarly to duodenum, there was no significant difference between control and 25 µg/rat 17β-estradiol groups of ileum. However, the application of 50 and 100 µg/rat 17β-estradiol decreased the spontaneous contractile tension of ileum (P<0.001).

It was clearly observed that the treatment of L-arginine (10^{-5} M) decreased the percentage inhibiton of spontaneous contractions of smooth muscle of duodenum, jejunum and ileum in 50 and 100 µg/rat 17β-estradiol groups as compared to control (P<0.001) *(Table 2)*. Moreover, 25 µg/rat 17β-estradiol showed a relaxant effect as same as 50 and 100 µg/rat 17β-estradiol groups in smooth muscle of jejunum. The treatment of L-arginine (10^{-5} M) applied after EFS did not show any significant difference between groups of jejunum but a decreasing percentage inhibiton of contractile activity was observed in 50 and 100 µg/rat 17β-estradiol groups of duodenum (P<0.001) and all 17β-estradiol groups of ileum (P<0.05).

It was seen that 8-Br-cGMP (10^{-6}) showed relaxant effect in all 17β-estradiol groups of smooth muscle of duodenum, jejunum and ileum *(Table 2)*. The percentage inhibiton was decreased in 17β-estradiol groups of smooth muscle of duodenum and ileum in a dose-dependent manner (P<0.001), while no significant difference obtained between all 17β-estradiol groups of jejunum. The treatment of 8-Br-cGMP (10^{-6}) applied after EFS caused a decreasing percentage inhibiton of contractile tension in all 17β-estradiol groups of smooth muscle of duodenum (P<0.001), jejunum (P<0.001) and ileum (P<0.01) as compared to spontaneous contractile tension.

It was clearly observed that the treatment of SNP (10^{-3} M) increased the percentage inhibiton of spontaneous contractions of smooth muscle of duodenum (P<0.001) and ileum (P<0.05) in 50 and 100 µg/rat 17β-estradiol groups, whereas the percentage inhibition was increased in 100 µg/rat 17β-estradiol group of jejunum, as compared to control group (P<0.05). The treatment of SNP (10^{-3} M) applied after EFS did not show any significant difference between groups of duodenum, jejunum and ileum *(Table 2)*.

Table 1. *Amplitude values of spontaneous contractile tension of duodenum, jejunum and ileum in groups (g)*
Tablo 1. *Gruplarda duodenum, jejunum ve ileumda spontan kasılım gerimlerinin amplitüt değeri (g)*

Amplititude of Contraction (g)	Control	25 µg 17β- estradiol	50 µg 17β-estradiol	100 µg 17β- estradiol	P<
Duodenum	0.66±0.04[a]	0.64±0.02[a]	0.58±0.03[ab]	0.49±0.01[b]	0.01
Jejunum	0.58±0.02	0.57±0.04	0.55±0.04	0.50±0.02	NS
Ileum	1.05±0.06[a]	1.09±0.06[a]	0.87±0.06[b]	0.78±0.04[b]	0.001

[a, b] Different letters in the same line are statistically significant, ***NS:*** *Not significant*

Table 2. *Percentage inhibition of contractility of smooth muscle of intestinum (%)*
Tablo 2. *Bağırsak düz kas kasılımlarının inhibisyon yüzdesi (%)*

Applications	Control	25 µg 17β- estradiol	50 µg 17β-Estradiol	100 µg 17β-estradiol	P<
Duodenum					
Arg (10^{-5})	25.8±4.11^{a}	20.8±1.72^{a}	9.66±2.41^{b}	7.46±1.65^{b}	0.001
8-Br-cGMP (10^{-6})	15.8±2.55^{a}	9.41±2.98^{b}	3.53±1.44^{bc}	1.61±0.39^{c}	0.001
SNP (10^{-3})	36.6±2.16^{b}	37.7±4.14^{b}	67.5±3.78^{a}	74.12±2.31^{a}	0.001
EFS + Arg (10^{-5})	3.07±0.22^{a}	2.72±0.20^{a}	2.07±0.06^{b}	1.97±0.12^{b}	0.001
EFS + 8-Br-cGMP (10^{-6})	13.2±1.84^{a}	1.72±0.20^{b}	1.07±0.06^{b}	0.97±0.12^{b}	0.001
EFS + SNP (10^{-3})	12.8±0.27	13.6±1.01	16.4±2.47	13.1±1.0	NS
Jejenum					
Arg (10^{-5})	15.8±1.41^{a}	7.30±1.31^{b}	5.94±1.21^{b}	4.47±1.45^{b}	0.001
8-Br-cGMP (10^{-6})	15.1±2.13^{a}	95.0±2.38^{b}	0.90±1.93^{b}	3.82±1.02^{b}	0.01
SNP (10^{-3})	23.8±3.24^{a}	32.9±2.23^{ab},	32.9±1.88^{ab}	38.6±5.39^{b}	0.05
EFS + Arg (10^{-5})	1.03±0.25	1.33±0.33	0.83±0.30	1.01±0.36	NS
EFS + 8-Br-cGMP (10^{-6})	7.33±0.95^{a}	1.40±0.36^{b}	0.70±0.22^{b}	0.80±0.20^{b}	0.001
EFS + SNP (10^{-3})	14.9±1.26	14.9±1.76	20.5±1.92	15.1±2.34	NS
Ileum					
Arg (10^{-5})	27.3±4.77^{a}	19.1±1.64^{a}	7.19±1.68^{b}	4.50±1.11^{b}	0.001
8-Br-cGMP (10^{-6})	9.66±1.28^{a}	5.80±1.41^{b}	2.36±1.20^{c}	0.75±0.40^{c}	0.001
SNP (10^{-3})	25.6±3.85^{b}	34.1±3.04^{ab}	39.3±2.12^{a}	43.3±4.33^{a}	0.01
EFS + Arg (10^{-5})	6.33±1.38^{a}	2.00±0.81^{b}	1.16±0.65^{b}	1.50±0.71^{b}	0.05
EFS + 8-Br-cGMP (10^{-6})	4.33±0.66^{a}	1.83±0.74^{b}	1.16±0.66^{b}	0.83±0.47^{b}	0.01
EFS + SNP (10^{-3})	11.8±1.30	10.3±1.56	9.83±1.07	7.16±2.10	NS

^{a,b,c} *Different letters in the same line are statistically significant,* ***NS:*** *Not significant*

DISCUSSION

It has been reported that 17β-estradiol can be injected at doses of 2-100 µg in rats and it peaks 18 h after administration [20,21]. Therefore, we used 25, 50 ve 100 µg/rat doses of 17β-estradiol and evaluated L-arginine/nitric oxide synthase (NOS)/cGMP pathway on isolated strips of duodenum, jejunum and ileum 18 h after different doses of 17β-estradiol administration.

In the present study, we clearly demonstrated that 17β-estradiol decreased the spontaneous contractile activity of duodenum and ileum in a dose-dependent manner *(Table 1)*. Importantly, 17β-estradiol did not alter the amplitude of spontaneous contractile tension of jejunum. Moreover, it has been reported in many studies that NO pathway directly affecting smooth muscles causes significant decrease in gastro intestinal motility [22,23]. In our study, it is possible to indicate that decreasing spontaneous contractility might be associated the interaction of estrogen with NO. Nitric oxide may directly or indirectly effect smooth muscle contractility of small intestinum. Therefore, the present study focused on the possible linkage between estrogen and NO that might be important in regulation of small intestine contractions.

It has been reported that NO is endogenously synthesized from L-arginine via NOS enzymes [24]. Physiologically, the concentration of L-arginine in mice sera is approximately 0.2-3.0 mM [25]. Moreover, L-arginine at around 0.01-1 mM concentrations totally removes the contractile activity of smooth muscles *in vitro* [26]. It has been clearly showed that the most effective dose of L-arginine is 10^{-5} M [18,27], therefore we preferred to use L-arginine at this dose range. It has been reported that estrogens increase NOS expression in intestine [28]. Therefore, if an endogenous NO system is involved in relaxation of intestinum via increasing NOS expression, smooth muscle relaxation would be expected after L-arginine treatment. In contrast, we observed that the relaxant effect of L-arginine (10^{-5} M) decreased, when the dose of 17β-estradiol increased in duodenum, jejunum and ileum *(Table 2)*. These results suggest that inhibition of relaxant effect of L-arginine by 17β-estradiol may be associated with the inhibition of cyclic guanylate cyclase (GC), cGMP and/or cGMP-dependent protein kinase (PKG 1) pathway rather than NO-NOS pathway. Moreover, we found that the treatment of L-arginine (10^{-5} M) after EFS similarly decreased the contractile tension in 17β-estradiol groups of duodenum and ileum but except jejunum. The deficiency of L-arginine on 17β-estradiol groups of jejunum may be explained by

a prevailing endogenous saturation of the L-arginine for NO synthesis as observed in myometrium [29] or low NOS activity in jejunum as suggested in oviduct [19].

Nitric oxide produced by either endogen or exogen NO donors via NOS enzymes increase the cGMP level by activating soluble guanylate cyclase. Thereafter, cGMP initiates the relaxation by phosphorilisation of cGMP-PK in smooth muscles. 8-Br-cGMP decreases the spontaneous mechanical activity [30]. Moreover, it has been reported that 8-Br cGMP has a relaxant activity in smooth muscles of uterus [18] blood vessels [31] and intestine [32]. Cyclic GMP-PK is an enzyme group and has two different forms which includes cGMP-PKI (cGMP-PK Ia/b) and cGMP-PKII [33-35]. It has been reported that cGMP-PKI produces relaxation in smooth muscles [35] and cGMP-PKII, the major form in gastrointestinal system, is responsible for ion transportation [33,34]. In the present study, we observed that the relaxant effect of 8-Br-cGMP was inhibited in all 17β-estradiol groups of smooth muscle of duodenum, jejunum and ileum. It has been reported that NO may be effective by stimulation of guaniline cyclase in various parts of intestine [30]. In human jejunal longitudinal smooth muscle, NO possibly shows depressor activity throughout guaniline cyclase mechanism rather than other mechanisms [36]. Furthermore, it has been reported that nNOS sourced NO gives rise to hyperpolarization by opening Ca^{+2} dependent K^{+} channels and that it leads to relaxation indipendent from cGMP [37]. This finding supports our previous suggestion that endogenously produced NO shows its relaxative effect mediated by estrogens via NO/NOS independent pathway. Furthermore, it suggests that estrogen inhibites the cGMP-dependent pathway.

Sodium nitroprusside, an exogen NO donor, causes a dose-dependent decrease in spontaneous ileal contraction. 1H-[1,2,4] oxadiazolol [4,3,a] quinoxalin–1-one (QDO), a cGC inhibitor, reduces the effects that are initiated by SNP [38]. The relaxing effect of SNP on smooth muscle can be seen at the highest dose of 10^{-3} M, however the doses higher than this concentration is advocated to be toxic [39]. Motility studies in digestive system indicates that this dose produces relaxation [3,19,32]. In our study, similar to other observations, SNP showed relaxing effect on all examined isolated tissues of intestine that this activity was increased by administration of estrogen. This finding supports our previous suggestion that exogenously produced NO shows its relaxative effect mediated by estrogens and the NO/NOS independent pathway may be associated with this effect. Moreover, SNP generates cGMP-dependent and independent relaxation in smooth muscles [18,27]. In our study, the relaxant activity of L-arginine and 8-Br-cGMP was decreased increasing dose-dependent manner of 17β-estradiol, whereas SNP increased the percentage inhibition of smooth muscle contractility *(Table 2)*. Furthermore, 17β-estradiol inhibited the cGMP-dependent pathway but activated the independent pathway.

In conclusion, above-mentioned results suggest that 17β-estradiol particulary inhibits the activity of cGMP-PK in duodenum and ileum. However, endogenous NO-NOS pathway mediated by 17β-estradiol may play a key role in secretory and/or ciliary activity of intestinum.

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RESEARCH ARTICLE

Farelerde 3-Metilkolantren İle İndüklenen Fibrosarkoma Üzerine Sisteaminin Etkileri: Genotoksisitenin Araştırılması

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Özet

Bu çalışmada, fare kemik iliği hücrelerinde 3-Metilkolantrenin genotoksisitesine karşı sisteaminin etkisi araştırıldı. Deneyde beyaz erkek fareler (*Mus musculus* albino) kullanıldı. Fareler her grupta 15 adet olacak şekilde beş gruba ayrıldı. Birinci grup negatif kontrol grubu olarak tutuldu. İkinci gruba susam yağı (0.2 ml, deri altı), üçüncü gruba sisteamin (%0.1 suda oral *ad libitum*), dördüncü gruba 3-Metilkolantren (1 mg 3-Metilkolantren/0.2 ml susam yağı çözeltisinden 0.2 ml deri altı yolla), beşinci gruba 3-Metilkolantren (1 mg 3-Metilkolantren/0.2 ml susam yağı çözeltisinden 0.2 ml deri altı yolla) ve sisteamin (%0.1 oranında suda hazırlanmış sisteamin çözeltisinden oral yolla *ad libitum* olarak verildi. Bütün gruplardaki hayvanlar 4 ay sonra eter anestezisi eşliğinde servikal dislokasyonla ötanazi edildi. Her gruptan 8 adet farenin femur kemik iliği hücrelerinde mitotik aktivite ile mikronükleus testi kullanılarak genotoksik ve sitotoksik etkiler belirlendi. 3-Metilkolantren'in mikronükleuslu polikromatik eritrositlerin sayılarında çoğalma ve kemik iliği hücrelerinde sitotoksik etki meydana getirdiği tespit edildi. Ayrıca mitotik aktiviteyi düşürdüğü gözlendi. Bu etkiler istatistiksel açıdan önemli bulundu ($P<0.01$). Sisteaminin ise genotoksik ve sitotoksik etki yapmadığı belirlendi. 3-Metilkolantren ile birlikte uygulanan sisteaminin yalnız başına verilen 3-Metilkolantrene göre mikronükleuslu polikromatik eritrosit sayılarında önemli derecede düşüş, mitotik aktivitede ise artış yaptığı gözlendi. Araştırma sonucunda sisteaminin farelerde 3-Metilkolantren ile indüklenen genotoksisiteye karşı koruyucu etki gösterdiği belirlendi.

Anahtar sözcükler: *3-Metilkolantren, Sisteamin, Genotoksisite*

The Effects of Cysteamine on the Mice with the 3-Methylcholanthrene - Induced Fibrosarcoma: Investigation of the Genotoxicity

Summary

In this study, the effect of cysteamine against the genotoxicity of 3 Methylcholanthrene's in the cell of mouse bone marrow was examined. In the experiment total of 75 white male mice were used. They were split into 5 groups. First group was kept as negative control group. Sesame oil to the second group (0.2 ml, subcutaneous), cysteamine to the third group (oral *ad libitum* 0.1% in water), 3-Methylcholanthrene to the forth group (1 mg 3- Methylcholanthrene /0.2 ml from the sesame oil solution 0.2 ml, subcutaneously) and cysteamine (*ad libitum* via oral route from the solution of cysteamine that was prepared in water at the rate of 0.1%) was given. Four months later animals in all groups were euthanasied with cervical dislocation (with ether anesthesia). Genotoxic and cytotoxic effects were defined by using mitotic activty and micronucleus in the cells of femur bone marrow of 8 mice from each group. It was determined that 3- Methylcholanthrene has brought about reproduction in the numbers of micronucleus polychromatic erythrocytes and cytotoxic effects in the cells of bone marrow. Furthermore, it was observed that it has slow down the mitotic activity. These effects were statistically considered as significant ($P<0.01$). On the other hand it was determined that the cysteamine did not has an genotoxic and cytotoxic effect. It was observed that when cysteamine used with 3- Methylcholanthrene caused to fall in the numbers of micronucleus polychromatic erythrocytes and upregulation in mitotic activity in comparison with 3- Methylcholanthrene that was given by itself. In the end of the study, it was observed that cysteamine had protective effect against the genotoxicity induced with 3- Methylcholanthrene on the mice.

Keywords: *The 3-Methylcholanthrene, Sisteamin, Genotoxicity*

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GİRİŞ

Günümüzde hızlı ve yanlış planlanan endüstrileşme çevre kirliliğine neden olmaktadır. Dolayısıyla insan, hayvan ve bitkiler çevre kirlenmesinde rol oynayan fiziksel ve kimyasal ajanlara maruz kalmaktadır. Bu nedenle maddelerin toksik etkileri ile zehirlenmelerin tedavi ve korunma yöntemlerinin bilinmesi büyük önem arz etmektedir. Zehirlerin özellikle alerjik, mutajenik, karsinojenik ve teratojenik özelliklerinin tespit edilmesi ve bu tip etkili maddelere karşı önlemler alınması kaçınılmaz görülmektedir [1]. Bu konuda oldukça yaygın araştırmalar yapılmakta olup mutajenik, kanserojenik, teratojenik ve alerjik testlere sıkça başvurulmaktadır. Bir maddenin mutajen ya da karsinojen, diğer bir değişle genotoksik etkiye sahip olup olmadığının tespit edilmesinde kardeş kromatid değişimi (KKD), kromozom aberasyonu (KA) ve mikronükleus (MN) testi, sık kullanılan testler olup kısa süre içerisinde sonuç vermektedirler [2-7]. Yetişkin kemiricilerde kemik iliği birinci derecede hematopoietik organdır. Hematopoietik hücrelerin bölünmeleri esnasında bazı kimyasal maddeler kromozom hasarına ya da mitozun engellenmesine neden olmaktadır. Kromozomlardaki aberasyon oranındaki artış, kanser riskinin artması anlamına gelmektedir. Kromozomal aberasyonların DNA'daki zincir kırıklarının yanlış onarılmasından kaynaklanabileceği ileri sürülmektedir. Kromozomal aberasyonlara neden olan kimyasal maddelere bağlı olarak ya tüm kromozomlar, ya da kromozom parçaları hücre bölünmesi esnasında ayrı bir bölüm olarak geride kalıp mikronükleusu oluşturmaktadır. Mikronükleus, mitozun anafaz safhasında kardeş çekirdeğe katılmayan tüm kromozom ya da kromozom parçalarından oluşmuş, genetik materyal (DNA) taşıyan küçük parçacıklardır. Normal hücre çekirdeğinin 1/5 ile 1/20'si arasında bir büyüklüğe sahiptir [8]. Ortaya çıkan mikronükleus sıklığının belirlenmesi mikronükleus testi ile yapılmaktadır. Bu mikronükleus testi, *in vivo* ve *in vitro* olarak mutajen, klastojen ve diğer kimyasal maddeler tarafından indüklenen DNA hasarının belirlenmesinde kullanılan sitogenetik bir testtir. Mikronükleus testi ile uygulanan kimyasal maddenin olgun olmayan polikromatik eritrositlerde (PCE) oluşturduğu kromozom kayıpları ve hasarları, ayrıca dolaşımdaki kanda olgun normokromatik eritrosit'in (NCE) oluşumu üzerindeki etkileri belirlenebilmektedir. Mikronükleus testi kromozomlarda ortaya çıkan sayısal ve yapısal düzensizliklerin dolaylı olarak gösterilmesinde kullanılmaktadır. Organizmayı etkileyen çeşitli fiziksel ve kimyasal ajanların sitogenetik etkilerinin büyük çaplı tarama çalışmalarıyla ortaya konulmasında kullanılabilen güvenli bir test olarak değerlendirilmektedir [1].

Kimyasal maddeler ya doğrudan ya da metabolizma reaksiyonları ile etkinleştikten sonra genetik materyal üzerine etki etmektedirler. Kimyasal karsinojenlerin hücrelerin nükleofillerine karşı affinite gösterip, onları bağlayarak genetik yapının bozulmasına neden olduğu bilinmektedir. Polisiklik aromatik hidrokarbonlar (PAH) en güçlü karsinojenler olarak bilinmektedir. PAH'ların içinde yer alan 3-Metilkolanren'in (3-MC) memeli sistemlerinde deneysel olarak toksik ve karsinojenik etkileri gösterilmiştir. 3-MC gibi PAH'lara zift, kurum, sıvı ve katı yakıtlar nedeniyle havada, ekzoz gazında, sigara dumanında, dumanlanmış yiyeceklerde sıklıkla rastlanmaktadır. Toksik etkileri nedeniyle 3-MC çevre sağlığı açısından büyük önem taşımaktadır. Vücuda alındığında, mikrozomal P-450 enzim sistemini stimüle ederek fizyolojik madde ve ilaçların metabolizma ve toksisitesini değiştirmektedir [9-12]. Yaptığı indüksiyon sonucu vücutta N-oksidasyon ve hidroksilasyon reaksiyonlarında hızlanmaya neden olmaktadır [10,11,13]. 3-MC ile indüklenen sitokrom P-4501A çok sayıda maddenin biyoaktivasyonunda rol oynamaktadır. Bu nedenle 3-MC hem doğrudan hem de diğer maddeleri aktive ederek mutasyona neden olduğu bilinmektedir. Yaptığı genotoksik etkiler sonucu teratojenite ve çeşitli kanser tipleri ortaya çıkmaktadır [10,11,14].

Sisteamin tiyoetanolamin, beta-merkaptoetilamin veya 2-aminoetantiyol yapısında olup, vücutta sisteinin dekarboksilasyonu ile oluşmaktadır. Koenzim A'nın yapısına girmesi nedeniyle enerji üretimi için gerekli olup antioksidan ve antiklastojenik bir maddedir. İlaç ve zehirlerin detoksifikasyonuna katılan, sistein, glutamin ve glisinden oluşan ve bir tripeptit olan glutatiyonun yapısında dolaylı olarak yer almaktadır [15-17]. Ayrıca genetik bir hastalık olan sistinozisin tedavisinde kullanılmaktadır. Sistein vücutta sistin halinde depo edilmektedir. Sistin redüktaz enzimiyle indirgenerek protein sentezinde kullanılmaktadır. Sistini indirgeyen enzim genetik yapıdaki mutasyona bağlı olarak bazı bireylerde bulunmamaktadır. Böyle bireylerde sistin özellikle böbrek, göz, kas ve beyin gibi organlarda hücre içine fazla miktarda çökerek sistinozis denilen hastalığa neden olur. Sisteamin sistin molekülündeki disülfid bağını koparark sisteamin-sistin ve sistein açığa çıkarır. Sisteamin dopamin beta-hidroksilaz enzimini inhibe ederek somatostatin sentezini bloke eder. İnsülin sekresyonunu artırır. Bu etkileri nedeniyle sisteaminin büyümeyi stimüle ettiği bilinmektedir [18-20]. Sisteamin hücre metabolizmasında görev alır. Yapısına girdiği glutatiyondan dolayı sisteaminin hücre savunma sistemlerinde bir rol oynayabileceği düşünülmektedir. Sisteamin vücutta hipotaurine dönüşerek idrarla atılmaktadır [21-23]. Bu araştırmada sisteaminin farelerde 3-MC ile indüklenen genotoksisite üzerine koruyucu etkisinin olup, olmadığı araştırılmıştır.

MATERYAL ve METOT

Bu araştırma Kafkas Üniversitesi Hayvan Deneyleri Yerel Etik Kurulundan izin alınarak yapıldı (KAÜ-HADYEK 26.11.2010/49). Araştırmada ağırlıkları 20±2 g olan *Mus musculus* albino fareler kullanıldı. Fareler standart diyet ve çeşme suyuyla *ad libitum* olarak 4 ay süreyle beslendi. Sisteamin (CAS: 156-57-0) Fluka ve 3-MC (CAS: 56-49-

5) Supelco firmalarından temin edildi. Toplam 75 adet fare her grupta 15 adet olacak şekilde 5 gruba ayrıldı. Birinci grup, kontrol grubu olarak tutuldu. İkinci gruptaki hayvanlara skapula hizasından sırt bölgesi derisi altına 0.2 ml susam yağı, üçüncü gruba ad libitum olarak içme suyuyla %0.1 oranında sisteamin 4 ay süreyle verildi (Sisteamin çözeltileri üç günlük hazırlandı). Dördüncü gruptaki hayvanlara sırt bölgesi derisi altına 1 mg 3-MC/0.2 ml susam yağı solüyonundan 0.2 ml bir kez enjekte edildi (bu gruba sisteamin içme suyuyla verilmedi). Beşinci gruptaki farelere sırt bölgesi derisi altına 1 mg 3-MC/0.2 ml susam yağı solüsyonundan 0.2 ml deri altı yolla bir kez enjekte edildi ve enjeksiyonu takiben içme suyuyla %0.1 oranında sisteamin 4 ay süreyle ad libitum olarak verildi. Süre sonunda hayvanlar tartıldı. Farelere, ötanaziden 2 saat önce 4 mg/kg dozunda kolşisin distile suda çözülerek intraperitonal olarak enjekte edildi. Hayvanlar eter anestezi altında servikal dislokasyon yöntemi ile öldürülüp, her gruptan 8 adet farenin femur kemikleri çıkartıldı. Kemikler kaslarından iyice temizlenerek görünür hale getirildi. Femur kemiklerinden biri mitotik indeksin tespitinde, diğeri ise mikronükleus testinde kullanıldı.

Mitotik aktivite için metafaz preparatları Preston'a göre laboratuvar ve çalışma koşulları göz önünde bulundurularak yapıldı [24]. Her iki femura ait kemik iliği enjektör yardımı ile santrifüj tüpüne aktarıldı (içerisinde 3 ml fötal dana serumu bulunan). Femur örneklerinden birine ait tüpler, 1.100 rpm'de 10 dak. santrifüj edildi ve süpernatant atıldı. Etüvde 0.075 M 5 ml KCl solüsyonu 37°C'de 30 dak. ısıtıldı. Isıtılan hipotonik çözeltide hücreler, 20-30 dak. inkübe edildi. İnkübasyondan sonra hücreler 1.100 rpm'de 10 dak. tekrar santrifüj edilerek, süpernatant atıldı. Hücreler taze olarak hazırlanmış 5 ml soğuk Carnoy's (metanol:glasiyel asetik asit 3:1) içerisinde fikse edilerek, santrifüjlenip, tekrar süpernatant kısmı atıldı. Bu fiksasyon işlemi 3 kez tekrarlanıp, santrifüjden sonra süpernatant kısım, tüpün dibinde 0.5 ml kalacak şekilde uzaklaştırıldı. Dipte kalan hücreler pastör pipeti yardımıyla süspanse edildi ve nemli temiz lamlara 3-4 cm yukarıdan damlatılarak yayıldı. Yayma işlemi bittikten sonra preparatlar oda sıcaklığında kurutuldu ve önceden hazırlanan %10'luk giemsa ile 10 dak. boyandı. Olympus CX21 marka ışık mikroskobunda, mitotik aktivite için x1000 büyütmede her hayvan örneğinden rastgele 1.000 hücre sayıldı. Sayılan hücrelerin içerisinde metafaz safhasında olanların adetleri tespit edilip, mitotik indeksleri belirlendi.

Mikronükleus tespiti için kemik iliği preparatları ilk kez Schmid tarafından geliştirilen ve laboratuvar koşullarına göre modifiye edilmiş yöntem ile hazırlandı [25]. Femur kemiği iki ucundan kesilerek, kemik iliği enjektör yardımı ile içerisinde 3 ml dana serumu bulunan santrifüj tüpüne aktarıldı. İçerisinde kemik iliği numunesi bulunan tüpler 2.000 rpm de 5 dak. santrifüj edildi ve süpernatantları atıldı. Tüpte kalan kısmın üzerine bir damla dana serumu konularak süspanse edildi. Bundan alınan bir damla örnek temiz lamlar üzerine yayıldı. Yayma işlemi tamamlanan lamlar havada kurutuldu ve metil alkolde 10 dak. fikse edildi. Fikse edilmiş preparatlar %0.25'lik May Grunwald boyası ile 5 dak. boyanarak saf su ile yıkandı. Daha sonra %0.125'lik May Grunwald boyası ile 5 dak. tekrar boyanarak saf suda yıkandı. En son %20'lik Giemsa boyası ile 30 dak. boyanıp, yıkandı ve kurumaya bırakıldı. Hazırlanan her preparattan rastgele 2.000 adet PCE sayıldı. Bunların içerisinde MNPCE'lerin sayıları belirlenerek, yüzdeleri çıkartıldı. Ayrıca her hayvan örneğinden 1.000 adet eritrosit (PCE+NCE) sayılarak PCE/NCE oranları tespit edildi.

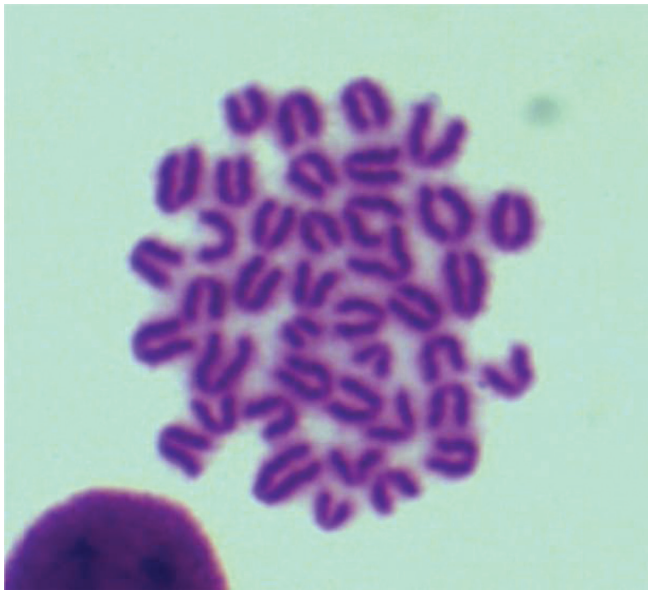
Gruplar arasında gözlenen faklılığın önemi (SPSS 18 paket programı) One - Way ANOVA Duncan testi ile istatistiksel açıdan değerlendirildi. $P<0.05$ önemli kabul edildi [26].

BULGULAR

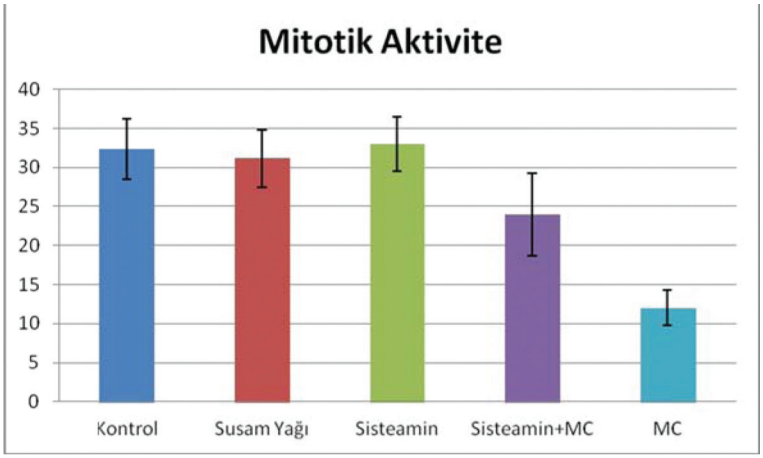
Deneyde 4 ay içerisinde dördüncü grupta üç, beşinci grupta ise bir adet fare olmak üzere toplam 4 ölüme rastlandı. Mitotik aktivite ve mikronükleus testi sağlam kalan ve ötanazi edilen hayvanlarda gerçekleştirildi.

Kontrol ve Deney Gruplarında Mitotik Aktivite

Araştırma *Mus musculus* ırkı albino erkek fare kemik iliği hücrelerindeki mitotik aktivite üzerine etkisinin olup olmadığını belirlemek için her gruptan yaşayan 8 farenin kemik iliği hücreleri üzerinde gerçekleştirildi. Her hayvandan rastgele belirlenen 1.000 hücre sayıldı ve metafaz evresindeki hücreler belirlenerek mitotik aktivite tespit edildi *(Şekil 1)*. Kontrol ve deney gruplarında mitoz görülen hücrelerin yüzde ortalamaları *Şekil 2* ve *Tablo 1*'de gösterilmiştir. Aynı şekilde kontrol ve deney gruplarından elde



Şekil 1. Fare metafaz örneği
Fig 1. Mouse metaphase example



Şekil 2. Mitotik aktivitenin gruplara göre değişimi

Fig 2. Mitotic activity change according to the groups

Tablo 1. *Kontrol ve deney gruplarının mitotik aktivite bakımından karşılaştırılması*
Table 1. *Comparison of the experimental and control groups in terms of mitotic activity*

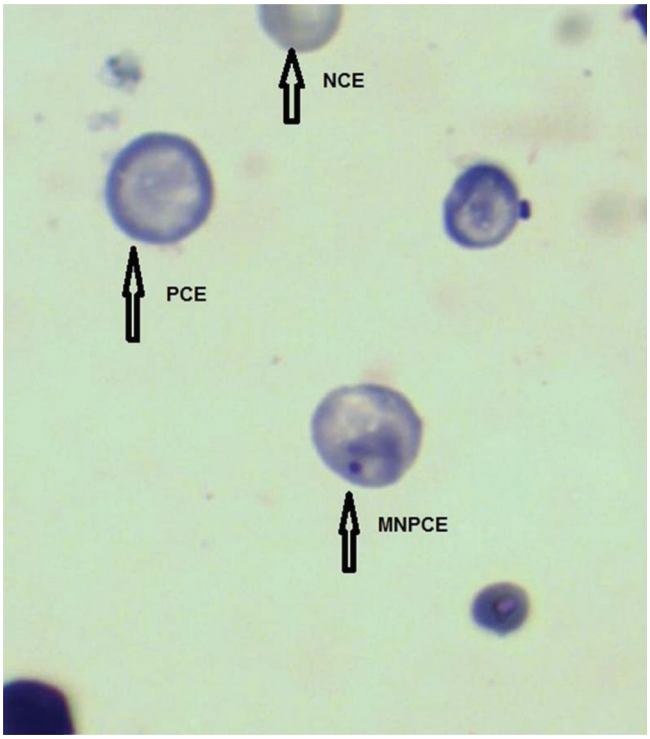
Gruplar	Denek Sayısı	Toplam Hücre Sayısı	İnterfaz Hücre Sayısı	Metafaz Hücre Sayısı	Grup Ortalaması	Metafaz Hücre Oranı Ortalaması (%)
Kontrol	8	8000	7741	259	32.38±3.89^{c}	3.238
Susam Yağı	8	8000	7751	249	31.13±3.68^{c}	3.113
Sisteamin	8	8000	7736	264	33±3.46 ^{c}	3.3
Sisteamin+MC	8	8000	7807	193	24±5.26^{b}	2.42
MC	8	8000	7904	96	12±2.27^{a}	1.2

Sütunlarda farklı harfler (a,b,c) istatistiksel olarak önemliliği göstermektedir (P<0.001) c-b=P<0.01, c-a=P<0.001, a-b=P<0.001

edilen metafaz evresindeki hücrelerin grup ortalamaları arasında gözlenen farklılığın anlamının belirlenmesi için Duncan Testi yapıldı ve elde edilen sonuçlar *Tablo 1*'de verildi (P≤0.001). Kontrol ve deney grupları arasında gözlenen farklılık istatistiksel açıdan önemine göre tablo üzerinde harflerle belirtildi. Kontrol, susam yağı ve sisteamin grupları arasında mitotik aktivitede istatistiksel açıdan anlamlı bir fark bulunmazken, 3-MC'nin mitotik aktiviteyi kontrol gruplarına göre anlamlı bir derecede düşürdüğü tespit edildi (P<0.001). 3-MC ile birlikte uygulanan sisteaminin (Grup 5) kontrol grubuna kıyasla mitotik aktiviteyi önemli ölçüde düşürdüğü fakat bu düşüşün yalnız başına verilen 3-MC'li grup (Grup 4) kadar olmadığı belirlendi (P<0.01).

Kontrol ve Deney Gruplarında Mikronükleus Sıklığı

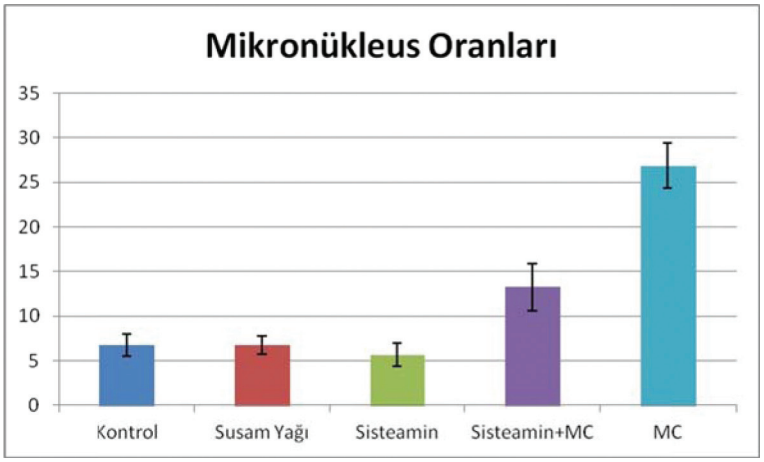
Mikronükleus testi için her bir hayvanın kemik iliği preparatlarında toplam 2000 PCE sayıldı. Sayılan bu 2.000 hücrede MNPCE sayısı belirlendi. Ayrıca her hayvan örneğinden toplam 1.000 eritrosit sayılarak PCE/NCE oranları hesaplandı. 3-MC *Mus musculus* ırkı albino farelerin kemik iliği polikromatik eritrositlerinde mikronükleus oluşturduğu *Şekil 3*'te gösterildi (MNPCE ve PCE/NCE). Kontrol ve deney gruplarından elde edilen MNPCE ve PCE/NCE oranları *Şekil 4* ve *Tablo 2*'de verildi. Gruplardan elde edilen sonuçlara Duncan Testi uygulanarak bulunan anlamlı sonuçlar (P≤0.001) *Tablo 2*'de gösterildi. 3-MC uygulanan grupta MNPCE sayısında istatistiksel açıdan önemli bir artış saptandı (P<0.001). Kontrol, susam yağı



Şekil 3. Fare eritrosit (PCE), (NCE), (MNPCE) örnekleri

Fig 3. Mouse erythrocyte (PCE), (NCE), (MNPC) samples

ve sisteamin gruplarında artış gözlenmezken, 3-MC + sisteamin uygulanan grupta kontrol gruplarına kıyasla mikronükleus sayısında önemli bir artış gözlendi. Fakat



Şekil 4. Mikronükleus sıklığının gruplara göre değişimi

Fig 4. Micronucleus frequency change according to the groups

Tablo 2. *Kontrol ve deney grupları arasında mikronükleus testinden elde edilen sonuçların karşılaştırılması (PCE: Polikromatik Eritrosit, MNPCE: Mikronükleuslu Polikromatik Eritrosit, NCE: Normokromatik Eritrosit)*

Table 2. *Between the control and experimental groups to compare the results of the micronucleus test (PCE: Polychromatic Erythrocytes, MNPC: Polychromatic Erythrocyte Micronucleus, NE: Normochromatic erythrocyte)*

Gruplar	Toplam PCE	MNPCE	MNPCE (%)	Grup Ortalaması	Toplam Eritrosit (PCE+NCE)	PCE Sayısı	NCE Sayısı	PCE/NCE Oranı
Kontrol	16000	54	0.33	6.75±1.28 ^{c}	8000	4993	3007	1.660 ^{b}
Susam Yağı	16000	54	0.33	6.75±1.04 ^{c}	8000	4996	3004	1.663 ^{b}
Sisteamin	16000	45	0.28	5.63±1.30 ^{c}	8000	4999	3001	1.665 ^{b}
Sisteamin+MC	16000	106	0.66	13.25±2.66^{b}	8000	3840	4160	0.923 ^{a}
MC	16000	215	1.34	26.88±2.53^{a}	8000	3523	4477	0.786 ^{a}

Sütunlarda farklı harfler (a,b,c) istatistiksel olarak önemliliği göstermektedir (P<0.001), c-b=P<0.001, c-a=P<0.001, b-a=P<0.001

bu artışın 3-MC uygulanan gruptakinden daha az olduğu tespit edildi.

TARTIŞMA ve SONUÇ

Genotoksik ajanlar hücre DNA'sı üzerinde olumsuz etkilere neden olan, diğer bir değişle genetik materyali bozan maddelerdir. Yaptıkları genotoksik etkiler mutasyona neden olur. Mutasyon normal somatik hücrelerinde kanserojen, germ hücrelerinde ise teratojen ve kanserojen etkilerle kendisini gösterebilir. Ayrıca çeşitli idiosinkratik ve alerjik reaksiyonlara neden olur. Bu olumsuz etkiler gelecek nesillere aktarılır. Bu nedenle mutasyon daima dikkate alınması gereken bir konudur. Sağlık açısından mutasyon yapıcı ajanların araştırılması ve ortaya konması büyük bir önem arz eder. Ancak her mutasyonun kötü sonuçlara neden olmayabileceğinin unutulmaması gerekir [27].

Bazı farmasötik (yüksek dozlarda) ve kimyasal maddeler, kanser tedavisinde kullanılan sitotoksik (antineoplastik) ajanlar, ayrıca radyoaktif ilaçlar veya böyle maddeleri ihtiva eden atıklar genotoksik etkilere neden olabilmektedirler. Bu tür genotoksik maddeler doğrudan etkili olabilecekleri gibi bazıları metabolik reaksiyonlarla aktif hale dönüşerek genotoksisiteye neden olmaktadır. Vücuttan atılan aktif formları aynı etkiyi diğer canlılar için gösterebilmektedir. İdrar, dışkı veya diğer vücut artıkları ile aktif formlarda atılan bu tip ajanlar çevredeki diğer canlılarda geno-toksisiteye sebep olabilmektedir. Bu tip canlılar arasında bakteri ve mantarlar da bulunur. Sonuçta bu durum mikroorganizmaların patojenisitesi değişebilir [27].

Deneysel kanser oluşturmada 3-MC fazla kullanılan ajanlar arasındadır. Kansere neden olan bu madde klastojenik etkilidir. Bakterilerde mutajenik etkinliği gösterilmiştir. Bu nedenle kimyasal karsinojen ve mutajen olarak tanımlanmıştır. Hayvanlarda DNA'yı uyardığı bildirilmiştir. Cyp-450 gen ekspresyonunu artırarak kanser gelişimine neden olmaktadır. Yapılan in vivo araştırmalarda 3-MC'nin 40 mg/Kg dozda intraperitonal olarak bir kez enjekte edilmesiyle 6 saat içerisinde Cyp1A gen transkripsiyon oranında 179 kez artışa neden olduğu belirlenmiştir. Yine Cyp 2C11 geninde transkripsiyon oranında kontrole göre %51 oranında redüklenmeye neden olduğu gösterilmiştir [28-31].

Yapılan bir çalışmada, dimetilnitrozamin, asetilaminofloren, aflatoksin B_1 ve 3-metilkolantrenin mikronükleus testi ile hem fare hem de hamsterlarda genotoksisitesi araştırılmıştır. Çalışmada kullanılan bu dört kimyasalın kemik iliği hücrelerinde mikronükleus frekansını artırdığı tespit edilmiştir [32].

Yapılan diğer bir çalışmada ise 3-MC'nin fare ve ratlarda fototoksisiteye neden olduğu ortaya konmuştur.

Yine aynı çalışmada 3-MC'nin mutasyona neden olduğu mikronükleus testi ile ispatlanmıştır [33].

Çeşitli araştırıcılar tarafından sisteaminin antikanserojenik ve antimutajenik etkileri de çalışılmıştır. Doğan ve ark.[34] yaptıkları araştırmada sisteaminin 3-MC ile indüklenen fibrosarkoma insidensinde azalma yaptığını belirlemişlerdir.

Bianchi ve ark.[35] tarafından yapılan bir başka çalışmada ise ultraviyole ve floresan ışığına maruz kalan hücrelerde sisteaminin etkisine bakılmıştır. Hücre kültürüne eklenen sisteaminin SCE frekansını artırmadığı, UV ve floresan ışığa maruz kalan hücrelerde SCE frekansında artış olduğu gözlenmiştir. UV ve floresan ışığı ile indüklenen hücrelerin sisteamin varlığında SCE frekansında önemli bir düşüş olduğu da tespit edilmiştir.

Tatsuta ve ark.[36] Wistar ırkı ratlara N-metil-N-Nitro-N-nitrozoguanidin verilerek indüklenen gastrik adenokarsinomaya karşı oral verilen sisteaminin etkisine bakılmış, oral olarak verilen sisteaminin adenokarsinoma sayısını önemli ölçüde azalttığı tespit edilmiştir.

Major ve ark.[37] normal periferal kan lenfositleri, in vitro kültürü yapılmış normal embriyo fibroblastları, down sendromlu hastaların periferal kan lenfositleri ve Trisomy 21'li embriyo fibroblastlarının 3-MC uygulamasından sonraki indüklenebilir kardeş kromatit değişimleri araştırılmıştır. Down sendromu bulunanların lenfositlerinde SCE frekansı sağlıklı kontrollerle karşılaştırıldığında 2.5 kat arttığı gözlemlenmiştir. Ancak Trisomy 21'li fibroblastlar normal fibroblastlarla karşılaştırıldığında, MC uygulamasına duyarlı bir yükseliş göstermemiştir.

Prasanna ve ark.[38] benzo[a]pyrene, 3-methylcholanthrene ve 3-methylcholanthrylene'nin Salmonella typhimurium TA98 ve TA100 s suşlarında oluşturduğu mutajenitenin selenyum ile inhibisyonunu çalışmışlardır. Bu maddelerin oluşturduğu mutajeniteyi selenyumun düşürdüğünü gözlemlemişlerdir.

Sozmen ve ark.[39] farelerde 3-MC ile indüklenmiş fibrosarkomalarda mitosis ve apoptozis ile Tunel metodu kullanarak siklin A nın ekspresyonunu araştırmışlardır. Sonuç olarak; siklin A ekspresyonunun önemli derecede pozitif koralasyon oluşturup 3-MC ile indüklenen fibrosarkomaların patogenesisinde önemli olabileceğini vurgulamışlardır.

Bu araştırmada 3-MC'nin genotoksik etkisi hem mikronükleus testi hem de mitotik aktivite yöntemi ile belirlenmiştir. 3-MC'nin mitotik aktiviteyi düşürdüğü, mikronükleus frekansını ise artırdığı tespit edilmiştir. Her iki durum kontrol grubuna göre istatiksel açıdan anlamlı bulunmuştur. Bu sonuçlar 3-MC'nin genotoksik etkili olduğunu göstermektedir. Çalışmada sisteaminin genotoksik etkilerinde azalma yaptığı görülmektedir. Tablolara bakıldığı zaman kontrol grubunda mitotik aktivite grup ortalaması 32.38 iken, 3-MC'li grupta 12 ve MC + sisteaminli grupta ise 24 olarak bulunmuştur. Yine kontrol grubunda mikronükleus sıklığı grup ortalaması 6.75 iken, MC'li grupta 26.88 ve MC + sisteaminli grupta da 13.25 olduğu tespit edilmiştir. Bu sonuçlar diğer araştırıcıların elde ettiği bulgularla bir paralellik göstermektedir.

Sonuç olarak, 3-MC'nin fare kemik iliği hücrelerinde genotoksisiteye neden olduğu, sisteaminin ise 3-MC tarafından oluşturulan bu genotoksik etkiyi azalttığı söylenebilir. Bu bulgular, sisteaminin 3-MC genotoksisitesine karşı kullanımı konusunda yapılacak yeni çalışmalara ışık tutacağı kanısındayız.

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RESEARCH ARTICLE

Molecular Typing of Methicillin Resistant *Staphylococcus aureus* Strains Isolated from Cows and Farm Workers [1] [2] [3]

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Summary

The aims of this study were to isolation and identification of methicillin resistant *Staphylococcus aureus* (MRSA) from mastitic cow milk and nasal swabs of cows and related workers, investigation presence of Panton-Valentine leucocidin (PVL) genes and molecular characteristics of these isolates. One hundred forty five mastitic milk samples were obtained from of 56 cows and nasal swabs were collected from these cows and 34 farm workers. MRSA isolates were recovered by pulsed field gel electrophoresis (PFGE), staphylococcal chromosomal cassette (SCC) *mec* typing, staphylococcal protein A (*spa*) typing, multilocus sequence typing (MLST). A total of 12 MRSA were identified. All strains were PVL genes negative. PFGE analysis of all 12 MRSA isolates produced four distinct pulsotypes (PT) designated as PT A-D. The PT A (7 strains) detected sequence type (ST) were ST 239, *spa* type t030 and SCC*mec* III except one. The PT B (3 strains) were ST1294, t459 and SCC*mec* III, the PT C (1 strain) was ST1940, t660, SCC*mec* III detected, the PT D (1 strain) was ST737, t542, SCC*mec* II. Consequently, it can be said that t030 and ST239 was the most common *spa* and MLST types which isolated from cow milk and nasal swabs and nasal swabs from workers of these farms in Aydın region, Turkey.

Keywords: *MRSA, PFGE, SCCmec, spa, MLST*

Sığırlardan ve Sektör Çalışanlarından İzole Edilen Metisilin Dirençli Stafilokokların Moleküler Tiplendirmesi

Özet

Bu çalışma mastitisli sığır sütleri ile bu sığırlar ve çiftlik çalışanlarının nazal sıvaplarından metisilin dirençli *Staphylococcus aureus* (MRSA) izolasyon ve identifikasyonu, izolatların moleküler özelliklerinin ve Panton–Valentine Leucocidin (PVL) genlerinin varlığının incelenmesi amaçlandı. Elli altı sığırdan 145 mastitisli süt ile 56 sığır ve 34 çiftlik çalışanına ait nazal sıvaplar alındı. MRSA izolatlarının moleküler özellikleri itilmiş alan jel elektroforezisi (PFGE), stafilokokal protein A (*spa*), çok lokuslu dizi tiplendirme (MLST) ve stafilokokal kromozom kaset *mec* (SCC*mec*) tiplendirmesi ortaya konuldu. Tüm suşlar PVL genleri bakımından negatif idi. Toplam 12 MRSA identifikasyonu yapıldı. PFGE analizi sonucunda 12 MRSA izolatında pulsotip A-D olamak üzere dört pulsotip belirlendi. Pulsotip A olan suşların (n=7) sekans tipi (ST) tipi ST 239, *spa* tipi t030 olarak bulunurken biri hariç hepsinin SCC*mec* III taşıdığı tespit edildi. Pulsotip B (n=3) ST1294, t459 ve SCC*mec* Tip III; pulsotip C (n=1) ST1940, t660, SCC*mec* Tip III ve pulsotip D (n=1) ST737, t542, SCC*mec* tip II olarak belirlendi. Çalışma sonucunda, Aydın yöresinde sığır süt, insan ve sığır burun sıvaplarından izole edilmiş olan en yaygın *spa* tipi t030, MLST tipinin ise ST 239 olduğu belirlendi.

Anahtar sözcükler: *MRSA, PFGE, SCCmec, spa, MLST*

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is a significant human pathogen that is also an emerging concern in veterinary medicine. It is present in an extensive range of animal species both as a cause of infection and

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in healthy carriers [1]. Methicillin resistance is caused by a modified penicillin binding protein PBP2a, which has a low affinity for all beta lactam antimicrobials, encoded by the *mec*A gene. Most of the methicillin resistant isolates show heterogeneous resistance therefore detection of the presence of *mec*A gene by PCR is accepted as "gold standard" and genotyping of MRSA is important in order to determine outbreaks [2].

Molecular typing techniques have been used with rising frequency in studies on epidemiology of methicillin resistant staphylococci (MRS), and also for a better understanding of the evolutionary relations among MRSA clones. The most important techniques used to investigate the molecular epidemiology of *S. aureus* are pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), *S. aureus* protein A (*spa*) and SCC*mec* typing [3]. The organization of SCC*mec* can be determined with a number of PCR based methods, such as *mec*A specific multiplex PCR by Oliveira and Lencastre. The SCC*mec* typing may supply to determination of hospital associated MRSA (HA MRSA) and community associated MRSA (CA MRSA) clones [4]. *spa* typing uses the DNA sequencing of a polymorphic 24 bp and *S. aureus* specific *spa* gene for discrimination of *S. aureus* [5]. The *S. aureus* MLST scheme uses the allelic profile of 7 housekeeping genes to establish the strain type [6].

MRSA infections in humans are an important healthcare problem and a serious economic burden. Several local studies showed an MRSA prevalence of 12-90% in humans [7-9]. In contrast to human isolates, few genotypic studies have reported that MRSA strains isolated from cow milks in Turkey [10-12]. Minimal information is available about MRSA colonization of healthy cattle [1]. It reported that this ratio is varied from 0 to 28% in different countries [13,14]. Clearly, more study of healthy cattle is required [1]. The aims of this study were to isolation and identification of MRSA from mastitic cow milk and nasal swabs of cows and related workers, investigation presence of Panton–Valentine leucocidin (PVL) genes and molecular characteristics using PFGE, SCC*mec*, *spa* and MLST typing of these isolates.

MATERIAL and METHODS

The Ethic Committee on Research Animal Care at Adnan Menderes University of Aydin, Turkey approved all procedures in this study (No: ADÜ-HADYEK-2010/97).

Bacterial Strains

The strains were isolated from nasal swabs of 34 farm workers, 145 mastitic milk and nasal swabs of these 56 cows obtained from 15 farms.

Diagnosis of Mastitis

The diagnosis of mastitis was done by veterinary practitioners, milk samples and cow nasal swabs were taken these cows. Clinical mastitis was diagnosed by changes in the udder and milk compositions. Changes in the udder included pain, swelling, warmth and abnormal appearance (blood tinged milk, watery secretions, clots, pus) of milk. Cows that did not have clinical mastitis were subjected to further investigation for subclinical mastitis by using CMT. The procedures and interpretations were performed previously [15]. For taken milk samples, teat ends were cleaned using 70% alcohol moistened swabs and allowed to dry. After discarding the first few streams, 2-5 ml of the milk samples were collected into sterile 5 ml glass flasks.

Nasal Swaps

Human nasal swaps were taken by a nurse. Nasal samples were taken for sampling from the medial septum area of both nostrils by gently rubbing mucosa approximately for 5 s with a cotton-tipped swab moistened with sterile water. Nasal swabs were transported to the laboratory in the day of sampling in Amies transport medium in cold chain.

Identification of MRSA Strains

The swabs were immediately suspended in 5 ml Tryptone Soya Broth (TSB, Oxoid) containing 7.5% sodium chloride and incubated for 48 h 37°C for selective enrichment of staphylococci. Enrichment cultures and milk samples were then streaked on to Mannitol Salt Agar (MSA, Oxoid) to isolate of staphylococci. The staphylococcal isolates were identified morphologically and biochemically by standard laboratory procedures. For discrimination of coagulase positive stahylococci (CoPS) from CoNS, the coagulase test was performed. Gram positive, catalase positive, coagulase positive, oxidase negative, bacitracin resistant, hemolytic, acetoin production positive, β galactosidase negative, mannitol-fermenting, and polymyxin B resistant staphylococci were identified as *S. aureus* [15].

S. aureus strains were tested for methicillin resistance using disc diffusion method outlined by the CLSI (2006). For this cefoxitin, discs (30 µg; Oxoid) were used. Zone sizes were read after incubation at 37°C for 24 h. Strains with zone sizes less than 19 mm were considered as methicillin resistant and studied further.

DNA Extraction

For genomic DNA from individual pure cultures of *E. coli* isolates were extracted with InstaGeneTM DNA extraction kit (Bio-Rad) according to the manufacturer's instructions.

Detection of the mecA, nuc and PVL Genes

In our study *S. aureus* specific thermonuclease and methicillin resistance genes were defined by presence of *nuc* and *mec*A genes, respectively. The oligonucleotide

primers described by and Kim et al.[17] and Oliveira and Lencastre [4] were used. Presence of PVL toxin genes are studied using method and primers described by previously [18].

Molecular Characterization of the MRSA Isolates

SCC*mec* types of the isolates were determined using methods and primers described by Oliveira and de Lencastre [4]. For the detection of *mec*A and SCC*mec* typing methicillin susceptible (*S. aureus* ATCC 29213) and methicillin resistanat (*S. aureus* HPV107, *S. aureus* N315, *S. aureus* HUSA304, *S. aureus* GRE14) reference strains used as negative and positive control in PCR, respectively. Visualization of PCR products was performed on 2% agarose gel stained with ethidium bromide.

PFGE was performed as previously described [19]. The SmaI digested DNA fragments were separated using CHEF DR III in 1% agarose gel for an initial time of 5 s and a final time of 25 s; at 14°C and during 18 h at 6.0 V with an angle of 120°. MLST and *spa* typing and analysis were performed as previously described in http://www.mlst.net and http://www.spaserver.ridom.de, respectively.

RESULTS

Isolation and Identification

From 235 (90 nasal swab, 145 mastitic milk samples) materials, 181 staphylococci were isolated and of these 92 were identified as *S. aureus* and 12 were detected as MRSA. Of these 12 materials originated from 6 (50%) were animal (2 bovine milk, 4 bovine nasal swab) and 6 (50%) were human (nasal swab). All MRSA strains were positive for *mec*A and *nuc* gene and but PVL gene negative. Fragments of expected sizes were 162 and 279 bp for the *mec*A and *nuc* genes, respectively (*Fig. 1* and *Fig. 2*). These MRSA strains were studied further.

Molecular Typing

Four SCC*mec* type from 12 MRSA isolate were determined using multiplex PCR. SCC*mec* Type II was found in 1, Type III in 10, Type IV in 1 *(Fig. 3)*. For this, one of the materials found as CA MRSA, while 11 were HA MRSA. The analysis of pulsed-field gel electrophoresis (PFGE) profiles revealed presence of 4 clusters *(Fig. 4)*. The first pulsotype (PT) A (7 isolates) clone strains were SCC*mec*

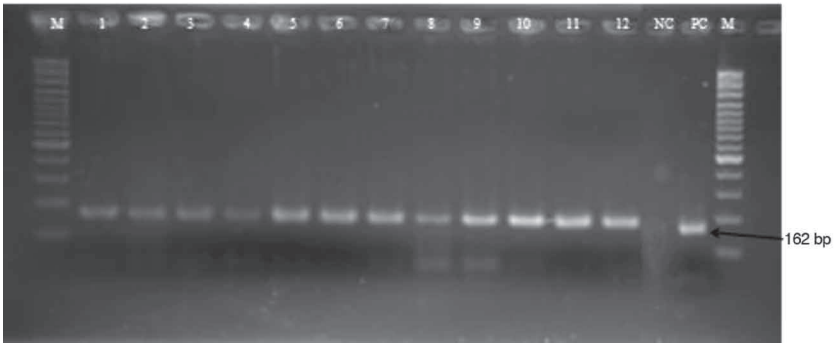
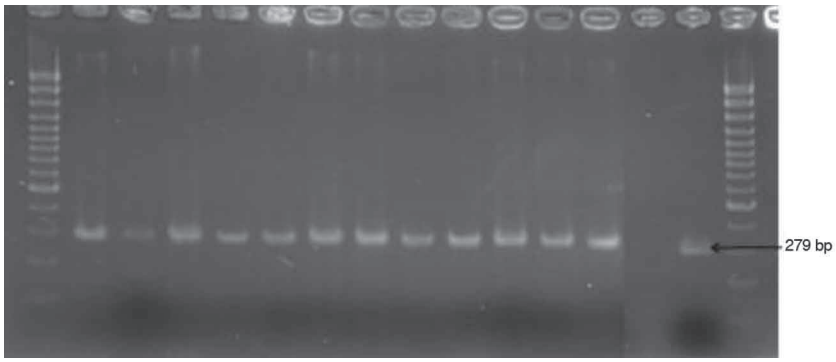


Fig 1. *mec* PCR M: Marker (100 bp DNA ladder) 1-12: *S. aureus* field isolates PC: Positive Control (*S. aureus* N315 strain, 162 bp positive) NC: Negative Control (without DNA master mix)

Şekil 1. *mec* PCR M: Marker (100 bp DNA ladder) 1-12: *S. aureus* saha izolatları PC: Pozitif Kontrol (*S. aureus* N315 suşu, 162 bp pozitif) NC: Negatif Kontrol (DNA'sız master miks)

Fig 2. *nuc* PCR M: Marker (100 bp DNA ladder) 1-12: *S. aureus* field isolates PC: Positive Control (*S. aureus* N315 strain, 279 bp positive) NC: Negative Control (without DNA master mix)

Şekil 2. *nuc* PCR M: Marker (100 bp DNA ladder) 1-12: *S. aureus* saha izolatları PC: Pozitif Kontrol (*S. aureus* N315 strain, 279 bp pozitif) NC: Negatif Kontrol (DNA'sız master mix)



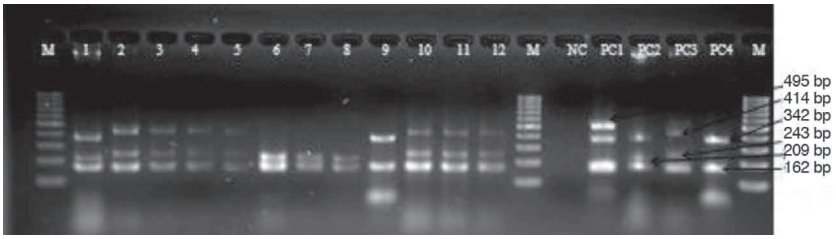


Fig 3. SCC*mec* types of isolates M: Marker (100 bp DNA ladder) 1-12: *S. aureus* field isolates PC: Positive Controls (PC1: *S. aureus* HPV107, PC2: *S. aureus* N315, PC3: *S. aureus* HUSA304, PC4: *S. aureus* GRE14) NC: Negative Control (*S. aureus* ATCC 29213)

Şekil 3. İzolatların SCC*mec* tipleri M: Marker (100 bp DNA ladder) 1-12: *S. aureus* İzolatları PC: Pozitif Kontroller (PC1: *S. aureus* HPV107, PC2: *S. aureus* N315, PC3: *S. aureus* HUSA304, PC4: *S. aureus* GRE14) NC: Negatif Kontrol (*S. aureus* ATCC 29213)

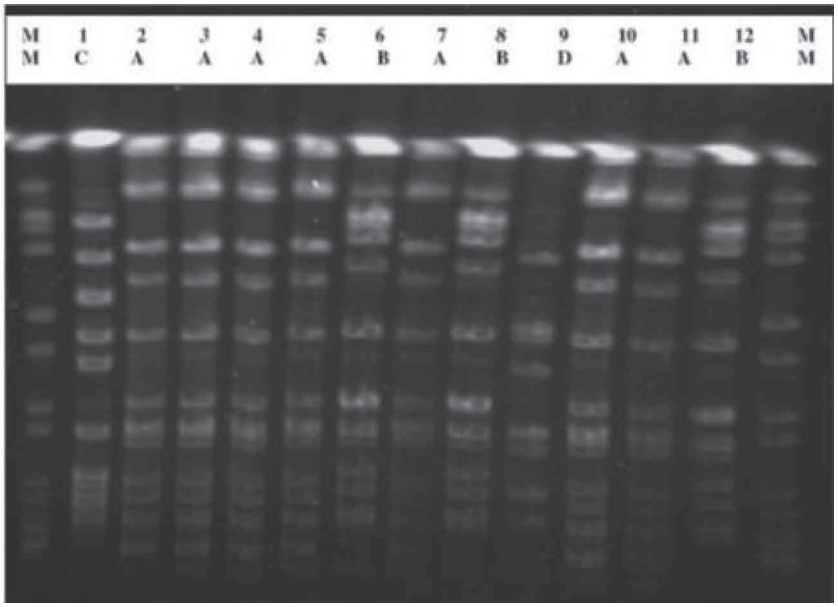


Fig 4. Pulsed-field gel electrophoresis analysis of 12 methicillin-resistant *S. aureus* strains.

PFGE profiles revealed presence of 4 clusters. M: Marker (*S. aureus* NCTC 8325 strain)

1: Pulsotype C; 2-5, 7, 10, 11: Pulsotype A; 6, 8, 12: Pulsotype B; 9: Pulsotype D

Şekil 4. On iki metisilin dirençli *S. aureus* suşunun İtilmiş Alan Jel Elektroforezisi. PFGE profilleri, 4 grup varlığını ortaya koydu. M: Marker (*S. aureus* NCTC 8325 suşu)

1: Pulsotip C; 2-5, 7, 10, 11: Pulsotip A; 6, 8, 12: Pulsotip B; 9: Pulsotip D

Table 1. *PFGE profiles, SCCmec, MLST and spa types of isolated 12 MRSA strains*
Tablo 1. *İzole edilen 12 MRSA suşunun , SCCmec, MLST ve spa tipleri ile PRGE profilleri*

Strain	Farm	Material	PFGE (PT)	SCC*mec* type	MLST (ST)	*Spa* (t)
1	1	HNS (FW)	D	II	737	542
2	2	BM	A	III	239	030
3	7	BNS	B	III	1294	459
4	7	BNS	B	III	1294	459
5	10	BM	A	III	239	030
6	10	BNS	A	III	239	030
7	11	BNS	B	III	1294	459
8	11	HNS (FW)	C	III	1940	660
9	12	HNS (FW)	A	IV	239	030
10		HNS (VP)	A	III	239	030
11		HNS (VP)	A	III	239	030
12		HNS (VP)	A	III	239	030

HNS: *Human nasal swab,* ***FW:*** *Farm Worker,* ***VP:*** *Veterinary Physician,* ***BNS:*** *Bovine Nasal Swab,* ***BM:*** *Bovine Milk*

type III (except one strain, the 9. strain SCC*mec* type IV), *spa*-types t030 (allelic profile 15-12-16-02-24-24) and MLST-types ST239 (allelic profile 15-12-16-02-24-24). The PT B (3 isolates) clone strains were type III with SCC*mec* typing, *spa*-types t459 (allelic profile 15-12-16-02-24) and MLST-types ST1294 (2-3-1-1-4-4-115). The third PT (C, 1 isolate) was SCC*mec* type III, *spa*-types t660 (allelic profile 11-12-41-20-17-12-12-17) and MLST-types ST1940 (allelic profile 213-1-4-1-5-5-4). The last, PT D (1 isolate), was SCC*mec* type II, *spa*-types t542 (allelic profile 26-23-13-23-13-23-31-05-17-25-17-25-16-28) and MLST-types ST737 (allelic profile 7-6-1-70-8-8-6). Distribution of SCC*mec*, *spa*, MLST types and PFGE profiles of 12 MRSA isolates is shown in *Table 1*.

DISCUSSION

MRSA is a growing problem in humans and animals [1,3]. While PFGE is accepted as a golden standard for determination of clonal relations between strains, SCC*mec* is used to detect the strains whether originated from hospital or community acquired. Recently, DNA sequence based methods for typing *S. aureus* such as MLST and *spa* typing have been developed and are widely used to establish clonal relationships between strains and to compare the geographical locations of MRSA clones [3,6]. In this study, the investigation of 12 MRSA strains with using PFGE, SCC*mec*, MLST, and *spa* typing methods which have been isolated from dairy cattle and farm

workers was aimed.

The description "methicillin-resistant" was first used in 1961 [20] in humans then the first MRSA strain in animals was reported at 1972 from dairy cattle with mastitis [21]. Since that time, MRSA has emerged as a significant problem worldwide. The number of cases was increased in the following years [1]. Vanderhaeghan et al.[21] reported that the share of MRSA in milk samples with clinic and subclinical mastitis was 10% in Belgium, while this share had been given as 17.2% by Turkyılmaz et al.[12] for Turkey. It reported that this ratio is varied from 0 to 60% in Turkey according to the limited number of studies in which methicillin resistance has been investigated with phenotypic methods in cow milk samples with mastitis [12]. It also claimed that it was not enough for determining the *mec*A gene only with phenotypic disc method. For that reason, results have to be confirmed with molecular methods against the probability of false positive and negative results [23].

The most common SCC*mec, spa* and MLST types show some differences according to the countries. For example, SCC*mec* Type I is common in Croatia [24] and Swiss [25]; Type II is common in Japan and Korea; Type III is common in Saudi Arabia, Singapore, China, Thailand, India [26], and also in Turkey [27]; Type IV is especially spread out in Spain [28], Greece [29]. As in humans, the most common SCC*mec* type in Turkey was Type III amongst *S. aureus* strains isolated from mastitic cow milks [11]. In this study, as parallel to above findings, the most common SCC*mec* type was found as Type III (83.3%) in both human and animals. It thought that there were a lot of hospital originated strains in animals and animals could have been infected with these strains because of poor hygienic conditions in these places.

In this study, just in humans in our country, it determined that the most common *spa* type was t030 (58.3%). t030 was isolated from strains which had been originated from human and animals between 1998 and 2011 years in Turkey, France, Iran, China, Swiss, South Africa, Check Republic, Norway, Spain, Denmark, Sweden, Lebanon, Romania, Croatia, Cyprus, Bulgaria, and France. t459 isolated from human originated samples collected between 2005 and 2011 years in Germany, Denmark, and China; t542 isolated from human originated samples collected between 2006 and 2010 years in Denmark and Germany; t660 isolated from human originated samples collected between 2007 and 2011 years in France, Holland, Spain, Sweden and Lebanon *(www.ridom.de/spa-server/)*.

MLST analysis revealed that the most common sequence type was ST239 (58.3%). This sequence type was also the most common sequence type for human in Turkey. Sequence types (ST737, ST1294, and ST1940) determined in this study had never found before in animals *(www.ridom.de/spa-server/)*. This can be aroused from there was no MLST typing before for *S. aureus* strains isolated from animals and these strains might have been originated from humans.

MRSA can be transmitted between people and animals during close contact [30]. It is certain that animals are a source of human MRSA infection in some circumstances, humans may also serve as sources of infection in animals. Some groups of individuals who work closely with animals, such as veterinarians, have high MRSA colonization rates. In our study, strains had been divided to four pulsotype. A total of 6 strains which consisted from 2 cow milk samples, 1 nasal cow nasal swaps and 3 nasal swaps taken from veterinarians who had treated these animals revealed that MRSA has clonal contagious traits. In this study, it was thought that the results of PFGE, MLST and *spa* typing methods support each other. Although it reported that animals can be a reservoir for MRSA which causes serious infections in humans [30], recent studies show that MRSA infections may be originated from humans [12,31]. In the study, the same pulsotype, MLST and *spa* typing results between two samples which had been taken from veterinarians and dairy cattle thought that veterinarians have important role in transferring of the strains to animals, and also thought that these infections may be originated from humans.

Consequently, it determined that the most common *spa* type and MLST type isolated from cow milk, cow nasal swap and human nasal swap samples in Aydın region were t030 and ST239, respectively. It recommended that a multipurpose epidemiologic study based on MLST and *spa* typing methods should be designed to compare MRSA isolates in animals in Turkey with isolates in the World.

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RESEARCH ARTICLE

Prevalence of Cryptosporidiosis and Molecular Characterization of *Cryptosporidium* spp. in Calves in Erzurum [1]

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Summary

This study was conducted to determine the prevalence of cryptosporidiosis and to identify *Cryptosporidium* species found in preweaned calves, in Erzurum, Turkey. Fecal samples were collected from 307 calves up to one month old from 5 dairy farms. Genomic DNA was obtained by DNA extraction (QIAamp DNA Stool kit). The prevalence of cryptosporidiosis was determined based on identification through a nested PCR protocol to amplify fragments of the *Cryptosporidium* SSU rRNA gene. 3.9% of calves were positive for *Cryptosporidium*. Calves that were subjected to traditional herd management, were female, aged 2 weeks, and had watery feces were affected by the disease at a greater incidence than those were subjected to planned herd surveillance program, were males, were older than 3 weeks, and had firm feces. DNA sequence analysis of the SSU rRNA gene on all of the PCR positive samples ascertained that *C. parvum* was the only species present. Further studies should be performed comprehensive fecal analysis for other causative agents for association *Cryptosporidium* species in calf diarrhea and mortality resulting in economic loss in the region.

Keywords: *Calf, Cryptosporidium, Nested-PCR, SSU rRNA, Erzurum*

Erzurum Yöresinde Buzağılarda Cryptosporidiosisin Prevalansı ve *Cryptosporidium* Türlerinin Moleküler Karekterizasyonu

Özet

Bu çalışma, Erzurum yöresindeki sütten kesim öncesi dönemdeki buzağılarda cryptosporidiosisin prevalansının ve *Cryptosporidium* türlerinin moleküler karekterizasyonunun ortaya konması amacıyla yapılmıştır. Bu amaçla beş süt işletmesinden, bir aydan küçük 307 buzağının dışkı örnekleri toplanmış ve DNA ekstraksiyonu (QIAamp DNA Stool kit) yapılarak genomik DNA elde edilmiştir. Nested PCR protokolü ile *Cryptosporidium* SSU rRNA gen bölgesinin kısmi amplifikasyonu yapılmış ve cryptosporidiosis prevalansı %3.9 olarak belirlenmiştir. Geleneksel yöntemlerle yetiştirilen, dişi, 2 haftalık yaşta ve sulu dışkıya sahip buzağıların modern işletmelerde yetiştirilen, erkek, 3 haftadan büyük ve katı kıvamlı dışkıya sahip olanlara göre hastalıktan daha çok etkilendiği saptanmıştır. PCR pozitif örneklerin SSU rRNA gen bölgesi hedef alınarak yapılan DNA dizi analizleri sonuçlarına göre *C. parvum*'un hayvanlarda bulunan tek tür olduğu anlaşılmıştır. Sonuç olarak, yörede ekonomik kayıplara neden olan buzağı ishalleri ve ölümlerinde *Cryptosporidium* türleri ile diğer hastalık etkenlerinin etkileşimlerinin ortaya konması amacıyla daha kapsamlı çalışmaların yapılmasının gerekliliği sonucuna varılmıştır.

Anahtar sözcükler: *Buzağı, Cryptosporidium, Nested-PCR, SSU rRNA, Erzurum*

INTRODUCTION

Cryptosporidiosis is a zoonotic protozoan disease that has a very broad and versatile geographic distribution including the Antarctic region [1]. *Cryptosporidium* is the causative agent and infects mainly the intestinal tract and rarely the respiratory system of diverse species including human, ruminant, feline, canine, rodent, avian, reptile

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and fish. Transmission usually occurs through the direct fecal-oral route or through ingestion of water or food contaminated with oocysts [2,3].

Bovines are the most common species of mammals, infected with *Cryptosporidium* and considered the major reservoir of *Cryptosporidium* for human infections [3]. Cryptosporidiosis in cattle is mainly caused by *C. parvum*, *C. andersoni*, *C. bovis* and *C. ryanae* [2,3]. At least 10 other *Cryptosporidium* species or genotypes such as *C. felis*, *C. meleagridis* and *C. suis* can also play role in etiology [4,5].

Bovine cryptosporidiosis is considered one of the most common causes of neonatal diarrhea in cattle, leading to economic losses. The severity of infection ranges from mild to severe depending on *Cryptosporidium* species as well as age, previous exposure and immune status of the host. Asymptomatic infection is common in yearling heifers and mature cows [6,7].

Cryptosporidium parvum is a zoonotic species and the predominant in preweaned calves, especially those at age of 1-4 weeks [8]. The agent is responsible for about 85% of cryptosporidiosis in preweaned calves but only 1% of the disease in postweaned calves and 1-2 year old heifers [6,9,10]. Among the other bovine species, *C. bovis* and *C. ryanae* were detected mainly in weaned calves, and *C. andersoni* in yearlings and adult cattle [9,11,12]. While *C. bovis* and *C. ryanae* are considered non-zoonotic, *C. andersoni* has recently been reported in few research involving humans in England [13].

The specific diagnosis of *Cryptosporidium* species is central to the control of the disease and to the understanding of the epidemiology. Lack of distinctive morphologic features of *Cryptosporidium* oocysts makes microscopical examination inconvenient in order to clearly differentiate species and genotypes [14]. Traditionally, *C. parvum* has been diagnosed by microscopy of fecal smears, with or without staining. However, two other species, *C. bovis* and *C. ryanae*, with similar oocyst morphology to *C. parvum*, can only be identified using DNA analysis. That is, microscopy cannot distinguish these three species [6]. Therefore, molecular analyses are required to detect and distinguish *Cryptosporidium* at species/genotype and subtype levels [3,14]. The most frequently used marker for *Cryptosporidium* species and genotype identification is the small subunit of ribosomal RNA (SSU-rRNA) gene [3].

The disease in calves has been studied in many countries, with prevalence ranging from 2.4 to 100% [3,7]. In Turkey, cryptosporidiosis was first diagnosed in calves in 1984 [15]. Since then, other surveys have revealed the prevalence of 7.2-63.9% in calves [16,17]. Most of the studies carried out in Turkey were based on microscopy of stained oocysts in feces [15,16,18-21]. Enzyme-linked immunosorbent assay (ELISA) [17,22] and PCR technique [23-25] have recently become more common to attain the prevalence of cryptosporidiosis. However, few studies have coped with genetic structure of *Cryptosporidium* species in Turkey [26-28]. This study was conducted to determine the prevalence of cryptosporidiosis and to characterize *Cryptosporidium* species based on PCR amplification and sequence analysis of SSU rRNA gene in younger than 1-month-old calves in Erzurum province, Turkey.

MATERIAL and METHODS

Sample Collection

A total of 307 fecal samples were collected from calves less than 1 month old in dairy farms (herd size ranging from 100 to 350 Brown Swiss, and Holstein cows in 3 professional dairy farms and from 40 to 85 Brown Swiss, crossbreed, and Anatolian Red cows in 2 traditional dairy farms) located in Erzurum province between April-2010 and October-2010. Samples were collected directly from the rectum with a gloved hand and transferred into a plastic cup. Fecal consistency was scored as firm, well formed, loose and diarrheic. Samples were kept at 4°C until laboratory analyses.

The study protocol was approved by the Animal Care and Use Committee at Ataturk University (4.4.2008-2008/8 decision number).

DNA Extraction and PCR Amplification

Oocysts were washed and concentrated from feces [29,30] prior to DNA isolation using a QIAamp DNA Stool kit (Qiagen, Maryland, USA). Before eluting, aliquots were added with 100 ml Buffer AE and stored at 20°C.

A nested PCR for the amplification of a fragment of SSU rRNA gene was performed using the protocols and primers as described by Xiao et al.[31] with the following modifications: At the first step of nested PCR, approximately 1.325 bp PCR product was amplified using primers 5′-TTCTAGAGCTAATACATGCG-3′ and 5′-CCCATTTCCTTCGAAA CAGGA-3′. The PCR contained 1x PCR buffer, 6 mM $MgCl_2$, 0.2 mM (each) dNTP, 200 nM (each) primer, 0.025 U of Taq DNA polymerase, and 1.5 µl of DNA template in a total 25 µl reaction mixture. A total of 35 cycles were carried out at 94°C for 45 s, 55°C for 45 s and 72°C for 1 min. There was also an initial hot start at 94°C for 3 min and a final extension at 72°C for 7 min. A secondary PCR was then performed to amplify 826-864 bp from 1 µl of the primary PCR mixture using primers 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5′-AAGGAGTAAGGAACAACCTCCA-3′. The PCR and cycling conditions were identical to the primary PCR. Amplification products were separated by electrophoresis on 1% (w/v) agarose gels, and visualized by ethidium bromide staining.

DNA Sequence Analysis and Phylogenetic Analysis

Successfully amplified samples were subjected to DNA sequence analysis for species determination. Sequencing

was performed using the ABI PRISM® BigDye terminator cycle sequencing kit in ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). Sequence data were then subjected to BLASTN (RefSeq) searches of the *Cryptosporidium* genome database at the National Center for Biotechnology Information *(http://www.ncbi nlm.nih.gov/)*. All sequence data were edited using BioEdit 7.0 *(http://www.mbio.ncsu.edu/BioEdit/bioedit.html)* and FinchTV Version 1.4.0 *(http://www.geospiza.com/finchtv)* following naked eye checking. Multiple sequence alignments were made with the Clustal W method with BioEdit 7.0 software [32]. The neighbor-joining (NJ) method as implemented in the MEGA5.1 program [33] was used for the phylogenetic analysis based on SSU rRNA, utilizing *Eimeria tenella* sequence (HQ680474) as out-group. The branch reliability was assessed by the bootstrap method with 1000 replications.

Data Analysis

The PROC MEANS and FREQ were computed to obtain descriptive statistics [34]. Animals were categorized by breed (culture, crossbreed and local), age (1-7, 8-15 and 16-30 days) and fecal consistency (firm, well formed, loose and diarrheic) before establishing cross-tables to reveal association of risk factors with cryptosporidiosis using Chi-square. The associations were considered significant at P<0.05.

RESULTS

The nested PCR showed a total of 12 (3.9%) positive amplfications in 307 fecal samples *(Table 1)*. The cryptosporidiosis prevalence among calves raised in modern farm was lower than those raised in farms with poor infrastructure (1.5 vs. 8.5%, P<0.003). The infection rate in culture breed was insignificantly higher (4.5%) than the other breeds. As the age advanced, the frequency of animals infected by *Cryptosporidium* increased quadratically, being the highest among calves aged 8-15 days (P<0.0001). Cryptosporidiosis was more common in females than males (6.8 vs. 1.3%, P<0.01). The cryptosporidiosis prevalence increased with the fecal water content (P<0.0001), 13.6% in calves with the diarrheic feces and 7.1% in calves with the loose feces *(Table 1)*.

All PCR-positive isolates in Erzurum were confirmed to be *C. parvum (Fig. 1)*. The sequences were deposited into GenBank under accession numbers KC437395 to KC437406, respectively. *C. parvum* [GenBank: JN245618, JQ250804, JX886767, DQ656355, AB513881, JX948126, JX416362, JQ413434, JQ182993], *C. andersoni* [GenBank: JX948125, JX437080], *C. bovis* [GenBank: JX515546, JX886773, JN245624] and *C. ryanae* [GenBank: JX886771, JN245623] were reference species, whereas *E. tenella* (HQ680474) was an out-group reference in comparisons. *Fig. 1* depicts phylogenetic relationship among *C. parvum* in Erzurum isolates and the other *Cryptosporidium* isolates as inferred by the NJ analysis of the partial SSU rRNA gene sequences. *C. parvum* in Erzurum isolates were grouped into the same clade with respective reference *C. parvum* sequences. In the present experiment, the percent identities were 99.3-100% among *C. parvum* in Erzurum isolates, 98.7-100% with other *C. parvum* isolates and 87.8-94% with other *Cryptosporidium* species from GenBank.

Table 1. *Factors affecting cryptosporidiosis in calves younger than one month in Erzurum (n=307)* *

Tablo 1. *Erzurum yöresinde bir aydan küçük buzağılarda cryptosporidiosise etki eden faktörler (n=307)* *

Variable	Infection Status	
	PCR – (n = 295, 96.09%)	PCR + (n = 12, 3.91%)
Enterprise		
Traditional (n=106, 34.53%)	97 (91.5)	9 (8.5)
Professional (n=201, 65.47%)	198 (98.5)	3 (1.5)
	X^2 = 9.05, P<0.003	
Breed		
Culture (n=264, 85.99%)	252 (95.4)	12 (4.5)
Crossbreed (n=8, 2.61%)	8 (100)	0
Local (n=35, 11.40%)	35 (100)	0
	X^2 = 2.03, P<0.36	
Age (d)		
1-7 (n=30, 9.77%)	28 (93.3)	2 (6.7)
8-15 (n=71, 23.13%)	62 (87.3)	9 (12.7)
16-30 (n=206, 67.10%)	205 (99.5)	1 (0.5)
	X^2 = 21.56, P<0.0001	
Sex		
Female (n=148, 48.21%)	138 (93.2)	10 (6.8)
Male (n=159, 51.79%)	157 (98.7)	2 (1.3)
	X^2 = 6.17, P<0.01	
Fecal consistency		
Firm (n=11, 3.58%)	11 (100)	0
Well formed (181, 58.96%)	181 (100)	0
Loose (n=56, 18.24%)	52 (92.9)	4 (7.1)
Diarrheic (n=59, 19.22%)	51 (86.4)	8 (13.6)
	X^2 = 24.00, P<0.0001	

* *Data are n (%)*

Fig 1. Phylogenetic relationship among *Cryptosporidium* isolates as inferred by neighbor-joining analysis of SSU rRNA nucleotide sequences. The sequence for *E. tenella* (HQ680474) was used as an out-group. Numbers on branches indicate percent bootstrap values from 1000 replicates. All of Erzurum isolates were identified as *C. parvum*

Şekil 1. *Cryptosporidium* izolatlarının SSU rRNA gen bölgelerinin nükleotid dizileri baz alınarak neighbor-joining metodu ile yapılan filogenetik analizi. *E. tenella* (HQ680474) sekansı grup dışı olarak kullanılmıştır. Filogenetik ağaçtaki numaralar 1000 tekrar sonucu elde edilen bootstrap değerlerini göstermektedir. Erzurum izolatlarının hepsi *C. parvum* olarak tanımlanmıştır

DISCUSSION

The prevalence of cryptosporidiosis in Turkey varies between 7.2-63.9% in calves [16,17]. To our knowledge, this study delivered the lowest prevalence rate (3.9%) among other reports from different locations of Turkey [17,19-22,24,26-28]. The difference could be due to a vast number of factors such as breed, age, management, environment, and season as well as diagnostic method [5,7,13]. The low prevalence could also be caused by spot fecal sampling instead of serial sampling, which may result in underestimation because of intermittent oocyst excretion [9,11].

The majority of *C. parvum* infections appear to be limited to dairy calves under eight weeks of age [10,35], being highest in calves up to 1-month-old [7,8,36]. In calves, the highest infection rates are reported in calves 7-14 days old [7,37], 8-14 days old [4,38] and 8-21 days old [39]. In accordance with the literature, in the present study, the infection prevalence was highest in calves aged between 8-15 days (12.7%), followed by those aged 1-7 days (6.7%) and 16-30 days (0.5%).

As previously reported by Trotz-Williams et al.[40] in Ontario, Canada, by Aysul et al.[26] in Aydın, Turkey and by Coklin et al.[13] in Prince Edward Island, Canada, *C. parvum* was the only species identified in calves less than 1 month old. On the other hand, the absence of *C. bovis*, *C. andersoni* and *C. bovis* in our study could be a result of the age group (≤ 1 months) because since *C. bovis* and *C. ryanae* are known to be more prevalent in weaned calves and *C. andersoni* in yearlings and adult cattle [6,9,11,12].

Calf diarrhea has a multifactorial etiology, and *C. parvum* is frequently associated with the disease [7,38,39,41]. Besides, viruses and bacteria are other causative agents that can cause this symptom simultaneously or individually. Of 12 *C. parvum* positive fecal samples, 8 were from diarrheic calves and 4 from calves with loose feces *(Table 1)*. In disagreement with some previous studies [35,39,41,42], our results proved an association of fecal consistency with the infection. Studies reporting relationship between fecal consistency and cryptosporidiosis are available [7,12,36]. Because other possible agents were not searched in the present study, it requires caution to make inference that

calves with watery feces are prone to cryptosporidiosis. Another factor to contribute fecal dry matter is feeding scheme because looser feces can be consequence of milk feeding [39]. These suggest that extensive sample analysis is required to confirm the relationship between fecal consistency and cryptosporidiosis.

The molecular characterization of *Cryptosporidium* species in Turkey has been published in three reports, in which *C. parvum* [26-28], *C. bovis* [27] and *C. ryanae* [27] were identified. In our study, homology search proved that all isolates in Erzurum were *C. parvum*. The partial SSU rRNA gene sequences had 100% similarity to reference sequences downloaded from the GenBank (DQ656355, AB513881, JX948126, JX416362, JQ413434, JQ182993 and JN245618). The NJ phylogenetic analysis based on the SSU rRNA *(Fig. 1)* showed that all sequences of *C. parvum* in Erzurum isolates clustered in the intestinal clade with reference *C. parvum* sequences (bootstrap value 92).

In conclusion, the current study elucidated the prevalence of cryptosporidiosis and the molecular characterization of *Cryptosporidium* species found in calves in Erzurum, Turkey. The prevalence of *Cryptosporidium* infection in dairy calves determined by nested PCR was at 3.9%. *C. parvum* was the only causative *Cryptosporidium* species in calves younger than 1 month in Erzurum province as ascertained by sequencing the amplified SSU rRNA regions.

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RESEARCH ARTICLE

Alterations in Haematological and Biochemical Parameters in Morkaraman Sheep with Natural *Psoroptes ovis* Infestation

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Summary

This study was carried out to determine possible alterations in some haematological and biochemical parameters in Morkaraman sheep naturally infested with *Psoroptes ovis*. For this purpose 22 sheep infested naturally with *P. ovis* and 10 clinically healthy sheep (control) were used. Serum total protein and globulin values were found to be high in sheep with *Psoroptes ovis* compared to control group while serum albumin value was low. Low blood haemoglobin concentration, low blood total Leukocyte (WBC), erythrocyte (RBC), lymphocyte, neutrophyle and monocyte and high eosinophil numbers were detected in sheep infested with *Psoroptes ovis* compared to control group. The results of this study, therefore, show that Psoroptic mange causes decrease in serum total albumin, increase in protein and globulin concentrations and decrease in total RBC, WBC, neutrophile numbers, increase in eusinophile numbers, decrease in haemoglobin concentration in Morkaraman sheep.

Keywords: *Morkaraman Sheep, P. ovis, Haematology, Biochemistry*

Morkaraman Koyunlarda Doğal *Psoroptes Ovis* Enfestasyonunda Hematolojik ve Biyokimyasal Değişiklikler

Özet

Bu çalışma *Psoroptes ovis* ile doğal olarak enfeste morkaraman koyunlarda bazı hematolojik ve biyokimyasal parametrelerdeki olası değişiklikleri saptamak amacıyla yapıldı. Bu amaçla *P. ovis* ile doğal olarak enfeste 22 koyun ve klinik olarak sağlıklı 10 koyun (kontrol) kullanıldı. *Psoroptes ovis* ile enfeste koyunlarda, kontrol grubuna göre serum total protein ve globulin değerleri önemli derecede yüksek bulunurken, serum albumin konsantrasyonu önemli derecede düşüktü. *Psoroptes ovis* ile enfeste koyunlarda kontrol grubuna göre, önemli derecede düşük hemoglobin konsantrasyonu, düşük total lökosit (WBC), eritrosit (RBC), lenfosit, nötrofil ve monosit değerleri saptandı. Kontrol grubuna göre, *Psoroptes ovis* ile enfeste koyunlarda euzinofil sayıları daha yüksek bulundu. Bu çalışmanın sonuçları, psoroptik uyuzun Morkaraman koyunlarda total serum protein ve globulin konsantrasyonunda artış, albumin konsantrasyonunda azalış ile kanda WBC, RBC, nötrofil ve lenfosit sayılarında azalma, hemoglobin konsantrasyonunda azalma, euzinofil sayısında artışa neden olduğunu göstermektedir.

Anahtar sözcükler: *Moraraman Koyun, P. ovis, Hematoloji, Biyokimya*

INTRODUCTION

Psoroptic scabies, caused by *psoroptes ovis,* is distributed world-wide. It is characterised chronic dermatitis, intense pruritis, rump, papules, crusts, excoriation and lichenification in sheep [1,2]. The economic impact include weight loss [3], hair and skin damage, decreased milk production, lower conception rates, alteration of energy metabolism, poor lamb growth and increased suscebility to the other disease and death [1,2,4,5]. Severe bacterial infections are involved commonly in severe cases. However, clinical signs of psoropic mange in sheep are similar to other scabies like sarcoptic and chorioptic mange. Therefore, clinical signs are not sufficient for its diagnosis and differentiation from other manges. It is essential that confirmatory diagnosis of *P. ovis* is needed to be made by microscopically.

In addition to dermatological and behavioural changes, systemic effects (haematological and biochemical changes)

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occurred due to lesions in cattle and sheep infested with *P. ovis* [4,6-10]. These systemic effects are neutrophilia, lypmphopenia, eosinophilia, reduce haemoglobin concentration and hypopoteinemia [11]. Systemic effects of *P. ovis* infestations are commonly reversed with appropriate treatment. The effects of *P. ovis* on haematological and biochemical constituents of cattle were well-studied [7-10]. But few studies have been reported on the effects of *psoroptes ovis* regarding biochemical and haematological changes in some breeds of sheep [4,11]. However, to the best of author knowledge, no report has been published up to date on the effects of *P. ovis* on the subject of haematological and biochemical parameters in Morkaraman sheep.

Therefore, the present study was conducted to determine the haematological and the biochemical changes in Morkaraman breed sheep naturally infested with *Psoroptes ovis*.

MATERIAL and METHODS

Animals

A total of 32 Morkaraman sheep, aged between 1-3 years, were used in this study. Twenty-two of these animals were infested with *P. ovis* naturally and 10 clinically healthy Morkaraman sheep (parasite free) control animals were used in this study.

Clinic Examinations

Routine clinical examination was carried out and body temperature, heart and respiratory rate were recorded for each animal.

Parasitological Examination

For parasitological examination, Scales from peripheral active lesions were scraped with a scalpel until the skin bleeds slightly. The material was then placed on a slide, treated with a 10% solution of potassium hydroxide and then gently warmed until the crusts were softened. It was then covered with thin layer of a cover-slip. Microscopic examination was then carried out under low light power (about 100 diameters) with diaphragm closed down.

Haematology

Peripheral blood samples were collected in dipotassium ethylenediaminetetraacetic acid (EDTA)- coated evacuated tubes and used to count total white blood cell (WBC), total red blood cell (RBC) numbers and measure packed cell volume and haemoglobin concentration (Hb). Differential leucocyte numbers and other hematological analyses were performed manually as described previously [12].

Biochemistry

Serum samples, collected from each animal, were used to determine the concentrations of serum total protein (TP), albumin, creatinine and activities of serum aspartatae amino transpherase (AST), alanine aminotranspherase (ALT) and concentrations of creatinine by using commerical kits by an auto analyser. The concentrations of globulin were calculated by substrating the albumin concentration from the concentration of total protein.

Statistical Analyses

All the values were expressed as the mean and standard deviation (Mean ± SD). Studendt's t-test was used to analyse the significance of the difference between the groups.

RESULTS

Clinical Findings

Common clinical signs in sheep were scratching, biting and liching at their own backs or flanks, pruritis and serous exudation and scab formation.

Parasitological Findings

P. ovis was detected with microscobical examination of scales taking from peripheral active lesions. Parasitological agents, which were found coincident with the morphological characteristics of *P. ovis* in microscopic examination of scales from peripheral active lesions in all sick animals. Morphological characteristics of *P. ovis* in microscopic examination were evaluated as described elsewhere [12].

Haematological Findings

There was a significant reduction in the number of RBCs ($P<0.05$), total leucocytes, lymphocytes, neutrophils, monocytes ($P<0.05$) and Hb concentration and increase eosinophil values ($P<0.01$) sheep infested with *P. ovis* compared to control group *(Table 1)*.

Biochemical Findings

There were significant increases in the concentrations of total protein ($P<0.01$), total globulin ($P<0.01$) and decreases in albumin concentrations ($P<0.01$) in psoroptic mange group comparing to control group. There are no significant differences in the concentrations of AST and ALT in psoroptic mange group compared to those of control group *(Table 2)*.

DISCUSSION

Sheep scab is caused by the non-burrowing mite; *Psoroptes* is the most important ectoparasite of sheep. It is very active in the keratin layer and causes direct damage to the skin result in chronic disease. The disease causes skin lesions, wool loss, reduction feed intake, suppression

Table 1. *Haematological values (Mean±SD) of Morkaraman sheep infested with P. ovis and control group*

Tablo 1. *P. ovis ile enfeste ve kontrol grubundaki koyunlarda Hematolojik değerler (Mean±SD)*

Parameters	Psoroptic Sheep (n=22) (Mean±SD)	Control (n=10) (Mean±SD)
WBC (10^9/l)	7.6±1.2*	10.7±0.4
Lymphocytes (10^9/l)	4.1±0.6*	6.2±0.5
Neutrophils (10^9/l)	1.2±0.3*	3.4±0.1
Monocytes (10^9/l)	0.4±0.1**	0.5±0.2
Eosinophils (10^9/l)	2.3±0.1**	0.6±0.1
RBC (10^{12}/l)	4.6±0.2*	6.3±0.3
Hb (g/dl)	7.6±0.7**	10.8±0.4
PCV %	21±0.3	35±0.1

*Significant differences in values between in sheep infested with P. ovis and control group were indicated * P<0.05, ** P<0.01*

Table 2. *Biochemical values (mean±SD) of Morkaraman sheep infested with P. ovis and control group*

Tablo 2. *P. ovis ile enfeste ve kontrol grubundaki koyunlarda biyokimyasal değerler (Mean±SD)*

Parameters	Psoroptic Sheep (n=22) (Mean±SD)	Control (n=10) (Mean±SD)
TP (g/l)	78.1±0.5**	67.3±0.5
Albumin (g/l)	19.4±0.2**	35.8±0.5
Globulin (g/l)	55.6±0.2**	32.2±0.2
Albumin/Globulin	0.34±0.01	1.09±0.04
AST (IU)	67.1±1.8	69.5±0.7
ALT (IU)	15.5±0.9	17.4±2.5
Creatinine (µmol/l)	97.2±0.1	97.6±0.3

*Significant differences in values between in sheep infested with P. ovis and Control Group were indicated ** P<0.01*

in weight gains in young animals and weight loss in adults. The majority of sheep become infected while mites are active. These infected sheep become reservoir and may introduce the mite to the healthy flocks during summer and autumn, and initiate outbreaks when the cold season arrives [1,2]. Therefore, differential diagnosis and its adequate treatment need to be done to prevent economical loss, new outbreaks and to apply control programmes in infected sheep. In this study, common clinical findings in sheep diagnosed *P. ovis* were scratching, biting and licking at their own backs or flanks, pruritis and serous exudation and scab formation. These findings are in agreement previous reports [2,13,14]. On the other hand common clinical signs reported herein are not definitive and differential diagnosis. Therefore, certain diagnosis has been made by a microscopic examination in this study.

Haematological findings showed significant decrease in total WBC, monocytes, lymphocytes and neutrophyle numbers in Psoroptic sheep as compared to control group. Decreased lymphocyte numbers should be attributed to stress leukogram during the course of the disease [11]. A decrease in total WBC, neutrophils and monocyte numbers in the peripheral blood may be closely associated with mite activity and caused by efflux of these cells from circulating granulocyte pool [4]. Furthermore, the decrease of lymphocyte numbers may be occurring due to stress which is occurring during dermatitis [4,15]. Decrease in this blood cells increase susceptibility to various viral and bacterial diseases, which may cause economical loses. Mean eosinophil numbers in psoroptic sheep were higher than control group. An increase in the numbers of circulating eosinophil has associated with parasitic infestations [16,17]. This could be due to allergic reactions caused by mite or their products of inflammatory reactions and due to activation of immune system. This supports the hypothesis that coetaneous response to mites is at least a part of hypersensitivity reaction [11].

P. ovis on cattle ingest erythrocytes [18]. But this does not occur in sheep [19]. Therefore, in this study, decreased RBC counts were suggested that it could result from a suppression of erytropoiesis and chronic dermal inflammation [11]. The Hb values were also significantly lower in infested animals, which because of significantly low erythrocyte counts, hametocryte and erythrocyte fragility or due to toxaemia caused by mites [17]. The decrease in packed cell volume in infested sheep may be contributed by the decrease in cellular components in blood due to infestation of mange mites. This may cause anaemia in psoroptic sheep.

Serum total protein and globulin concentrations were higher in psoroptic sheep than control group. Serum albumin concentration was lower in psoroptic group than control group. Decreased serum albumin concentrations may have been due to anorexia reported herein which is a feature of severe scab [4,11]. Furthermore, decline in serum albumin concentration in sheep infested with *P. ovis* may attribute to leakage of serum proteins through the more permeable hypertrophic or damaged epitelium of the skin [4]. An increase in serum globulin concentration can indicate the development of an antibody response to the present antigen [4,11,13]. Furthermore, in the study, increase the total protein, and the decrease in A/G ratio were likely due to increased globulin concentration that is associated with chronic inflammatory reaction in the skin. No significant difference found in AST, ALT activities and creatine concentrations values between two groups. This shows that *P. ovis* infestation has no effect on the liver and kidney functions in sheep. On the contrary, Fisher and Crookshang [8], reported that kidney and liver may be damaged and serum concentrations of cholesterol may be decreased during course of *P. ovis* infestation in calves.

In conclusion, the result of this study indicates that psoroptic mange may cause increase in serum total protein and globulin, decrease in albumin concentrations, decrease in total RBC, WBC, neutrophil numbers, increase in eusinophil numbers, decrease in haemoglobin concentration in Morkaraman sheep.

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RESEARCH ARTICLE

Hekzavalent Kromun *Capoeta capoeta* (Guldenstaedt 1773) ve *Squalius cephalus* (Linnaeus 1758) Üzerine Olan Etkisinin Histopatolojik ve Elektroforetik Yöntemlerle Saptanması [1]

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Özet

Çalışmada, Kars Çayı'ndan yakalanan Siraz balığı (*Capoeta capoeta*) ve Tatlısu kefali (*Squalius cephalus*) üzerine hekzavalent kromun (CrVI) (potasyum dikromat olarak) etkileri histopatolojik ve elektroforetik yöntemlerle incelendi. Balıklar çeşme suyunda 10 gün süreyle bekletilerek ortama adaptasyonları sağlandı. Daha sonra, her bir balık türü için her grupta 10'ar adet olmak üzere 3 grup oluşturuldu. I. gruptaki (kontrol) balıklar çeşme suyu içeren tankta, II. gruptaki balıklar 10 gün süreyle, III. gruptaki balıklar ise 20 gün süreyle 10 mg/L dozunda CrVI içeren tanklarda bekletildi. Bu süre sonunda, balıklardan kan ve doku örnekleri alındı, daha sonra analizler yapıldı. Histopatolojik inceleme sonucunda CrVI'a maruz kalan *Capoeta capoeta* ve *Squalius cephalus*'ların karaciğer dokularında fokal nekroz alanları, hidropik ve vakuolar dejenerasyonlar tespit edildi. Bu dejenerasyonların şiddetinin hekzavalent kroma maruz kalma süresiyle artış gösterdiği gözlemlendi. Serum proteinlerinin SDS-PAGE'inde ise 10 gün süreyle CrVI uygulamasına bağlı olarak *Capoeta capoeta*'nın bazı protein bantlarında hafif kalınlaşma olduğu, *Squalius cephalus*'un protein bantlarında ise belirgin bir değişikliğin şekillenmediği saptandı. Hekzavalent kromun 20 gün süreyle uygulandığı *Capoeta capoeta*'nın birçok protein bantlarında incelmede, bazı protein bantlarında ise belirgin derecede kalınlaşmalar gözlendi. *Squalius cephalus*'da ise bazı protein bantlarında hafif derecede kalınlaşma meydana geldiği tespit edildi. Sonuç olarak; potasyum dikromat uygulamasının zamana bağlı olarak artan derecelerde *Capoeta capoeta* ve *Squalius cephalus* karaciğer dokularında ve protein ekspresyonlarında bozulmalara neden olduğu sonucuna varıldı.

Anahtar sözcükler: *Capoeta capoeta, Squalius cephalus, KromVI, SDS-PAGE, Karaciğer dejenerasyonu*

Detection of the of Effects of Hexavalent Chromium by Histopathological and Electrophoretic Methods on *Capoeta capoeta* (Guldenstaedt 1773) and *Squalius cephalus* (Linnaeus 1758)

Summary

In this study, effects of hexavalent chromium (CrVI) (as potassium dichromate) on *Capoeta capoeta* and *Squalius cephalus* obtained from Kars Creek were investigated by histopathological and electrophoretic methods. Fish were adapted into the medium for 10 days. Then three groups were made in which for each fish species ten fish were included. The fish in the 1st group were held in normal water, 2nd and 3rd groups were held in the water containing 10 mg/L CrVI, respectively for 10 and 20 days. At the end of this period, tissue and blood samples were taken. These samples were analyzed subsequently. After histopathological examination occasional necrosis, hydropic and vacuolar degenerations in liver of the fish were found which were exposure to 10 mg/L CrVI. An increase in the level of degeneration was observed in the livers tissues of the experimental fish groups in parallel to the increase of the duration. In SDS-PAGE of serum proteins, compared to control, slight thickening of some protein bands were detected in the *Capoeta capoeta* exposed to CrVI for 10 days. There were no significant changes in protein bands of *Squalius cephalus*. Thinning of some protein bands and a significant thickening of some protein bands in the *Capoeta capoeta*. Slight thickening of some protein bands were detected in the *Squalius cephalus* exposed to hexavalent chromium for 20 days. In exposure to depending on the time of hexavalent chromium on *Capoeta capoeta* and *Squalius cephalus,* it is concluded that liver tissue and serum protein expressions of these fish were increasingly degenerated.

Keywords: *Capoeta capoeta, Squalius cephalus, ChromiumVI, SDS-PAGE, Degeneration of Liver*

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GİRİŞ

Krom doğal çevrede saf metalik olarak bulunmayan en yaygın elementlerden biridir. Kromun divalent (CrII), trivalent (CrIII) ve oksidasyonu sonucu oluşan hekzavalent (CrVI) formları bulunmaktadır [1]. Krom çok sert olması ve erime noktasının 1900°C ve kaynama noktasının 2642°C olması nedeniyle, metallere sertlik sağlanması ve zırhlı araç yapımı için kullanılır. Başlıca kullanım alanı nikel ile beraber paslanmaz çelik yapımıdır. Krom doğada +3 yüklüdür, indirgenme reaksiyonuyla +6 değerlik alır [2]. Krom doğal ya da insan kaynaklı yollarla CrIII ve CrVI şeklinde hava, su ve toprağa karışır. En toksik olanı CrVI'dır. Hekzavalent kromun karsinojenik, mutajenik ve teratojenik etkileri bulunmaktadır [3].

Kromun balıklar üzerine akut toksisite çalışmalarında, Vutukuru [4], *Labeo rohita*'ya 24-96 saat süreyle CrVI'ya maruz bırakmış ve LD50 değerlerini sırasıyla 111.45 ve 39.40 mg/L olarak hesaplamıştır. Bunun yanı sıra, hematolojik parametrelere göre anemi gözlemiştir. Biyokimyasal parametrelerden total glikojen, lipit ve protein içeriklerinde azalmalar olduğunu tespit etmiştir. Boge ve ark.[5], *Salmo gairdneri* ve *Dicentrarchus labrax* üzerine CrVI kromun letal olmayan dozlarını uygulayarak bağırsak enzimleri üzerine etkilerini incelemiş, 13 ve 21 günlük krom maruziyeti sırasıyla 18 mg/L *(Salmo)* ve 5 mg/L *(Dicentrarchus)* olarak uygulanmış ve Alkalin fosfataz'da azalma, Na/K ATPaz aktivitesinde artış gözlemlemişlerdir. Kuykendall ve ark.[6], *Oncorhynchus mykiss,* hibrit *Lepomis macrochirus* ve *Ictalurus punctatus* üzerine kromu 4 gün süreyle uygulamış ve DNA-protein çapraz bağlarını (DPXs) gözlemişlerdir. Sonuç olarak kromun *Lepomis* ve *Ictalurus*'ların eritrositlerinde DPX oluşumunun arttığını belirtmişlerdir.

Krom deri, tekstil, paslanmaz çelik, elektrokaplama gibi birçok endüstri alanında su kaynaklarına boşaltılmakta ve başta balık popülasyonları olmak üzere sucul ekosistem için oldukça ciddi tehdit oluşturmaktadır [7-9]. Kromun balıkların karaciğer, solungaç, böbrek gibi organları üzerine doz ve zamana bağlı olarak morfolojik olarak değişikliklere neden olduğu bilinmektedir. Bu çalışmaların ışığı altında, *Capoeta capoeta* (Guldensttead 1773) ve *Squalius cephalus* (*Linnaeus* 1758) üzerine hekzavalent kromun histopatolojik ve elektroforetik etkilerinin araştırılması amaçlandı.

MATERYAL ve METOT

Deney Gruplarının Oluşturulması

Bu araştırmada, Kars Çayı'ndan yakalanan *Capoeta capoeta* (250-320 g ve *Squalius cephalus* (150-230 g) balık türleri kullanıldı. Balıkların toplandığı suyun kalitesi; pH: 7.9-8.3, O_2: 5.0-8.6, konduktivite: 210 ms/cm^2, NH_3: 420 µg/L, PO_4: 55.2 µg/L, NO_3: 0.263 µg/L, sıcaklık: 17.5-19.0°C olarak tespit edildi. Laboratuvar ortamında 500 L'lik tanklara alınarak çeşme suyunda 10 gün süreyle ortama adaptasyonları sağlanan balıklar daha sonra her grupta 10'ar adet *Capoeta capoeta* ve *Squalius cephalus* bulunan 3 gruba ayrıldı. I. gruptaki (kontrol) balıklar çeşme suyu içeren tankta, II. gruptaki balıklar 10 gün süreyle, III. gruptaki balıklar ise 20 gün süreyle 10 mg/L dozunda potasyum dikromat ($K_2Cr_2O_7$) içeren tanklarda bekletildi. Çalışma esnasında ise tanklardaki su sıcaklığı termostatik termometre aracılığıyla 18±0.2 ve O_2 miktarı ise 5±0.4 olarak ayarlandı. Çalışma süresi sonunda elektroforetik inceleme için balıkların kuyruk venlerinden kan örnekleri ve daha sonra da histopatolojik çalışmalar için karaciğer doku örnekleri alındı.

Histopatoloji

Deneklerden alınan doku örnekleri %10'luk fosfat buffer formaldehit solusyonuna alınıp 48 saat tespit edildikten sonra, doku örnekleri bir gece akarsuda yıkandı. Daha sonra sırasıyla alkol, ksilol serilerinden geçirildi ve 65°C'ye ayarlanan etüvde bir gece parafinde bırakıldıktan sonra kasetlerde parafine gömüldü. Hazırlanan bu parafin bloklardan 3-5 mm kalınlığında kesitler alınarak, hematoksilen ve eozin (HE) boyama metoduna göre boyanarak ışık mikroskobunda (Olympus BX51, JAPAN) değerlendirildi.

Sodyum Dodesil Sülfat Poliakrilamid Jel Elektroforezi (SDS-PAGE)

Alınan kan numuneleri +4°C ve 3.000 rpm'de 10 dk santrifüj edilerek serumun ayrılması sağlanmıştır. Alınan serumlar analizler yapılıncaya kadar -20°C'de saklandı. Numunelerin protein konsantrasyonları biüret yöntemi ile ölçülerek [10], SDS-PAGE işlemi Laemmli ve O'Farrell metotlarına göre yapıldı [11,12]. Proteinler, 16x10 cm boyutlarında ve 1 mm kalınlığında slab jelde sepera edildi. Slab jel, proteinlerin stoklandığı yoğunlaştırıcı ve daha sonra da proteinlerin sepera edildiği ayırıcı jel kısımlarından meydana gelmektedir. %10 akrilamid içeren ayırıcı jel elektroforez işleminden 12 saat önce hazırlanarak polimerize edilerek bir gece buzdolabında saklandı. %4 akrilamid içeren yoğunlaştırıcı jel ise elektroforez işleminden 2 saat önce hazırlanarak polimerize edildi. Her numune ve standart %10 gliserol, %2 merkaptoetanol (2-ME), %2 sodyum dodesil sülfat (SDS), %0.01 brom fenol blue (BFB) içeren numune tamponu ile karıştırılarak protein konsantrasyonları sırası ile 2 µg/µl ve 0.2 µg/µl'ye ayarlandı ve daha sonra numuneler ve standart proteinler kaynar suda 3 dk bekletilerek proteinlerin denatürasyonu sağlandı.

Yoğunlaştırıcı jelden her bir jel çukuruna 20 µl serum numunesi ve standart protein uygulanarak brom fenol blue jelin en alt kısmına gelinceye kadar jele 200 voltluk gerilim verildi. Elektroforez işlemi sonrası jeller, %0.125 coomassie blue R-250, %40 metanol ve %7 asetik asit bulunan boya çözeltisi içerisinde su banyosunda 56°C'de 20-30 dk.'da boyandı. Jeldeki fazla boya %5 metanol ve %7.5 asetik asit içeren çözelti ile su banyosunda 56°C'de her 20 dk.'da

bir çözelti değiştirilmek suretiyle 1 saatte dekolore edildi. Elektroforez uygulamasında protein standardı olarak, sığır albumini (66 kD), yumurta albumini (45 kD), gliseraldehit-3-fosfat dehidrojenaz (36 kD) ve tripsinojen (24 kD) kullanıldı.

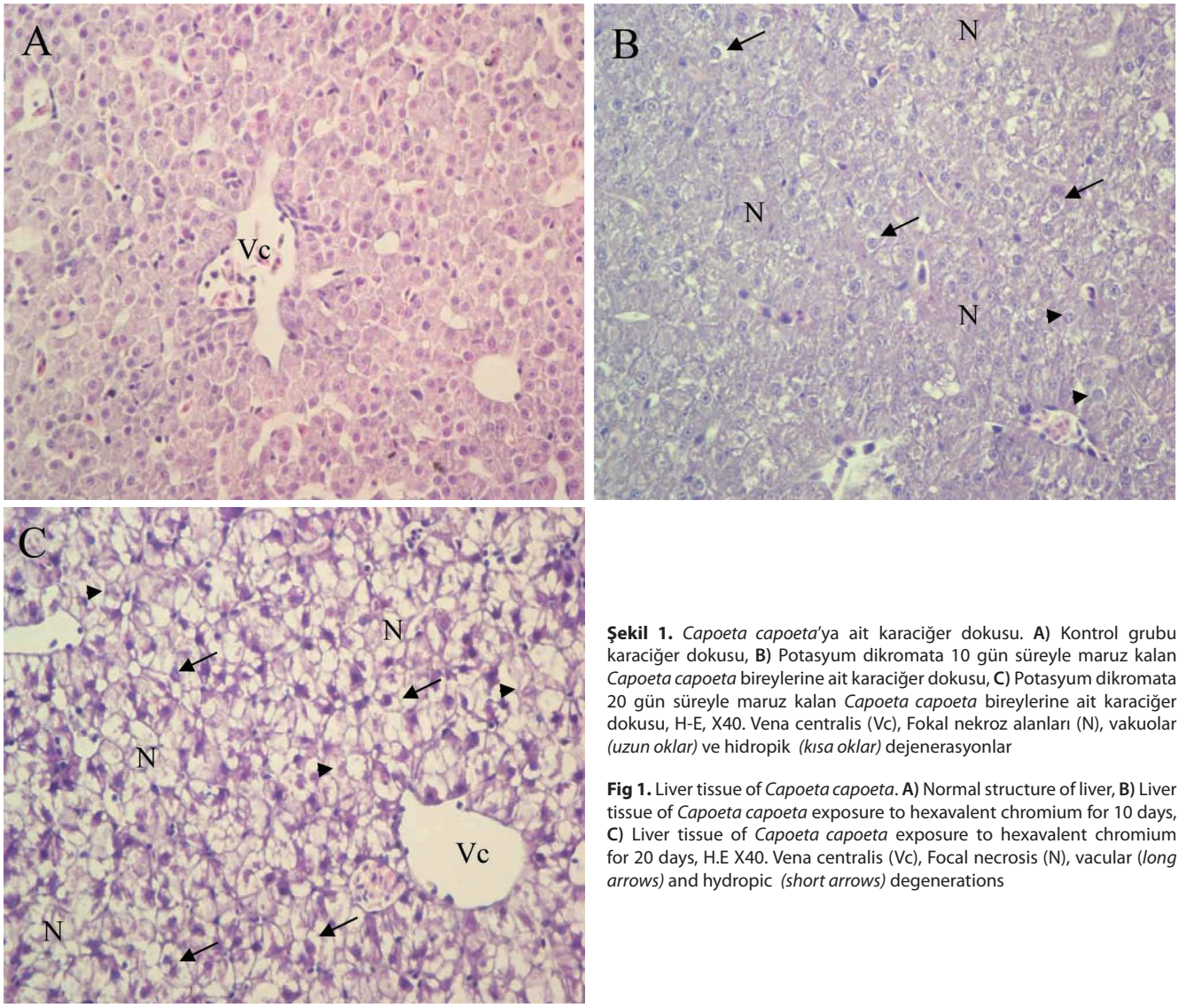
BULGULAR

Histopatolojik Bulgular

Capoeta capoeta ve *Squalius cephalus*'da kontrol grubuna ait karaciğer dokularının mikroskobik incelemesinde herhangi bir histopatolojik bulguya rastlanmamış olup, sinozoidal yapının ve hepatositlerin normal görünümde olduğu gözlemlendi *(Şekil 1A, Şekil 2A)*. Potasyum dikromatın 10 mg/L dozuna 10 gün süreyle maruz kalan II. gruptaki *Capoeta capoeta* ve *Squalius cephalus*'a ait karaciğer dokularının mikroskobik incelemesinde ise fokal nekroz alanları ile birlikte vakuolar ve hidropik dejenerasyonlar tespit edildi *(Şekil 1B, Şekil 2B)*. Aynı dozda potasyum dikromata 20 gün süreyle maruz bırakılan III. gruptaki balıkların karaciğer dokularında da nekroz alanları, vakuolar ve hidropik dejenerasyonlar saptanmış olup bu dejenerasyonların şiddeti uygulanan süreyle birlikte artış gösterdiği belirlendi *(Şekil 1C, Şekil 2C)*.

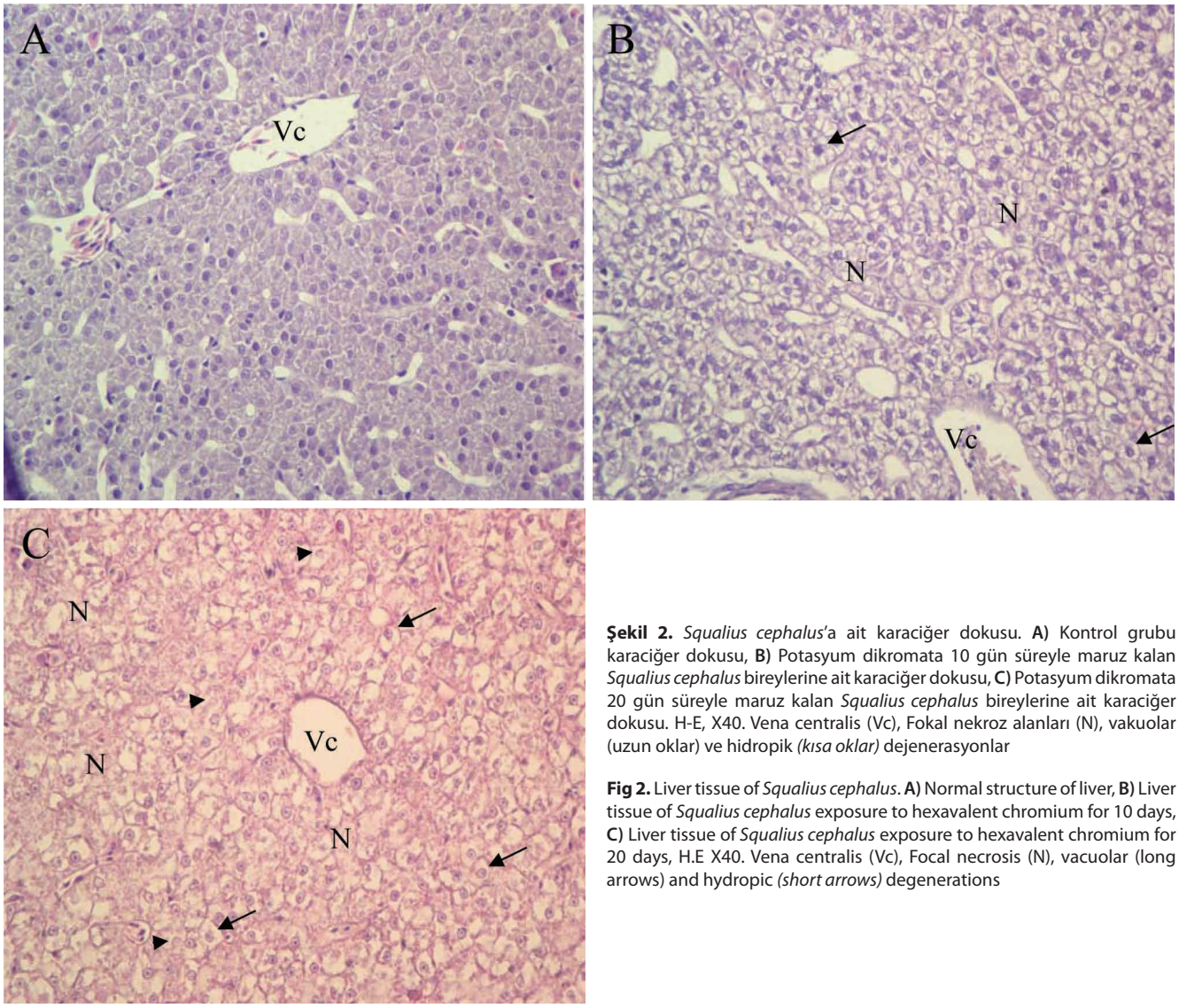
Elektroforetik Bulgular

SDS-PAGE'den elde edilen elektroferograma göre, 10 gün süreyle potasyum dikromat uygulamasına bağlı olarak *Capoeta capoeta*'nın 95 kD, 38 kD ve 30 kD'luk protein bantlarında hafif kalınlaşma olduğu, *Squalius cephalus*'un protein bantlarında ise belirgin bir değişikliğin şekillenmediği saptandı *(Şekil 3)*. Potasyum dikromat'ın 20 gün süreyle uygulandığı *Capoeta capoeta*'nın 77 kD, 62 kD ve 15 kD'luk protein bantlarında incelme, 47 kD ve 38 kD'luk protein bantlarında ise belirgin derecede kalınlaşma gözlendi. *Squalius cephalus*'da ise 80 kD, 62 kD ve 38 kD molekül ağırlığına sahip protein bantlarında hafif derecede kalınlaşma meydana geldiği tespit edildi *(Şekil 4)*.



Şekil 1. *Capoeta capoeta*'ya ait karaciğer dokusu. **A)** Kontrol grubu karaciğer dokusu, **B)** Potasyum dikromata 10 gün süreyle maruz kalan *Capoeta capoeta* bireylerine ait karaciğer dokusu, **C)** Potasyum dikromata 20 gün süreyle maruz kalan *Capoeta capoeta* bireylerine ait karaciğer dokusu, H-E, X40. Vena centralis (Vc), Fokal nekroz alanları (N), vakuolar *(uzun oklar)* ve hidropik *(kısa oklar)* dejenerasyonlar

Fig 1. Liver tissue of *Capoeta capoeta*. **A)** Normal structure of liver, **B)** Liver tissue of *Capoeta capoeta* exposure to hexavalent chromium for 10 days, **C)** Liver tissue of *Capoeta capoeta* exposure to hexavalent chromium for 20 days, H.E X40. Vena centralis (Vc), Focal necrosis (N), vacular (*long arrows)* and hydropic *(short arrows)* degenerations



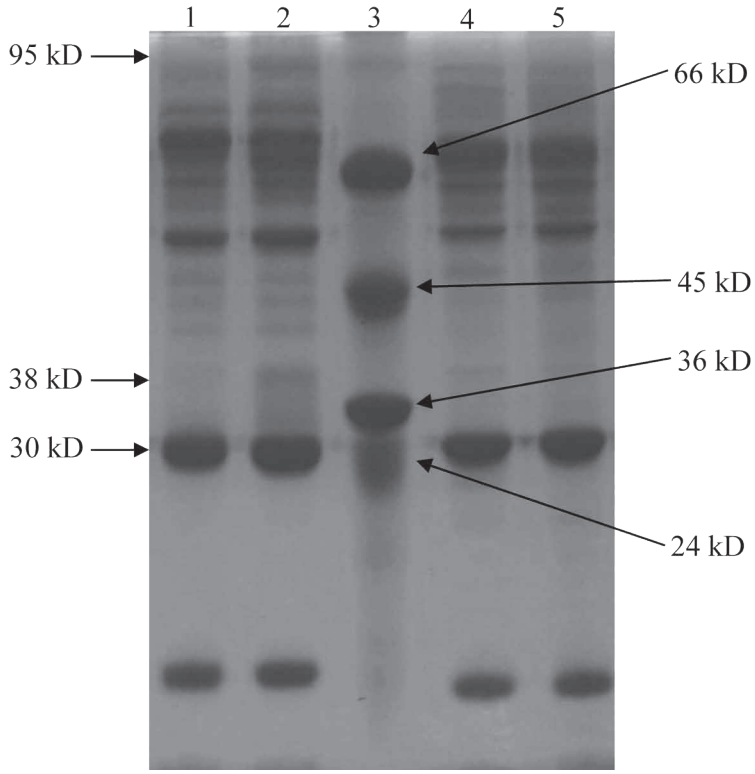
Şekil 2. *Squalius cephalus*'a ait karaciğer dokusu. **A)** Kontrol grubu karaciğer dokusu, **B)** Potasyum dikromata 10 gün süreyle maruz kalan *Squalius cephalus* bireylerine ait karaciğer dokusu, **C)** Potasyum dikromata 20 gün süreyle maruz kalan *Squalius cephalus* bireylerine ait karaciğer dokusu. H-E, X40. Vena centralis (Vc), Fokal nekroz alanları (N), vakuolar (uzun oklar) ve hidropik *(kısa oklar)* dejenerasyonlar

Fig 2. Liver tissue of *Squalius cephalus*. **A)** Normal structure of liver, **B)** Liver tissue of *Squalius cephalus* exposure to hexavalent chromium for 10 days, **C)** Liver tissue of *Squalius cephalus* exposure to hexavalent chromium for 20 days, H.E X40. Vena centralis (Vc), Focal necrosis (N), vacuolar (long arrows) and hydropic *(short arrows)* degenerations

TARTIŞMA ve SONUÇ

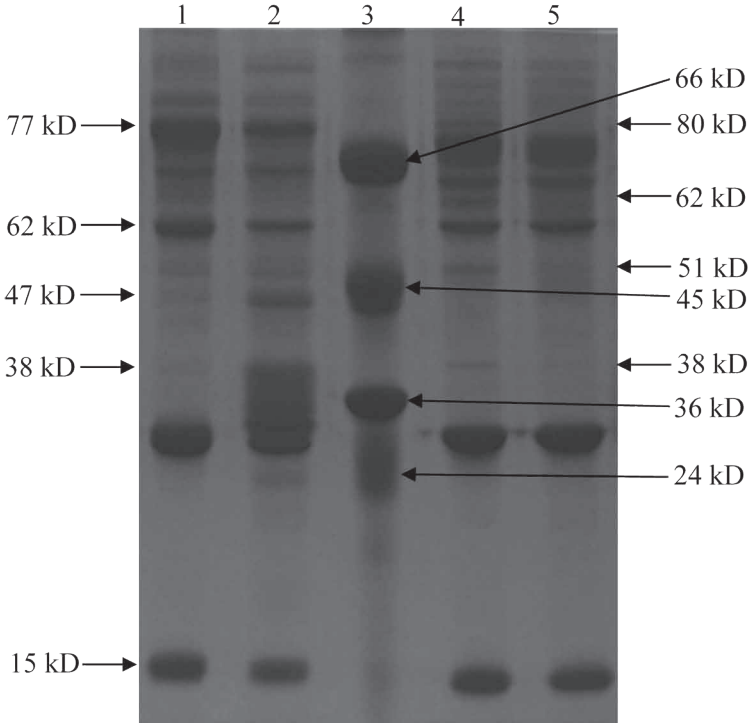
Mutajenik ve teratojenik özellikleriyle beraber karsinojen etki gösteren krom endüstriyel bir kirleticidir. Krom balıklarda çeşitli düzeylerde patolojik, fizyolojik, biyokimyasal ve genetik olarak ters etkili olabilmektedir. Krom VI'nın balıklar üzerine etkilerini belirlemek amacıyla birçok araştırmacı çalışmalar yapmış ve kromun toksik bir ağır metal olduğunu açıkça belirlemişlerdir. Yapılan bir çalışmada hekzavalent kromun subletal dozları sazan *(Cyprinus carpio)*'a uygulanarak histolojik değişimler gözlenmiştir. Böbreklerde nekroz, piknotik nukleusla birlikte, hematopoetik dokunun hücrelerinde bozulma ve glomerolusun dejenerasyonu ile birlikte renal tubüllerde atrofi tespit edildiği bildirilmektedir [13]. Krom VI'nın farklı dozlarının (50, 100 ve 200 mg/L) gökkuşağı alabalığı *(Oncorhynchus mykiss)* üzerine uygulanması sonucu beyin ve solungaçlarda Na/K ATPaz'ın yanı sıra, süperoksit dismutaz (SOD) ve glutatyon redüktaz (GR) antioksidan enzimleri üzerine etkileri incelenmiştir. Beyin ve solungaçlarda SOD ve GR'lerde azalmalar tespit edilmiştir. Solungaçlarda Na/K ATPaz aktivitelerinde önemli derece inhibisyonlar olduğu belirtilmiştir [14]. Tilapia türü olan *Oreochromis aureus* üzerine yüksek konsantrasyonlarda potasyum dikromat ve deterjan uygulanmış ve bu toksikanların meydana getirebileceği genetik değişimler gözlenmeye çalışılmıştır. Deneme sonunda ölen ve hayatta kalan örneklerin tamamının genetik yapısı Malic Enzyme *(ME)*, Isocitrate Dehydrogenase *(ICD),* Aspartate Amino Transferase *(AAT),* Phosphoglucose Isomerase *(PGI),* Malate Dehydrogenase *(MDH),* Glycerophosphate Dehydrogenase *(G3PDH)* enzimleri kullanılarak alloenzim elektroforez metoduyla analiz edilmiş ve *PGI, MDH* ve *G3PDH* enzimlerinde genotip tayini gerçekleştirilememiştir. *ME, ICD* ve *AAT* enzimlerinin genotip tayini yapılarak, genotip tayini sonunda, *AAT* enziminde bir çeşit homozigot genotip elde edilirken, *ME* ve *ICD* enzimlerinde iki çeşit homozigot genotip tespit edilmiştir [15].

Memelilerde olduğu gibi balıklarda da karaciğer dokusu önemli bir detoksifikasyon merkezidir. Burada bulunan



Şekil 3. Potasyum dikromatın 10 mg/L dozunda 10 gün süreyle uygulama sonrası balıkların kan serumlarının SDS-PAGE'de yürütülmesi ile elde edilen elektroferogram. **1-** *Capoeta capoeta* kontrol grubu, **2-** *Capoeta capoeta* Potasyum dikromat uygulanan grup, **3-** standart proteinler, **4-** *Squalius cephalus* kontrol grubu, **5-** *Squalius cephalus* Potasyum dikromat uygulanan grup

Fig 3. Serum proteins electropherograms of *Capoeta capoeta* and *Squalius cephalus* exposure to hexavalent chromium for 10 mg/L 10 days. **1. lane:** control group of *Capoeta capoeta,* **2. lane:** group of *Capoeta capoeta* exposure to hexavalent chromium, **3. lane:** standard proteins, **4. lane:** control group of *Squalius cephalus,* **5. lane:** group of *Squalius cephalus* exposure to hexavalent chromium



Şekil 4. Potasyum dikromatın 10 mg/L dozunda 20 gün süreyle uygulama sonrası balıkların kan serumlarının SDS-PAGE'de yürütülmesi ile elde edilen elektroferogram. **1-** *Capoeta capoeta* kontrol grubu, **2-** *Capoeta capoeta* Potasyum dikromat uygulanan grup, **3-** Standart proteinler, **4-** *Squalius cephalus* kontrol grubu, **5-** *Squalius cephalus* Potasyum dikromat uygulanan grup

Fig 4. Serum proteins electropherograms of *Capoeta capoeta* and *Squalius cephalus* exposure to hexavalent chromium for 10 mg/L 20 days. **1. lane:** control group of *Capoeta capoeta,* **2. lane:** group of *Capoeta capoeta* exposure to hexavalent chromium, **3. lane:** standard proteins, **4. lane:** control group of *Squalius cephalus,* **5. lane:** group of *Squalius cephalus* exposure to hexavalent chromium

methallotiyonin, SOD, glutatyon peroksidaz, katalaz gibi birçok enzim ağır metallerin toksik etkilerine karşı koruma sağlamada görevlidirler [16]. Farklı balık türleri üzerinde yapılan araştırmalarda da kromun karaciğer dokusu üzerine etkileri ortaya konmuştur. Mishra ve ark.[7], *Channa punctatus* üzerine hekzavalent kromun kronik ile subletal dozlarını uygulamışlar ve karaciğerde hepatosit hücrelerinde vakuolizasyon, piknotik nukleus ve sinuzoidal boşluklarda artışlar olduğunu bildirmişlerdir. Aynı araştırıcılar başka bir çalışmada hekzavalent kromu *Channa punctatus* üzerine uygulayarak akut toksik etkilerini incelemişler ve krom tuzunun 96 saatlik LC50 değerini 41.75 mg/L olarak tespit etmişlerdir. Bununla birlikte krom toksisitesine bağlı olarak hepatositlerde atrofi ve sinozoidal boşluklarda artış gözlenmiştir [8]. Başka bir çalışmada, Kroma maruz bırakılan Japon balığı *(Carassius*

auratus)'larda krom maruziyeti neticesinde karaciğer dokularında dejenerasyon ve vena centraliste nekrozlar gözlemlendiği bildirilmiştir [9]. Mevcut çalışmada, 10 mg/L dozunda potasyum dikromata 10 gün süreyle maruz kalan *Capoeta capoeta* ve *Squalius cephalus*'a ait karaciğer dokularının mikroskobik incelemesinde ise fokal nekroz alanları ile birlikte vakuolar ve hidropik dejenerasyonlar tespit edildi. Yine 20 gün boyunca potasyum dikromata maruz bırakılan balıkların karaciğer dokularında da nekroz alanları, vakuolar ve hidropik dejenerasyonlar saptanmış olup, bu dejenerasyonların şiddetinin uygulanan süreyle birlikte artış gösterdiği belirlendi. Bulguların yukarıda bahsedilen literatürlerle paralellik gösterdiği tespit edildi.

Yapılan literatür incelemelerinde, hekzavalent kromun serum protein ekspresyonları üzerine etkileri ile ilgili bilgiye rastlanamamıştır. Ancak yapılan diğer ağır metal çalışmalarında *Capoeta capoeta* ve *Squalius cephalus*'un serum protein ekspresyonlarında değişiklikler meydana geldiği bildirilmektedir. Yılmaz ve ark.[17], kobalt parahidroksibenzoat toksisitesi neticesinde *Capoeta capoeta capoeta*'nın büyük molekül ağırlıklı protein bantlarında kalınlaşmalar ve düşük molekül ağırlıklı protein bantlarında ise incelmeler meydana geldiğini belirtmişlerdir. Benzer bir araştırmada Bayram ve ark.[18], *Capoeta capoeta capoeta*'nın serum proteinleri üzerine 1 mg/L ve 2 mg/L dozlarında kobalt (II) klorür'ün etkilerini araştırmışlar ve kontrol grubuna göre deney gruplarındaki birçok protein bandında incelmeler olduğunu, bununla birlikte 1 mg/L'lik grupta 32.4 kD, 2 mg/L'lik grupta ise 33.3 kD, 30.6 kD ve 28.2 kD'luk yeni proteinlerin sentezlendiği saptamışlardır. Başka bir araştırmada da, *Squalius cephalus* üzerine 1 mg/L ve 2 mg/L kadmiyum sülfat uygulama sonrası $CdSO_4$ konsantrasyonundaki artışa bağlı olarak protein bantlarında kademeli bir incelme meydana geldiği, 1 mg/L kadmiyum sülfat uygulanan grupta 35.3 kD ve 100.5 kD'luk proteinlerde, 2 mg/L kadmiyum sülfat uygulanan grupta da 44.5 kD ve 47.3 kD'luk proteinlerde inhibisyon tespit edildiği belirtilmiştir [19]. Yapılan bu çalışmada ise 10 gün süreyle hekzavalent kroma maruz kalan *Capoeta capoeta*'ların bazı protein bantlarında kalınlaşmalar meydana geldiği, *Squalius cephalus* bireylerinin protein bantlarında ise belirgin bir değişikliğin şekillenmediği saptandı. Denemenin II. grubunda 20 günlük hekzavalent krom maruziyeti neticesinde ise *Capoeta capoeta* ve *Squalius cephalus* bireylerinin serum protein bantlarında kalınlaşmalarla birlikte *Capoeta capoeta* bireylerine ait bazı protein bantlarında incelmeler meydana geldiği tespit edildi. Yukarıdaki literatürlerde [17-19] de görüldüğü gibi, ağır metallerin doza ve süreye bağlı olarak serum protein ekspresyonları üzerinde farklı etkiler gösterdiği düşünülmektedir.

Sonuç olarak, *Capoeta capoeta* ve *Squalius cephalus* balıklarında 10 ve 20 gün süreyle 10 mg/L dozunda uygulanan hekzavalent kromun toksik etki meydana getirdiği tespit edilmiştir. Ayrıca, elektroforezden elde edilen protein bantlarındaki değişikliklerin krom maruziyeti için belirteç olabileceği düşünülmektedir.

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RESEARCH ARTICLE

The Effectiveness of Hesperidin in the Prevention of Bacterial Translocation Caused by Methotrexate in the Gastrointestinal Tract

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Summary

Methotrexate (MTX) is an antimetabolite that it is widely used in childhood cancers. Gastrointestinal toxicity stemming from oxidative damage is an important factor limiting its use. MTX causes morphological damage in the mucosa of the small intestine and serious barrier function disorder. Bacterial translocation can be seen when intestinal barrier functions are deteriorated. The aim of this study was to investigate the effect of hesperidin, a powerful antioxidant, in the prevention of bacterial translocation caused by MTX. Rats were given a single intraperitoneal dose of MTX at 20 mg/kg body weight. Hesperidin was given with oral gavage at 200 mg/kg body weight through 5 days. On the 6th day, biopsy specimens from the ileocecal region, ascending colon and mesenteric lymph nodes were placed in culture media. Increased intestinal bacteria growth was found and prominent bacterial translocation were determined in the MTX group ($P<0.05$). Hesperidin significantly reduced the growth load and bacterial translocation. This study showed that hesperidin protects against translocation by preventing damage caused by MTX.

Keywords: *Methotrexate, Bacterial translocation, Hesperidin*

Metotreksatın Neden Olduğu Gastrointestinal Kanaldan Bakteriyel Translokasyonun Önlenmesinde Hesperidinin Etkinliği

Özet

Metotreksatı (MTX) çocukluk çağı kanserlerinde yaygın olarak kullanılan antimetabolittir. Oksidatif hasardan kaynaklanan gastrointestinal toksisite MTX için önemli bir sınırlayıcı faktördür. MTX'in ince barsak mukozasında morfolojik hasar ve ciddi bariyer fonksiyon bozukluğuna neden olur. Barsak bariyer fonksiyonları bozulduğu zaman, bakteriyel translokasyon görülebilir. Bu çalışmanın amacı, MTX'in neden olduğu bakteriyel translokasyonun önlenmesinde güçlü bir antioksidan olan hesperidinin etkisini değerlendirmektir. Sıçanlara MTX tek doz intraperitoneal yoldan 20 mg/kg/gün verildi. İntestinal bakteriyel translokasyonu önlemek için ardışık 5 gün hesperidin 200 mg/kg/gün gavaj ile uygulandı. Çalışmanın 6. günü ileoçekal bölge, asendan kolon ve mesenterial lenf nodlarından biyopsi örnekleri kültür vasatlarına alındı. MTX grubunda, barsakta daha fazla üreme ve aynı zamanda belirgin bakteriyel translokasyon saptandı ($P<0.05$). Hesperidin üreme yükünü ve bakteriyel translokasyonu belirgin azalmaktadır. Bu çalışmada hesperidinin MTX'ın oluşturduğu hasarını önleyerek translokasyonu engellediği görülmüştür.

Anahtar sözcükler: *Metotreksat, Bakteriyel translokasyon, Hesperidin*

INTRODUCTION

MTX is the most widely used an anti-metabolite in cancer chemotherapy. It also plays a crucial role in the treatment of a range of diseases, including lymphocytic leukemia, non-Hodgkin's lymphoma, osteosarcoma, choriocarcinoma, head and neck cancer and breast cancer. MTX also has major toxic effects, including intestinal injury and enterocolitis. Administration of MTX compromises mucosal barrier function, leading to gut flora invading the circulation [1,2].

Mucosal injury and compromise of the intestinal barrier can result in nonspecific and unlimited translocation of intestinal microorganisms. Although MTX has been shown

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to cause morphological injuries included intestinal barrier function damage in the mucosa of small intestine its association with bacterial translocation is still unclear [3]. Reducing mucosal damage is important in order to lower the side-effects in patients receiving chemotherapy to a minimum. Neutrophil infiltration and oxidative stress have been shown to be involved in intestinal damage stimulated with MTX [4]. Recent studies have concentrated on antioxidant substances that may prevent the undesired side-effects of MTX in intestinal tissue. Several studies have been performed using antioxidant agents for the purpose of preventing MTX-related damage [5-8].

Hesperidin (flavanone) (HES) is a member of the bi-flavonoid group. It is a potent antioxidant with effects similar to those of Vitamin E and has various biological effects in the nervous systems of numerous mammals. In vitro and in vivo studies have demonstrated antimicrobial, antiviral, antihypertensive, hypolipidemic, antiulcerogenic, antineoplastic, anti-inflammatory, antioxidant and anti-hepatotoxic effects [9]. HES is reported to eliminate free oxygen radicals. It also inhibits the effects of pro-inflammatory mediators that induce neutrophil chemotaxis, such as prostaglandins [10]. In general, citrus bioflavonoids containing HES are known to be safe and have no side-effects, even in pregnancy [11].

This study was planned to determine the effect of HES on translocation that may occur as the result of an increase in certain bacterial groups and changes in the intestinal flora due to MTX on intestinal barrier functions.

MATERIAL and METHODS

Experimental Model

This study was planned in order to investigate the effect of HES on translocation that may occur with a rise in certain bacteria groups and the impairment of mucosal integrity caused by MTX. Forty age and race-matched 16-week-old male Wistar albino rats weighing 275-420 g were included. All rats were fed under identical conditions. Rats were housed 6 or 7 to a cage and maintained in a 12-hour light/dark cycle, at a constant temperature of 21±2°C and relative humidity of 40%-60%. They were divided into four groups: (1) a control group (n = 10), (2) rats receiving MTX alone (n = 10), (3) rats receiving HES alone (n = 10), and (4) rats receiving MTX plus HES (n = 10). All experimental protocols were approved by the Committee on Animal Research at Cukurova University, Turkey. Experimental animals were cared for and used in accordance with the National Institute of Health Guide (8.10.2012, Number - 1).

Study Protocol

Control Group: Serum saline was administered orally through an intragastric tube for five days.

MTX-Group: A single dose (20 mg/kg body weight) of MTX (MTX 500 mg in 20 ml vehicle, F.H. Faulding & Co. Ltd., Australia) was given intraperitoneally to each rat. Serum saline was applied as a placebo through intragastric tube, 5 days after MTX injection, and continued daily until the rats were sacrificed.

HES-Group: HES (SIGMA, USA) 200 mg/kg dissolved in distilled water was administered orally through an intragastric tube for five days and continued until rats were sacrificed.

MTX plus HES-Group: Five days after administration of a single intraperitoneal dose of MTX (20 mg/kg), HES (Hesperidin, 200 mg/kg body weight dissolved in distilled water) was administered orally through an intragastric tube every day and continued until the rats were sacrificed.

On the 6th day after injection of MTX, rats were sacrificed using intraperitoneal ketamine HCl (Ketalar, Parke Davis and Eczacıbaşı, Istanbul) (50 mg/kg) and xylazine HCl (Rompun, Bayer Health Care) (5 mg/kg body weight) injection anesthesia.

Microbiological Analysis

Specimens were taken to the laboratory within 1 h at +4°C in previously tared broth medium containing 1 ml Brain-Heart Infusion Broth (BHIB) and analyzed in terms of microbial load. Specimens brought to the laboratory were re-weighed, the weight of the biopsy specimens was recorded. Tissue samples were subsequently broken down in the BHIB with sterile glass rods. Specimens were weighed again at the end of the breaking down process. Specimens were homogenized by vortexing; 0.5 ml of specimen was taken and 1/10 dilutions in 4.5 ml BHIB were prepared. Subsequently, $10^{-2,-8}$ dilutions on a log10 base were obtained. In order to count bacteria in the form of cfu/ml from each dilution, 0.1 ml was inoculated into five McConkey broths, and the inoculated broths were left to incubate for 18 h at 37°C. Following incubation, mean colony numbers with a standard deviation ±10 from slides with countable colony numbers from 10 to 100 and determined as the figure for cfu/mg calculation. The figure was multiplied by the dilution coefficient, and ratios were determined first with the ml specimen and then with the tissue in ml, thus giving the figure in the mg tissue specimen. The study was based on Gram-negative bacteria colonization, regardless of species.

Statistical Analysis

Values were presented as means (minimum-maximum). Analysis of bacterial density data was performed by using SPSS-15. The Mann-Whitney U test was used to compare the results obtained from the four groups; $P<0.05$ was regarded as statistically significant.

RESULTS

Bacterial load was determined as cfu/g in the tissue samples. Although no bacterial growth was seen in any specimens, the highest value in the mesenteric lymph node specimens was 0.60 mg in the control group, The highest specimen weights and growing bacterial densities were 0.48 mg and $5.5X10^3$ cfu/g, in MTX group, 0.50 mg and 0 cfu/g in HES group, and 0.45 mg and $2x10^3$ cfu/ respectively g in MTX plus HES-Group *(Table 1)*.

The highest weight of ileocecal region was 0.31 mg in control group, and the highest bacterial density was 4.3 x 10^5 cfu/g in one specimen. Specimen weight and growing bacteria were found as 0.48 mg and 5.5 x 10^6 cfu/g in MTX group, 0.50 mg and $4.4x10^3$ cfu/g in HES group, 0.45 mg and 9.2 x 10^4 cfu/g respectively in MTX plus HES-Group *(Table 1)*.

The highest value of bacteriological load was found as 1.82 mg in the ascending colon, in control group, and the highest bacterial load was $5.5x10^5$ cfu/g. The highest weight and growing bacteria densities from this group specimens were 1.35 mg and 5.8 x 10^6cfu/g in MTX group, 1.18 mg and 2.7x 10^5 cfu/g in HES group, and 1.29 mg and $5.9x10^5$ cfu/g respectively, in MTX plus HES-Group *(Table 1)*.

There was no growth in mesenteric lymph node specimens in either control or HES groups. In other words, no bacterial translocation was seen in the intestine. Although the bacterial load of ascending colon was similar to the control and HES groups, significantly fewer bacteria grew in the ileocecal region in HES group. Serious translocation was seen in MTX group in the mesenteric lymph node specimens. However translocation was also seen in the group given MTX plus HES-Group but translocated bacteria density was 10 times lower than that of MTX group (P= 0.028). In other words, HES reduced but did not completely prevent the mucosal damage caused by MTX *(Table 1)*.

Ileocecal tissue specimens showed that MTX significantly increased the bacterial load comparing to MTX plus HES-Group (P=0.009, P=0.009). In the ascending colon, the bacterial load was higher, up to Log10, in the MTX group than that of HES and MTX plus HES-Group. The comprehensive information about the mean weight and bacteria densities in groups lymph node specimens was presented in *Table 1*.

DISCUSSION

Intestinal mucositis remains a major concern during cancer chemotherapy in more than 40% of cancer patients after standard doses of treatment, and in almost 100% of patients treated with high doses. In the gut, mucosal damage and barrier function alterations have been described as consequences of different processes: apoptosis, hypo-proliferation, inflammatory response, altered absorptive capacity, and bacteria proliferation and colonization [12,13].

Bacterial translocation is the passage of viable indigenous bacteria from the gastrointestinal tract to extra-intestinal sites. These include the mesenteric-lymph-node complex, liver, spleen and bloodstream. Three major mechanisms are involved in bacterial translocation: excessive intestinal bacterial growth, deficiencies in host immune defenses and increased intestinal mucosal barrier permeability or damage [14,15].

Recent research has concentrated on the role of the gastrointestinal tract as a reservoir for pathogens that can translocate to the circulation, initiating the septic process and eventually resulting in multiple organ failure. Until recently, most experimental studies have been performed in animal models and have quantified translocation by the recovery of viable micro-organisms from the mesenteric lymph nodes and other tissue by means of culture techniques [16]. Bacterial translocation has been reported in cases of burns [17], hemorrhagic shock [18], endotoxemia [19] and Crohn's disease [20]. The impairment of intestinal barrier functions caused by chemotherapy has been investigated in some studies, but there is a lack of research providing definitive evidence of chemotherapy-associated bacterial translocation [1,21-24].

Table 1. *Mean weight and bacteria densities in groups lymph node specimens*
Tablo 1. *Grupların lenf nodu örneklerinin ortalama ağırlık ve bakteri yoğunlukları*

Specimen	Control (n: 10)		MTX (n: 10)		HES (n: 10)		MTX plus HES (n: 10)		p1	p2	p3
	mg Mean (min-max)	cfu/g Mean (min-max)	mg Mean (min-max)	cfu/g Mean (min-max)	mg Mean (min-max)	cfu/g Mean (min-max)	mg Mean (min-max)	cfu/g Mean (min-max)			
Mesenteric-lymph-node	0.46 (0.34-0.60)	0.0 (0.0-0.0)	0.41 (0.35-0.48)	$4.0x10^3$ ($1.8x10^3$-$5.5x10^3$)	0.42 (0.34-0.50)	0.0 (0.0-0.0)	0.38 (0.29-0.45)	$9.1x10^2$ (0.0-$2.3x10^3$)	0.005	0.054	0.028
Ileocecal region	0.24 (0.18-0.31)	$4.0 x 10^5$ ($4.0x10^4$-$4.3x10^5$)	0.41 (0.35-0.48)	$5.0x10^6$ ($4.3x10^6$-$5.5x10^6$)	0.42 (0.34-0.50)	$4.0x10^3$ ($3.3x10^3$-$4.4x10^3$)	0.38 (0.29-0.45)	$8.0x10^4$ ($7.0x10^4$-$9.2x10^4$)	0.009	0.12	0.009
Ascending colon region	1.34 (0.88-1.82)	$5 x 10^5$ ($4.6x10^5$-$5.5x10^5$)	1.13 (0.90-1.35)	$5.0x10^6$ ($4.5x10^6$-$5.8x10^6$)	0.92 (0.55-1.18)	$2.0x10^5$ ($1.6x10^5$-$2.7x10^5$)	0.95 (0.72-1.29)	$5.5x10^5$ ($5.2x10^5$-$5.9x10^5$)	0.009	0.046	0.009

p1: *Comparison of control group and MTX group bacterial density,* ***p2:*** *Comparison of control group and MTX plus HES group bacterial density,* ***p3:*** *Comparison of MTX group and MTX plus HES group bacterial density*

HES enhances epidermal permeability barrier homeostasis. This is at least partly the result of stimulation of epidermal proliferation and differentiation [25]. Xu et al.[26] showed that HES is effective in an experimental study in which they induced experimental colitis with dextran sulphate. We investigated the effect of HES on MTX-induced bacterial translocation in rats. Oral administration of HES significantly decreased bacterial translocation. These results demonstrate that HES could ameliorate MTX-induced intestinal epithelial damage, our knowledge our study is the first study that MTX-induced bacterial translocation could be ameliorated by HES treatment in the intestine. The culture techniques used in this study were performed to confirm whether intestinal bacteria can translocate to extraintestinal organs such as the MLNs, ileocecal region and ascending colon region in a rat model of chemotherapy. One recent study showed that MTX induced severe damage of small intestinal mucosa and barrier functions in rats. Granulocyte colony-stimulating factor (G-CSF) given subcutaneously to rats is significantly effective in the preventing of bacterial translocation and morphological distortion after complete mechanical intestinal obstruction [3].

There was no growth in mesenteric lymph node specimens in the control and HES groups in this study; in other words, no bacterial translocation occurred. At the same time, while the two groups were similar in terms of ascending colon bacterial loads, significantly few bacteria grew in the ileocecal region in rats belonging to the HES group. Serious translocation was observed in the MTX group, and while translocation was seen in the MTX plus HES group rats, the translocated bacterial load was 10 times less that in the MTX group. In conclusion, our data suggested that HES could ameliorate MTX-induced bacterial translocation. However, it is difficult to explain the possible mechanism of HES based on the present data. Further studies are therefore needed to clarify the exact mechanism.

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RESEARCH ARTICLE

An Investigation into the Prevention of Dark Cutting in Cattle Due to the Effects of Altitude and Silage [1] [2] [3]

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[1] *This project was supported by the Turkish Scientific and Technical Research Council (Project no: TBAG 106 T 746)*
[2] *This study is a partial summary of the PhD dissertation of the first author*
[3] *This study was presented at the IIIrd Turkish National Congress of Veterinary Animal Science July 15-17,2010, Afyon Kocatepe University, Afyonkarahisar, Turkey as an oral abstract*

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Summary

The objective of the study was to investigate methods for the prevention of dark cutting in meat from cattle due to high altitude origin and silage use in fattening. Brown Swiss male vealers (n=83) were sourced from the highlands of the Eastern Anatolian Region of Turkey, where the altitude is approximately 1.800 m, and transported to Antalya province. Holstein male vealers (n=43) were sourced from Antalya province. Both Brown Swiss and Holstein male veal were divided into four groups. Group-I: Basic ration plus sunflower meal (SFM); Group-II: Basic ration(BR) plus sunflower meal(SFM) and 2.000 IU vitamin E/head/day; Group III: Basic ration(BR) plus cottonseed meal(CSM) and 2.000 IU vitamin E/head/day and Group IV: Basic ration(BR) plus cottonseed meal. Vitamin E supplements were provided for 120 day before slaughter. Mean musculus longissimus dorsi dark carcass rates measured in Brown Swiss and Holstein male vealers were 55.40% and 67.40% at pH≤6.01, respectively, at 24 h after slaughter. Vit-E supplementation lessened the dark carcass problem so meat color traits of bulls transported from high altitude to low altitude and raised with high levels of silage can be improved with cottonseed meal plus Vit-E supplementation.

Keywords: *Cattle, Altitude, Silage, Vitamin E, Gossypol*

Sığır Karkaslarında Yüksek Bölge ve Silaj Etkisiyle Oluşan Koyuluğun Önlenmesi İmkanlarının Araştırılması

Özet

Bu araştırma, sığır karkaslarında yüksek bölge ve silaj etkisiyle oluşan koyuluğun önlenmesi imkânlarının araştırılması amacıyla yapılmıştır. Esmer ırk erkek danalar (n=83) Doğu Anadolu bölgesinden, yaklaşık 1.800 m yükseklikten, temin edilip Antalya ilindeki bir besi işletmesine nakledilmişlerdir. Holştayn ırkı erkek danalar (n=43) ise Antalya ilinden temin edilmiştir. Esmer ve Holştayn ırkı erkek danalar dört gruba ayrılmıştır. Her iki genotip için Grup I: Temel rasyon+ayçiçeği tohumu küspesi; Grup II: Temel rasyon + ayçiçeği tohumu küspesi + 2.000 IU E vitamini/gün/baş; Grup III: Temel rasyon + pamuk tohumu küspesi + 2.000 IU E vitamini /gün/baş; Grup IV: Temel rasyon+pamuk tohumu küspesi vitamini grupları oluşturulmuştur. Rasyona kesimden 120 gün önce vitamin E katkısı yapılmaya başlanmıştır. Kesim sonrasındaki 24. saatte pH≤6.01 olduğunda Esmer ve Holştayn ırkı erkek danalarda normal karkas oranları sırasıyla %55.40 ve %67.40 olarak tespit edilmiştir. Vitamin E katkısı koyu karkas problemini azaltmıştır. Yüksek rakımdan düşük rakıma nakledilen ve yüksek seviyede silajla beslenen sığırlarda et rengi özelliklerinin pamuk tohumu küspesi ve E vitamini ile birlikte iyileştirilebileceği söylenebilir.

Anahtar sözcükler: *Sığır, Yükseklik, Silaj, Vitamin E, Gossipol*

INTRODUCTION

Fattening of beef cattle occurs worldwide, and more recently, the production of high quality meat has become increasingly important. Meat color, which is a criterion for determining meat quality, is one of the most important traits for both the market and customers. Furthermore, the correct meat colour convinces customers of the freshness

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of meat and is pre-eminent in the decision to purchase it. Owing to its colour, dark cutting meat negatively affects purchasing decisions by customers and is therefore reluctantly purchased from slaughter houses by both wholesalers and butchers [1,2].

Dark cutting carcass is attributable to various factors, including genotype [3,4] decreases in muscle glycogen levels and stress factors such as abnormal ambient temperature, humidity changes, animal density, social group changes, lariage time at pre-slaughter, season and feeding regime [2,4-8].

The use of high quality silage has been widely adopted to reduce the costs of feeding. However, dark cutting has become a problem for fattening companies worldwide. O'Sullivan et al.[9] reported that the feeding of beef cattle mainly on forage negatively affects meat colour and quality traits.

Holstein and Brown Swiss genotype bulls are commonly used for the production of meat in Turkey. Fattening companies have sourced Brown Swiss crossbred bulls from the East Anatolian Region, much of which is at an altitude of more than 1.800 m. However, the fattening companies have regularly voiced complaints about problems in the marketing of dark cutting carcasses from Brown Swiss bulls. The number of red blood cells per unit of volume in both humans and animals living at high altitudes is higher than in those living at low altitudes. When red blood cells are broken down, the residual iron is released and transferred from the blood to the body tissues by transferrin [10].

Gossypol, like cotton seed meal(CSM), has been widely used as a protein supplement in cattle fattening rations. *In vitro,* gossypol complexes with iron cations at 1:1 or 1:4 rates [11]. Plasma gossypol levels *in vivo* decline linearly with increasing supplementary dietary Fe [12,13]. Such results indicate that gossypol may be a useful dietary supplement for reducing blood iron levels and consequently reducing the problem of dark cutting.

Therefore, the current study compared the effects of cotton seed meal(CSM) and sun flower meal(SFM), with and without supplementary Vit-E, on the colour and pH of musculus longissimus dorsi meat, and also their effects on dark cutting.

MATERIAL and METHODS

Animals, Diets and Housing Type

A total of 126 Brown Swiss (n=83) and Holstein (n=43) male vealers, aged from 6 to 8 months, were used in the study. The Brown Swiss meal vealers were transported to Antalya province from the highlands of the Eastern Anatolian Region of Turkey at an altitude of approximately 1.800 m. The Holstein meal vealers were obtained from Antalya province. The treatment groups were kept in sheltered housing with an eternit roof and fencing. The allocated area for each animal was 7 m^2. Bulls from both genotypes were randomly allocated to two separate subgroups to form 4 groups in total. The treatment groups were as follows: Group I, Basic ration(BR) + sunflower meal (SFM) (G-I); Group II, Basic ration(BR) + sunflower meal (SFM) and 2.000 IU Vit-E/day/per bull (G-II); Group III, Basic ration (BR) + cotton seed meal (CSM) and 2.000 IU Vit-E/ day/per bull (G-III), and Group IV, Basic ration(BR) + cotton seed meal(CSM) (G-IV). The Vit-E supplement used in the animal feed was supplied by BASF Ireland, Ltd. (Ekol Food and Agricultural Co, Istanbul). Animals were fed concentrate and maize silage *ad libitum*. The rations included 50.00% maize silage, 41.10% barley, 3.30% wheat bran and 2.70% SFM or CSM. The same management regime was applied for the duration of the study. Bulls were fattened over the course of 10 months and Vit-E was supplied for 120 d prior to slaughter. The animals were slaughtered at about 16-18 months of age, without stunning or electroshock. They were slaughtered by shackling the right leg and exsanguination while hanging.

pH and Meat Colour

pH and colour of the fresh longissimus dorsi muscle samples were determined. A digital pH meter (Mettler-Toledo SG2 with Inlab 427) was used for that purpose as soon as possible after dressing, and at 45 min, 24 h, 72 h, 120 h and 168 h after slaughter. The colour parameters, L* (lightness), a* (redness) and b* (yellowness), were measured with a Minolta CR 400 colorimeter at 0 min, 45 min, 24 h, 72 h, 120 h and 168 h after slaughter.

*Determination of Muscle α-Tocopher*ol

Meat samples collected at 24 h postmortem were frozen at -18°C prior to the determination of the alfa-tocopherol content. The level was determined with a method modified slightly from Salvatori et al.[14]. The mobile phase was 4% 1-4 dioxane and 0.04% acetic acid in hexane and had a flow rate of 1 ml/min at 25°C. Alfa-tocopherol content was determined with high-performance liquid chromatography (HPLC); (Agilent 1100 model LC MSD (Liquid Chromatography Mass Spectrofotometry)). The detection wavelengths were 292 nm for UV and 292-330 nm for fluorescence.

Determination of CSM Gossypol Amount

CSM in the feed was sampled at the beginning, middle and end of the fattening period. The gossypol content was determined with a method adapted from Hron et al.[15]. The mobile phase contained 80% acetonitrile and 20% 10 mM KH_2PO_4 and had a flow rate of 1 ml/min. The flask was filled to volume with the mobile phase and the gossypol amount was determined by HPLC (Agilent 1100 LC MSD). A 20 µl sample was injected into an Inertsil ODS3 (25 cm × 4.6 cm) column and the gossypol content was calculated from the peak area response of a standard curve

established by chromatography and referenced against known amounts of pure gossypol.

Measurement of Non-transferrin Bound Iron (NTBI)

Blood samples were collected in vacutainer collection tubes by venipuncture from the same animals at the beginning, midpoint and endpoint of the fattening period. They were centrifuged for 5 min at 3.000 rpm. Serum samples were collected and stored frozen at -20°C until analysis. NTBI content in the blood serum was determined using a method modified from Gostriwatana et al.[16]. The non-transferrin bound iron (NTBI) level was measured by using a graphite furnace and atomic absorption spectrophotometry (Perkin Elmer, Analyst 800).

Statistical Analyses

Using a factorial design, the color parameters (L*, a*, b*) and pH were analyzed with two-way analysis of variance (ANOVA) to reveal genotype, treatment and genotype × treatment interactions [17].

RESULTS

pH and Meat Colour

The means of the pH values for the Brown Swiss and Holstein groups are summarized in *Table 1*. The ultimate pH (24 h) in the musculus longissimus dorsi ranged from 5.87 to 5.97 for Brown Swiss and from 5.92 to 5.98 for the Holstein. Among treatments, no differences were determined at 0 min, 45 min, 24 h, 72 h, 120 h and 168 h for the Brown Swiss. However, significant differences were determined 0 min (P<0.05), 45 min (P<0.01), 72 h (P<0.05), 120 h (P<0.01) and 168 h (P<0.01) for the Holstein *(Table 1)*.

The pH values of Brown Swiss and Holstein bulls are presented for normal and dark carcass rate and meat quality traits (L*, a*, b*) for pH≤6.00 in *Table 2* and *3*. The normal carcass rate (pH≤6.00) at 24 h was 55.40% for Brown Swiss and 67.40% for Holstein bulls (*Table 2* and *3*). In the present study, G-III had the highest normal carcass rate of pH≤6.00 among groups, with Brown Swiss at 66.70% and Holstein at 90.00% (*Table 2* and *3*). Overall, the interactions of pH, genotype and treatment were not significant (P>0.05) *(Table 4)*. The L* values ranged from 33.11 to 35.79 for Brown Swiss and from 29.52 to 37.94 for Holstein bulls for pH≤6.00 (*Table 2* and *3*), and both the Holstein and Brown Swiss CSM groups had higher numbers of animals with a pH≤6.00 than the SFM groups (*Table 2* and *3*). The a* value ranged from 20.92 to 23.79 for Brown Swiss and from 18.54 to 24.18 for Holstein bulls when the pH was≤6.00 (*Table 2* and *3*). In the present study, the effects of genotype, ration and genotype x ration interaction were significant for a* values *(Table 4)*. The b* value ranged from 10.90 to 13.45 for Brown Swiss and 8.65 to 14.69 for Holstein bulls when the pH was ≤6.00 (*Table 2* and *3*).

Muscle α-tocopherol (Vitamin E) Concentration and Gossypol Amount in CSM

The Vit-E concentration was determined in musculus longissimus dorsi samples with HPLC. Vit-E amounts for G-I, G-II, G-III and G-IV Brown Swiss vealers were 0.65, 1.02, 1.09 and 0.46 µg/g, respectively, and 0.65, 1.18, 1.75 and 0.47 µg/g for the Holstein vealers, respectively *(Table 5)*. The Vitamin E amount was not significantly different between genotypes in the treatment groups (P>0.05). Muscle α-tocopherol concentration was not significanly different for treatment groups of the Brown Swiss genotype. However, it was significant for treatment groups of the Holstein genotype (P<0.001) *(Table 5)*.

Table 1. *Least square means for pH for Brown Swiss and Holstein feeding groups*
Tablo 1. *Esmer ırk ve Holştayn ırkında deneme gruplarında pH ortalamaları ve standart hataları*

Breed	Characters	Treatment				P
		Mean SE	Mean SE	Mean SE	Mean SE	
		I	II	III	IV	
Brown Swiss	pH 0 min	6.81±0.06	6.80±0.04	6.86±0.06	6.56±0.04	-
	pH 45 min	6.69±0.05	6.63±0.04	6.56±0.04	6.56±0.04	-
	pH 24 h	5.95±0.11	5.89±0.11	5.87±0.08	5.97±0.09	-
	pH 72 h	6.19±0.12	6.16±0.12	5.86±0.09	6.06±0.10	-
	pH 120 h	6.20±0.12	6.15±0.12	5.87±0.09	6.13±0.11	-
	pH 168 h	6.31±0.12	6.24±0.13	5.89±0.10	6.10±0.12	-
Holstein	pH 0 min	7.08±0.12^{b}	6.73±0.04^{a}	6.82±0.09^{ab}	7.05±0.11^{b}	*
	pH 45 min	6.97±0.11^{b}	6.58±0.05^{a}	6.66±0.14^{a}	6.98±0.05^{b}	**
	pH 24 h	5.98±0.09	5.92±0.16	5.97±0.14	5.97±0.12	-
	pH 72 h	6.06±0.08^{a}	6.24±0.17^{ab}	6.56±0.17^{b}	5.96±0.12^{a}	*
	pH 120 h	6.04±0.07^{a}	6.12±0.18^{a}	6.63±0.15^{b}	5.96±0.13^{a}	**
	pH 168 h	6.10±0.10$^{a.}$	6.18±0.17^{a}	6.63±0.17^{b}	6.00±0.13^{a}	**

a,b: *Means within a row with different superscripts significantly differ (P<0.05), - non-significant (P>0.05); * P<0.05; ** P< 0.01;* ***I:*** *BR+SFM,* ***II:*** *BR+SFM+vit-E,* ***III:*** *BR+CSM+vit-E,* ***IV:*** *BR+CSM*

Table 2. *pH values, 24th h color traits for Brown Swiss*
Tablo 2. *Esmer ırkta 24. saatte renk ve pH özellikleri*

Characters	Brown Swiss						
	Group	n	Carcass Rate (%)	pH	L*	a*	b*
pH≥ 6.01	I	11	52.40	6.22±0.76	29.27±0.59	17.20±1.19	7.74±0.93
	II	8	40.00	6.16±0.06	30.32±1.11	20.35±1.45	9.54±1.05
	III	7	33.30	6.32±0.12	32.91±1.22	21.12±1.18	11.36±1.17
	IV	11	52.40	6.14±0.07	32.42±1.38	17.09±1.61	7.92±1.38
	Total	37	44.60	6.20±0.04	31.12±0.59	18.58±0.74	8.87±0.61
pH≤ 6.00	I	10	47.60	5.65±0.04	35.79±1.57	23.79±1.57	13.45±1.11
	II	12	60.00	5.71±0.06	33.11±1.00	21.57±0.84	10.90±0.85
	III	14	66.70	5.65±0.03	35.33±0.80	22.70±0.84	12.17±0.73
	IV	10	47.60	5.80±0.04	34.80±1.24	20.92±0.99	11.62±0.80
	Total	46	55.40	5.70±0.02	34.74±0.48	22.26±0.53	11.99±0.44

L: *Lightness,* ***a:*** *Redness,* ***b:*** *Yellowness,* ***I:*** *BR+SFM,* ***II:*** *BR + SFM + vit-E,* ***III:*** *BR + CSM + Vit-E,* ***IV:*** *BR + CSM*

Table 3. *pH values, 24 h color traits for Holstein*
Tablo 3. *Holştayn ırkında 24. saatte renk ve pH özellikleri*

Characters	Holstein						
	Group	n	Carcass Rate (%)	pH	L*	a*	b*
pH≥ 6.01	I	5	50.00	6.19±0.11	36.00±1.22	23.39±1.21	13.99±1.07
	II	4	36.40	6.17±0.05	30.72±0.05	21.10±1.08	10.62±0.71
	III	1	10.00	6.02±0.00	33.14±0.00	18.76±0.00	8.48±0.00
	IV	4	33.30	6.49±0.14	32.99±1.99	20.05±1.79	11.10±1.50
	Total	14	32.60	6.26±0.68	33.43±0.94	21.45±0.82	11.81±0.74
pH≤ 6.00	I	5	50.00	5.77±0.05	37.94±1.19	24.18±1.14	14.69±0.90
	II	7	63.60	5.77±0.38	33.53±1.00	22.19±1.08	12.17±1.20
	III	9	90.00	5.97±0.01	29.52±0.57	18.54±1.75	8.65±1.19
	IV	8	66.70	5.72±0.04	36.23±0.77	23.84±1.20	14.12±0.57
	Total	29	67.40	5.81±0.02	33.79±0.72	21.86±0.81	12.05±0.67

L: *Lightness,* ***a:*** *Redness,* ***b:*** *Yellowness,* ***I:*** *BR+SFM,* ***II:*** *BR + SFM + vit-E,* ***III:*** *BR + CSM + Vit-E,* ***IV:*** *BR + CSM*

Table 4. *Effects of genotype (Brown Swiss and Holstein) and treatment on quality traits of MLD samples*
Tablo 4. *MLD kaite özellikleri üzerine genotip (Esmer ırk ve Holştayn) ve denemenin etkisi*

Variaton Source	pH			L*			a*			b*		
	df	ms	F	df	ms	F	df	ms	F	df	ms	F
Genotype	1	1.92	3.09	1	74.65	2.47	1	325.69	12.19***	1	112.21	7.64**
Treatment	3	0.17	0.28	3	19.03	0.63	3	160.40	6.01***	3	56.58	3.85*
Interaction	3	1.51	2.43	3	286.58	9.49***	3	331.56	12.41***	3	183.30	12.48***
Error	118	0.62	-	118	30.20	-	118	26.71	-	118	14.69	-

*Values are degrees of freedom (df), mean squares (ms) and significant (F) from two way variation analysis, * P<0.05, ** P<0.01, *** P<0.001,* ***L:*** *Lightness,* ***a:*** *Redness,* ***b:*** *Yellowness*

The gossypol amount of 4 847.63 mg/kg in cotton seed meal was determined with HPLC *(Table 6)*. Cotton seed meal supplement was used as 400 g/day/per velaer into ration. Gossypol consumption was 1 939.05 mg/d for the cotton seed groups.

NTBI Amount

NTBI was high in all groups at the beginning of the fattening period, with the exception of those receiving Vit-E supplementation, which had lower values. In pre-slaughter blood values, differences in NTBI amount between the genotypes were determined to be significant. NTBI values moderately increased in groups with Vit-E supplementation. However, for those Holstein groups without Vit-E supplementation, there was a dramatic increase *(Fig. 1)*.

DISCUSSION

pH and Meat Colour

The ideal range of pH values at 24 h postmortem has

Table 5. *Vitamin E value in MLD samples from Brown Swiss and Holstein vealers*
Tablo 5. *Esmer ve Holstayn MLD kasında E vitamini değerleri*

Genotype	Groups				P
	Group - I n (6) X±Sx	Group - II n (6) X±Sx	Group - III n (7) X±Sx	Group - IV n (7) X±Sx	
Brown Swiss	0.65±0.21	1.02±0.21	1.09±0.27	0.46±0.63	-
Holstein	0.65±0.14^{a}	1.18±0.29^{ab}	1.75±0.42^{b}	0.47±0.63^{a}	***
P	-	-	-	-	

*Group I: Basic ration + sunflower meal; Group II: Basic ration + sunflower meal + Vitamin E; Group III: Basic ration + cotton seed meal + Vitamin E; Group IV: Basic ration + cotton seed meal; ^{a,b} Means within a row with different superscripts differ (P<0.05), non-significant (P>0.05); ***P<0.001, MLD: Musculus longissimus dorsi*

Table 6. *Gossypol amount into the cotton seed meal*
Tablo 6. *Pamuk tohumu küspesindeki gossypol miktarı*

Sample	Gossypol (mg/kg)
1st sample	4789.90
2nd sample	4898.50
3th sample	4854.50
Total	4847.63

colour traits (L*, a*, b*) for pH≤6.00 in *Table 2* and *3*. In the present study, when the silage and concentrate balance of the diet was 1:1, L* values ranged from 29.27 to 35.79 for Brown Swiss and 29.52 to 37.94 for Holstein bulls. L* values ranged from 38.30 to 40.00 in Brown Swiss bulls fed with concentrated rations available *ad libitum* [20]; they were higher than the range demonstrated in the present study. The L* values (30.51-32.98) reported by Baublits et al.[21] were close to the results of the present study for the roughage groups. Sunflower meal and cotton seed meal supplements were used in this study. The redness value of the sunflower meal groups was higher than for the cotton seed meal groups in groups without vitamin E supplementation *(Table 2, Table 3)*. In the present study, the 24 h redness values were similar to those of Baublits et al.[21] who reported 19.34 to 20.47 and the 20.20 to 22.40 of Bruce et al.[22]. However, the values from the present study were lower than those of Belgian Blue cattle (23.00 - 23.10) that were fed rations supplemented with Vit-E [23]. In the present study, the effects of genotype, ration and genotype x ration interaction were significant for b* values. The b* value of the sunflower meal groups was higher than for the cottonseed meal groups in those groups not

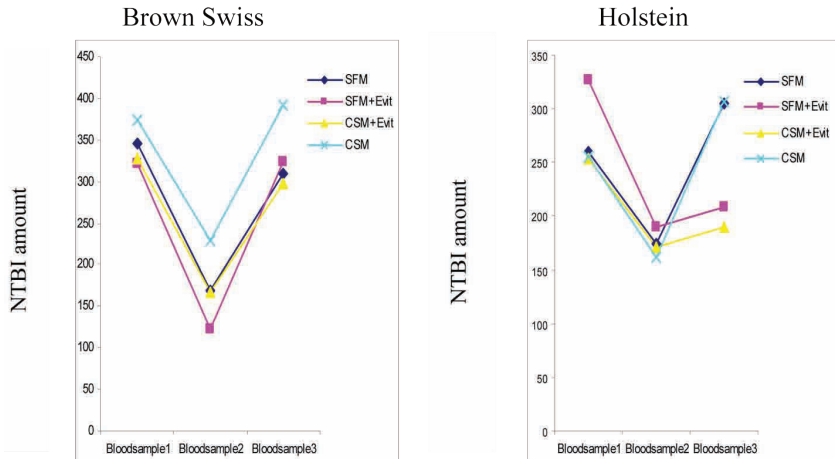

Fig 1. NTBI values for Brown Swiss and Holstein groups in blood sera samples

Şekil 1. Kan serumu örneklerinde Esmer ve Holştayn ırkında TBDM değerleri

been reported as 5.6 to 5.8 [1,18]. However, no meat quality problems have been reported up to pH 6.00 [19]. In the present study, pH values≤6.00 at 24 h ranged from 5.87 to 5.98 and were considered acceptable. The dark carcass rate for the Brown Swiss genotype was higher than for the Holstein genotype, so genotype clearly has a role in meat color (*Table 2* and *3*). Hence, the Brown Swiss genotype was considered predisposed to dark-colored meat [4]. In the present study, G III had the highest normal carcass rate of pH≤6.00 among groups, with Brown Swiss at 66.70% and Holstein at 90.00%. Accordingly, it appears that there was a synergistic effect between vitamin E and CSM in reducing dark cutting. Hence, it may be feasible to use vitamin E and cottonseed meal in combination to reduce the dark cutting problem.

The pH values of Brown Swiss and Holstein bulls are presented for normal and dark carcass rate and meat supplemented with Vit-E. The b* values in the present study approximated those of Baublits et al.[20] who reported values of 7.71 to 9.65 for different rations and, Abril et al.[24] who reported 10.68 for pH≤6.10 and 6.73 for pH>6.10 at 2 d. These results show that supplementation with different protein sources and Vit-E in rations can affect b* values.

Muscle α-tocopherol (Vitamin E) Concentration and Gossypol Amount in CSM

Vit-E content in meat samples in the current study was lower than in several other studies [25-27] but was similar to others [23,28]. The Vit-E amount in Brown Swiss meat (0.46-1.09 µg/g) was lower than in Holstein meat (0.47-1.75 µg/g) in the current study. Dufrasne et al.[23], Lanari et al.[25] and Yang et al.[29] reported that increasing the amount of maize silage in the diet may decrease the vitamin E level in Brown Swiss meat.

The average gossypol consumption was 1 939.05 mg/d for the cotton seed groups. A safe amount of gossypol for cattle per day was reported to be 24 g [30]. Hence gossypol consumption by both genotypes in the present study was well under the reported safe maximum amount and no toxic or harmful effects were observed in the test cattle.

NTBI Amount

NTBI was high in all groups at the beginning of the fattening period, with the exception of those receiving Vit-E supplementation, which had lower values. For pre-slaughter blood values, differences in NTBI amount were determined to be significant between the genotypes. NTBI values moderately increased in groups with Vit-E supplementation. Marwah et al.[31] noted a negative correlation between vitamin E and NTBI. Compared with the control group, Vit-E supplementation dramatically decreased the level of NTBI for Holstein bulls *(Fig. 1)*. Similarly, Marwah et al.[31] found a negative correlation between Vit-E and NTBI. However, in the present study, NTBI values dramatically increased in Brown Swiss groups at the third blood sampling, with or without vitamin E supplementation. The binding of Fe to transferrin protein may be related to environmental conditions or genotype characteristics in Brown Swiss bulls. Transferrin is responsible for the transportation of free iron from blood to tissues. Ferric ion is bonded to N and C regions within transferrin. The N region is endothermic and the C region is exothermic. Ferric ion is bound to the C region in higher amounst than the N region [32].

The current study determined that cottonseed plus Vit-E supplementation can be used to reduce the formation of particularly dark carcass in Holstein and Brown Swiss genotypes. More specifically, the meat color traits of bulls transported from high altitude to low altitude and raised with high levels of silage can be improved with cottonseed plus Vit-E supplementation. To meet the consumer demand for bright red meat, meat producers should pay careful attention to counteracting the effects of silage on blood iron levels by administering appropriate levels of Vit-E to Brown Swiss. It can also be concluded that, according to the results for CSM groups, cotton seed may be used as an alternative feed material to help reduce dark cutting in bulls predominantly fed silage or forage.

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RESEARCH ARTICLE

Normal Echocardiographic Findings in Four Month Old Male Ostrich *(Struthio camelus)*

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Summary

Echocardiography in birds is a useful and competent technique for morphological and functional cardiac assessment. The aim of this study was to establish normal reference echocardiographic values for ostrich. Echocardiographic parameters from 25 apparently healthy male and almost 4 month old ostrich were chosen for this experiment. Echocardiography was prepared from the second and third intercostals space and over the sternum. The mean and standard deviation was calculated for each parameter. The values obtained are: The left ventricular internal diameter at end systole and end diastole was 1.50±0.2 and 2.70±0.16 cm; left ventricular free wall at end systole and end diastole was 0.89±0.04 and 0.62±0.03 cm; inter ventricular septum at end systole and end diastole was 0.99±0.81 and 0.65±0.11 cm, respectively. The stroke volume was 20.98±2.56 cm^3 and fractional shortening was 44.41±0.53%. Normal echocardiographic values in the healthy birds may be used as early diagnostic and prognostic values in ostriches with cardiac diseases.

Keywords: *Ostrich, Echocardiography, Male, Age*

Dört Aylık Erkek Devekuşu *(Struthio camelus)* Normal Ekokardiyografik Bulguları

Özet

Kuşlarda ekokardiyografi morfolojik ve fonksiyonel olarak kalbin değerlendirmesinde yararlı ve geçerli bir tekniktir. Bu çalışmanın amacı devekuşu için normal ekokardiyografik değerleri ortaya koymaktır. Çalışmada görünüşte sağlıklı 4 yaşlı 25 adet erkek devekuşunun ekokardiyografik bulguları kullanıldı. Ekokardiyografi sternumun üzerinden ikinci ve üçüncü interkostal aralıktan sağlandı. Her parametre için ortalama ve standart sapma hesaplandı. Elde edilen değerler: sol ventrikül iç çapı sistol ve diastol sonunda sırasıyla 1.50±0.2 ve 2.70±0.16 cm; sol ventrikül serbest duvarı sistol ve diastol sonrasında sırasıyla 0.89±0.04 ve 0.62±0.03 cm; interventriküler septurm sistol ve diastol sonunda sırasıyla 0.99±0.81 ve 0.65±0.11 cm olarak kaydedildi. Kalp atım hacmi 20.98±2.56 cm^3 ve fraksiyonel kısalma 44.41±0.53% olarak belirlendi. Sağlıklı devekuşlarının normal ekokardiyografik değerleri bu hayvanların kalp hastalıklarında erken tanı ve prognostik amaçlı kullanılabilir.

Anahtar sözcükler: *Devekuşu, Ekokardiyografi, Erkek, Yaş*

INTRODUCTION

Cardiac ultrasonography or echocardiography is considered as a noninvasive and valuable method for evaluating structural and functional values of the cardiovascular system and has been used in veterinary medicine since about 30 years ago and echocardiography is a useful technique for diagnosing cardiovascular disease

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in animals [1]. Whereas, vascular diseases in birds are not uncommon, according to findings from postmortem surveys but cardiovascular diseases are common in avian species [2-6] which can be diagnosed with echocardiography in antemortem period. In veterinary medicine, echocardiographic examination has become a very important diagnostic tool, and is indicated for assessment of cardiac function and the structure of the heart. Several years ago echocardiography of birds due to position of the air sac and anatomical peculiarities was difficult but in recent years it is used for the diagnosis of cardiac complications in birds [7,8], horses [9,10], dogs [11], and cats [12]. Echocardiography as a noninvasive method for assessment of cardiac turkeys and other species of birds have been used [13]. Also reference Values from clinically normal animals by use of 2D and M mode echocardiography have been reported in human [14] and a variety of animals, including ferrets [15,16], chinchillas [17], hamsters [18] and rabbits [19], cats [20,21], dogs [22-25], rat [26], horse [27], however, to the author's knowledge, reference values for ostrich have not been published in English veterinary literature. The aim of the study was to establish normal reference echocardiographic value in the ostrich.

MATERIAL and METHODS

In this study, 25 healthy male almost 4 month years old ostrich were selected (In this study only four months old male ostriches has been used). Echocardiographic examinations were performed with an EX8000 Medison ultrasound system, using a 2-4 MHz Phased array transducer. Echocardiography was prepared from the second and third intercostals space and over the sternum (as a new approach in author's experience). Fasting and general anesthesia have not be needed and the birds were enclosed in standing position. Left ventricular end-diastolic and end-systolic measurements were taken at the largest and at the smallest dimensions between the interventricular septum and the left ventricular free wall, respectively.

The following M-mode measurements were performed: Interventricular septal thickness at end-diastole (IVSd), interventricular septal thickness at end-systole (IVSs), left ventricular internal diameter at end-diastole (LVIDd), left ventricular internal diameter at end-systole (LVIDs), left ventricular posterior wall thickness at end-diastole (LVPWd), left ventricular posterior wall thickness at end-systole (LVPWs). Right ventricular internal diameter at end-diastole (RVIDd), right ventricular internal diameter at end-systole (RVIDs). In addition, fractional shortening (FS) was calculated [28-30].

Statistical Analysis

Maximum, Minimum, Mean, Range and Standard deviation of measurements were calculated.

RESULTS

Echocardiography was prepared from the second and third intercostals space and over the sternum (*Fig. 1* and *Fig. 2*). Values obtained with 2-dimensional and M-mode in all 25 ostrich were summarized *(Table 1)*. Fractional shortening was significantly lower than the reference value.

DISCUSSION

Echocardiography is considered as noninvasive and valuable method for evaluating structural and functional values of the cardiovascular system. It has been used for many animals and birds but there is limited information about normal echocardiographic measurements in

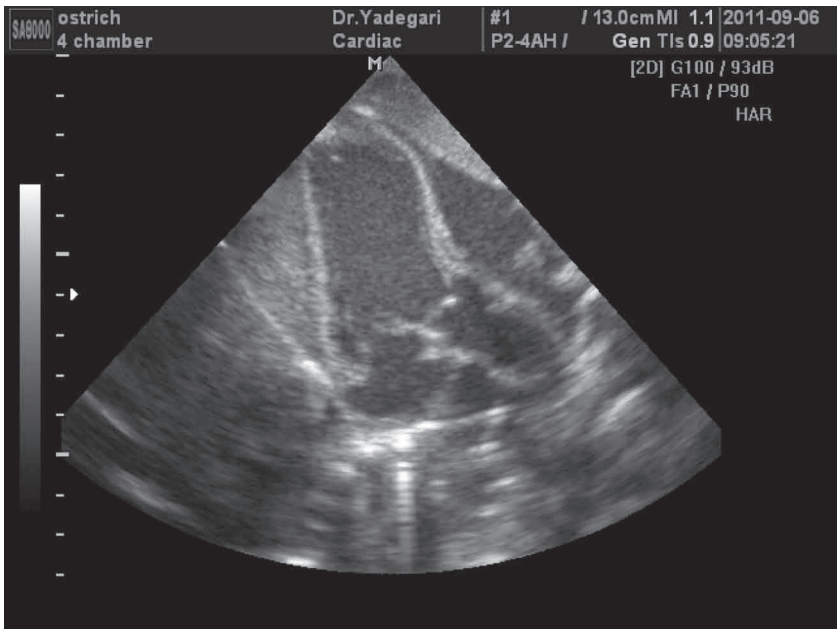

Fig 1. Left ventricle inflow tract and four-chamber view

Şeki l. Sol ventrikül giriş yolu ve dört-odası görünümü

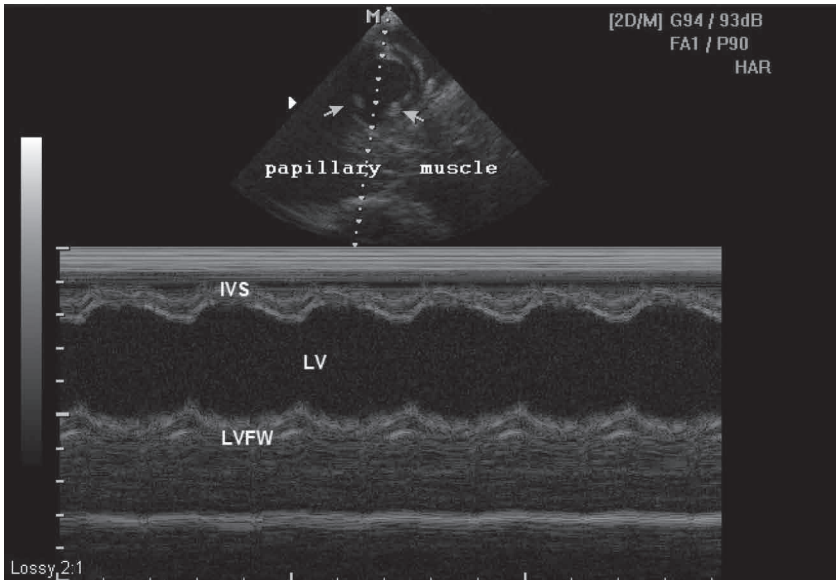

Fig 2. 2D and M-mode echocardiography

Şekil 2. 2D ve M-mode ekokardiyografi ile değerlendirilmesi

Table 1. *Mean and Standard deviation (S.D) of the total echocardiographic parameters calculated for 25 healthy ostriches*

Tablo 1. *25 sağlıklı devekuşu için hesaplanan total ekokardiyografik parametrelerin ortalama ve standart sapmaları (S.D)*

Parameters	Min	Max	±S.D	Mean
Weight (kg)	36.24	44.96	4.36	40.6
RVIDd (cm) [1]	1.38	1.5	0.06	1.44
RVIDs (cm) [2]	0.33	0.43	0.05	0.38
IVSd (cm) [3]	0.81	1.03	0.11	0.92
IVSs (cm) [4]	1.29	1.61	0.16	1.45
LVIDd (cm) [5]	2.76	3.12	0.18	2.94
LVIDs (cm) [6]	1.17	1.39	0.11	1.28
LVFWd (cm) [7]	0.84	1.02	0.09	0.93
LVFWs (cm) [8]	1.59	1.79	0.10	1.69
FS% [9]	53.61	58.97	2.68	56.29

1*- Right ventricular internal diameter at end-diastole,* ***2****- Right ventricular internal diameter at end-systole,* ***3****- Interventricular septal thickness at end-diastole,* ***4****- Inter ventricular septal thickness at end-systole,* ***5****- Left ventricular internal diameter at end-diastole,* ***6****- Left ventricular internal diameter at end-systole,* ***7****- Left ventricular posterior wall thickness at end-diastole,* ***8****- Left ventricular posterior wall thickness at end-systole,* ***9****- Fractional shortening*

ostriches. The heart is the one vital organ in living things. In ostrich it one of the biggest organs in respect body, because in adult ostrich, height receive to 1.5-2 meters thus heart should be able to pumping the blood to this distance. One of the confident procedures for notification about structural and functional status of the heart is echocardiography. Existence of having the normal echocardiographic indicators values in any breed of birds has special importance.

The recognition of a high incidence of atherosclerosis in birds [2,3,31] has generated an interest in developing imaging modalities that will help identify lesions antemortem. Atherosclerosis has been reported in many avian orders, but Psittaciformes [32] and Anseriformes such as ducks and geese [32] appear to be particularly susceptible. Atherosclerosis has also been seen in other species such as ostrich, penguins, cormorants, free-ranging owls and various Passeriformes, including birds of paradise [32,33]. As well as aortic aneurysms are also found occasionally in poultry, specifically chickens and turkeys and anecdotally in ostriches [34,35] can be diagnosed with echocardiographic procedure.

Echocardiography may be useful for diagnosis of the arteriosclerotic processes of the large vessels close to the heart, but no systematic studies have been done to date. In a white cockatoo, an aneurysm of a coronary artery associated with arteriosclerosis has been diagnosed by echocardiography [36].

Martinez Lemus et al.[37], in 1998 studied on echocardiographic evaluation of cardiac structure and function in broiler and Leghorn chickens. This study was conducted to validate echocardiography in chickens and to compare cardiac structures and function between broiler and Leghorn chickens. Diameters of the right and left ventricles and the thicknesses of the left ventricular free wall and the interventricular septum were measured echocardiographically in 5 and 7-wk-old chickens from both lines. In this study suggested the broiler chickens have relatively smaller structural and functional heart than Leghorn chickens. Also right ventricular fractional shortening did not differ between the chicken lines, but difference in left ventricular fractional shortening was established Pees et al.[38] Belief 2D-echocardiography measurements demonstrate a strong correlation between the sternal length/the body mass and the length of the left ventricle in psittacines. Standardized views and protocols for mammals recommended by the American College of Veterinary Internal Medicine cannot be used in birds and comparisons of the measurements with those in mammal

or human medicine are not valid. However, in recent years, case reports regarding the use of echocardiography for diagnosis of cardiac disease in birds have demonstrated the potential of this diagnostic procedure. Initial studies have been conducted and standardized protocols for echocardiographic examination in avian patients have been established [38]. There is scant information about the cardiovascular system in ostrich. However, MacAlister [39], Bezuidenhout [40] studied ostrich heart and its associated arteries and veins. Krautwald-Junghanns et al.[41] examined the heart of 108 racing pigeons of the breed Bricoux. The birds were divided in two groups. One group those kept in aviaries (untrained) and other group, racing birds. Heart dimension and/or the heart work in these 2 groups have significant differences. Thus, for the first time, prove the information of athletics' heart in birds and the adaptation of the avian heart to appropriate performance were requirements. Pees et al.[42] in one examination of using echocardiography in 39 pigeons, which divided in 3 groups (control group, receiver 5 mg/kg enalapril group and receiver 10 mg/kg enalapril group) show an improvement of cardiac function from 26.8% (day 1) to 36.6% (day 140) could be seen, that means right ventricular FS increased. The effect of age on fractional shortening was reported. In avian species, previous reports of fractional shortening exist such as turkeys, reported left ventricular fractional shortenings varied from 38.8% to 81.9% in apparently healthy turkeys ranging in age from 17 to 161 days, with the lowest percentage occurring in the oldest birds [43]. Gyenai et al.[44] use echocardiography in turkey poults from hatch to 28 days of age. The parameters evaluated included Left Ventricular Internal-Diastolic dimension (LVIDd), Left Ventricular Internal-Systolic dimension (LVISd), Interventricular Septal End-Diastolic (IVSEd), Interventricular Septal End-Systolic (IVSEs), Left Ventricular Wall End-Systolic (LVWEs) and Left Ventricular Wall End-Diastolic (LVWEd). To induce DCM, feed containing Furazolidone (Fz) was fed to turkey poults from one to 28 weeks-of-age and compared between the LVIDd and LVISd were the most consistent indicators of DCM [44].

To our knowledge, there is no previous study on normal echocardiographic findings in ostrich. In one study exhibited, because of the avian anatomy suitable echocardiographic windows to the heart are limited. There are two possibilities: the ventromedian and the parasternal approach [45], but in our study we try to exert another simple and convenient style approach for this reason. Therefore, we suggested carrying out echocardiography procedure from the second and third intercostals space and over the sternum is an applicable method and it may be stated that the achieved values presented here, are primary echocardiographic parameters of the heart in ostrich.

Obtained data in this research demonstrates that it is possible to use a new simple and different approach for achieve suitable echocardiogram in ostrich and perhaps other birds. Normal echocardiographic values in the healthy birds may be used as early diagnostic and prognostic values in ostriches with cardiac diseases.

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RESEARCH ARTICLE

The Microbiological Quality of Ready to Eat Salads Sold in Afyonkarahisar, Turkey

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Summary

This study was designed to evaluate the microbiological quality of RTE salads sold at retail in Afyonkarahisar. Total of 261 Ready-to-eat (RTE) salad samples (58 Russian salad, 52 sesar salad, 45 tuna fish salad, 57 Mediterranean salad and 49 cig kofte) collected from 7 different private restaurants, cafes and shopping centers were microbiologically analysed during 2011-2012. Total viable count (TVC), *Staphylococcus aureus, Enterobacteriaceae* and yeast and mold counts of the samples were determined. Of the total of 261 samples, 55.1% and 54% were found to be contaminated with >6 log cfu/g TVC and >4 log cfu/g *Enterobacteriaceae* respectively whereas 13% of which were found to be contaminated with >2 log cfu/g *S. aureus*. All of Mediterranean salads were contaminated with >6 log cfu/g TVC and >4 log cfu/g *Enterobacteriaceae* and similarly, most of tuna fish and sesar salad samples were found to be contaminated with these agents with the contamination levels of 83.3% and 86.6%, 71.1% and 75% respectively. The results of this study revealed that the high contaminations of these foods may be a potential hazard for public health.

Keywords: *Microbiological Quality, RTE Foods, Salads*

Afyonkarahisar'da Satışa Sunulan Tüketime Hazır Bazı Salata/Mezelerin Mikrobiyolojik Kalitesi

Özet

Bu çalışma Afyonkarahisar'da satışa sunulan tüketime hazır bazı salata/mezelerin mikrobiyolojik kalitesini belirlemek amacıyla planlanmıştır. Çalışma kapsamında 2011-2012 döneminde Afyonkarahisar'da restaurant, cafe ve çeşitli satış merkezlerini içeren 7 farklı özel işletmeye ait 261 salata/meze örneği (58 rus salatası, 52 sezar salatası, 45 ton balıklı salata, 57 akdeniz salatası, 49 çiğ köfte) mikrobiyolojik yönden incelenmiştir. Örneklerde Aerob Mezofil Genel Canlı (AMGC), *Staphylococcus aureus, Enterobacteriaceae* familyasına ait türler ve maya-küf sayıları belirlenmiştir. Bu çalışma sonucunda toplam 261 örneğin %55.1'inin >6 log kob/g düzeyinde AMGC içerdiği belirlenirken, %54'ünün >4 log kob/g düzeyinde *Enterobacteriaceae* familyasına ait türleri ve %13'ünün >2 log kob/g *S. aureus* içerdiği ortaya konmuştur. İncelenen örneklerden akdeniz salatası örneklerinin tamamında >6 log kob/g düzeyinde AMGC ve >4 log kob/g düzeyinde *Enterobacteriaceae* familyasına ait türlerin bulunması benzer şekilde ton balıklı salata ve sezar salatası örneklerinin de söz konusu bakteriyel ajanlarla sırasıyla %83.3 ve %86.6, %71.1 ve %75 oranında kontamine bulunması, çalışma kapsamında incelenen örneklerin halk sağlığı açısından risk teşkil edebileceğini ortaya koymaktadır.

Anahtar sözcükler: *Mikrobiyolojik Kalite, Salata, Tüketime Hazır Gıdalar*

INTRODUCTION

During harvest the superficial microbiota of vegetables comprises mainly Gram negative saprophytes, and pathogenic microorganisms. Vegetables may harbour enteric pathogens involved in foodborne outbreaks world-wide causing symptoms of gastroenteritis and chronic infections [1,2]. Ready-to-eat (RTE) foods can be described as the foods being ready for immediate consumption at the point of sale. RTE foods could be raw or cooked, hot or chilled and can be consumed without heat treatment [3].

Total viable count (TVC) results give knowledge about the food processing conditions. Series of results over time

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generally provide a better understanding. The presence of coagulase positive staphylococci (a subgroup of *S. aureus*), is an indication of human contact. Even minimal handling of foods can result in coagulase positive staphylococci being present in foods at low levels. Extensive handling and/or temperature abuse may result in increased levels and increased food safety risk if toxin production occurs [4].

European Scientific Committee [5] has reported that most of the outbreaks linked to fresh products have been associated with members of *Enterobacteriaceae* group. In RTE foods that are fully cooked, *Enterobacteriaceae* are used as an indication of either post-processing contamination or inadequate cooking [4]. Mycotoxins released by some molds, (*Aspergillus*, *Fusarium*, *Penicillium*, *Chaetomium* and *Stachybotrys*) during their metabolic activities may cause toxic effects ranging from short-term mucous membrane irritation to suppression of the immune system and cancer [6].

In Turkey, large supermarkets are responsible for most of the total sales of the RTE foods. The salads on retail sale in Turkey include fresh or boiled vegetables with or without cooked chicken meat or canned tuna fish and mayonnaise. The vegetables can be obtained from the fresh products through selection, washing, peeling, cutting, rinsing and packaging [2] but these processes may not be enough hurdles for the contamination and growth of pathogens and spoilage microorganisms during storage under refrigeration [7].

This study provides an overview of prevalence data related to some of foodborne pathogens for different categories of RTE foods; Russian salads, sezar salads, tuna fish salads, Mediterranean salads and cig kofte. The major objective of this study was to assess the microbiological safety of these RTE foods and to document the occurrence of some indicator and pathogenic microorganisms. The results can be used for risk assessment and for designing more effective methods.

MATERIAL and METHODS

RTE Food Samples

In this study, a total of 261 samples were collected, from August 2011 to June 2012 in Afyon Turkey. A total of 261 RTE food samples consisting of 58 Russian salad (salad mix containing boiled carrot, potato, peas and mayonnaise), 52 sezar salad (salad mix containing boiled or fried chicken meat, fried bread, lettuce, parsley, tomato, cucumber, boiled corn) 45 tuna fish salad, (salad with canned tuna fish meat, parsley, lettuce tomato cucumber and boiled corn) 57 Mediterranean salad, (salad mix including tomato, Turkish white cheese, olive, boiled corn, lettuce, black cabbage, carrot, cucumber) and 49 cig köfte (with cracked wheat, parsley, fresh onion, tomato paste salt and other spices) samples were randomly acquired from 7 different restaurants, cafes and supermarket chains in city of Afyon located in the middle region of Turkey. Ingredients of RTE foods and their conditions at retail level are presented in *Table 1*.

Microbiological Analyses

- Total Viable Count (TVC)

Total Viable Counts were enumerated by the pour plating method on plate count agar (PCA), followed by incubation at 37°C for 48 h; colonies were recorded as cfu/g [8].

- S. aureus

A direct method was used to determine *S. aureus* counts: 0.1 ml of the appropriate dilution was inoculated on propoured and dried Baird-Parker agar plates supplemented with egg yolk-tellurite emulsion (Oxoid). The plates were then incubated at 37°C for 24 to 48 h. Each typical colony of *S. aureus* (black zone with clearing of egg yolk) was subcultured in tryptone soy agar (37°C, 24 h; Oxoid). Colonies obtained on the last agar were examined microscopically, tested for Gram and catalase reactions, and confirmed by coagulase activity (rabbit plasma-EDTA, MERCK) [9].

- Enterobacteriaceae

The spread plate technique was used to prepare duplicate plates for determination of *Enterobacteriaceae* counts. *Enterobacteriaceae* counts were determined in duplicate in poured plates of violet red bile glucose agar (Oxoid) incubated for 24 hour at 37°C. The typical colonies were confirmed by oxidase test and by the fermentation of glucose in Kligler medium [10].

- Yeast and Mold

Enumeration of yeasts and molds was done using Chloramphenicol glucose yeast extract agar by the pour

Table 1. *Ingredients of RTE foods and their conditions at retail level*
Tablo 1. *Tüketime hazır gıdaların içerikleri ve saklama koşulları*

Samples	Ingredients	Conditions at Retail Level
Russian salad	Boiled carrot, potato, peas and mayonnaise	Stored in fridge
Sezar salad	Boiled or fried chicken meat, fried bread, lettuce, parsley, tomato, cucumber and boiled corn	Stored in fridge
Tuna fish salad	Canned tuna fish meat, parsley, lettuce, tomato, cucumber and boiled corn	Stored in fridge
Mediterranean salad	Tomato, Turkish white cheese, olive, boiled corn, lettuce, black cabbage, carrot and cucumber	Stored in fridge
Cig kofte	Cracked wheat, parsley, fresh onion, tomato paste, salt and other spices	Stored in fridge

plate technique and incubation at 25°C for 3-5 days according to ISO 7954:1999 standard [11].

RESULTS

Table 1 summarizes the ingredients of RTE foods and their conditions at retail level. All of the RTE salad samples were under refrigeration temperature at point of sale. Some of RTE salads contained cooked materials where some of them include mayonnaise, raw vegetables and other raw ingredients.

Table 2 gives the incidences, and the distribution of incidence levels of all analysed parameters (>6 log cfu/g for TVC, >4 log cfu/g for *Enterobacteriaceae* and >2 log cfu/g for *S. aureus*, Yeast and Mold counts) in the analysed RTE food samples.

The TVC of the analysed samples ranged from 3.0 log to 6.0 log cfu/g for Russian salad, 3.0 log to 7.0 log cfu/g for sezar salad, 4.0 log to 7.0 log cfu/g for tuna fish salad, 6.0 log to 7.0 log cfu/g for Mediterranean salad and 3.0 log to 6.0 log cfu/g for cig kofte samples, respectively. The highest proportion of samples (38.69%) fell in the range between 6.0 log and 7.0 log cfu/g for TVC *(Table 3)*. The highest proportion of samples (27.58%) fell in range between 10^5-10^6 log cfu/g for *Enterobacteriaceae* counts.

Regarding the distribution of microbial populations, 55.1% of the samples were found have TVC of >6 log cfu/g, 54% of samples >4 log cfu/g for *Enterobacteriaceae*, 13% of samples >2 log cfu/g for *S. aureus*, 61.3 % of samples >2 log cfu/g yeast and 9.5% of samples with >2 log cfu/g mold *(Table 2)*. The highest incidence of TVC greater than 6 log cfu/g was detected from Mediterranean salad (100%) followed by tuna fish salad (83.3%) and sezar salad (71.1%). Mediterranean salad also had the highest incidence (100%) of *Enterobacteriacea* greater than 4 log cfu/g followed by tuna fish salad (86.6%) and sezar salad (75%). The percentage of samples over contaminated with *S. aureus* (greater than 2 log cfu/g) were, cig kofte samples (20.4%) followed by tuna fish salad (15.55%) and sezar salad (13.46%) *(Table 3)*.

DISCUSSION

Many RTE food products has been developed prepared by various cooking processing and packaging. To provide higher microbiological quality RTE food products, it is important to collect and analyse all the information regarding foodborne outbreak surveys. The principle known factors that contributed to foodborne diseases are reported to be inadequate hand washing (31%) and cross contamination between raw materials and cooked foods.

The presence of high TVC *Enterobacteriaceae*, *S. aureus* and yeast and molds in RTE products are of special concern as RTE products are not usually subjected to sufficient heat treatment before consumption. High counts of these bacteria suggest contamination resulted from poor conditions of processing, insufficient heating or contaminations from contaminated cutting boards, knives and serving wates [12]. Occurrence of *S. aureus* results from poor hygiene practices of operators, cross contamination during preparation or improper storage [13]. High risk conditions during food preparation should be well explained to the operators [14].

Several studies regarding the microbiological quality of various RTE foods have been reported in Brazil [15], in Taiwan [12,16], in Argentina [17], in United Arab Emirates [18], in Italy [19] and in UK [20].

Although TVC does not define the microbiological safety of especially raw products, it is still a very useful tool to monitor the effect of different technologies on the microbiological quality of the products. Also, the determination of TVC should be used as a simple and inexpensive parameter to control the effectiveness of the process in the HACCP monitoring plan [19]. In this study, 55.1% of the RTE samples were found have TVC of >6 log cfu/g which is similar to that reported by Tessi et al.[17] reporting that 74.3% of RTE samples were over contaminated with aerobic counts (>10^5 CFU/g). Likewise, De Giusti et al.[19] also reported high contamination levels (10^6-10^9 CFU/g) from 60.9-82.5% of RTE vegetables in different producers. On the contrary, Patricia and Azanza [21].

Table 2. *Occurrence of TVC, Staphylococcus aureus, Enterobacteriaceae, Yeast and Mold in RTE food samples.*
Tablo 2. *Tüketime hazır gıdalarda TVC, Staphylococcus aureus, Enterobacteriaceae, maya ve küf varlığı*

Sample	Na	TVC (>6 log cfu/g)		*Enterobacteriaceae* (>4 log cfu/g)		*S. aureus* (>2 log cfu/g)		Yeast (>2 log cfu/g)		Mold (>2 log cfu/g)	
		n	(%) b	n	(%) b	n	(%) b	n	(%) b	n	(%) b
Russian salad	58	9	15.5	4	6.8	6	10.34	19	32.7	9	15.5
Sezar salad	52	37	71.1	39	75	7	13.46	44	84.6	9	17.3
Tuna fish salad	45	35	83.3	39	86.6	7	15.55	39	86.6	7	15.5
Mediterranean salad	57	57	100	57	100	4	7.01	57	100	-	-
Cig kofte	49	6	12.2	2	4	10	20.40	1	2.0	-	-
Total	261	144	55.1	141	54	34	13.02	160	61.3	25	9.5

a *Number of samples analysed;* **b** *Number of percentage of positive samples*

Table 3. *Microbiological evaluation and percentage of samples in the following ranges (%)*
Tablo 3. *Örneklerinin mikrobiyolojik olarak değerlendirilmesi ve ilgili kontaminasyon aralıklarında dağılımı (%)*

Sample	Microorganism Levels (cfu/g)	TVC		*Enterobacteriaceae*		*S. aureus*		Yeast		Mold	
		n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Russian Salad (n=58)	$<1.0x10^2$	3	5.3	44	76.0	52	89.7	34	58.9	47	81.0
	10^2 to $<10^3$	-	-	3	5.3	4	6.9	5	8.6	2	3.5
	10^3 to $<10^4$	13	22.5	7	12.1	1	1.7	14	24.1	5	8.6
	10^4 to $<10^5$	18	31.0	2	3.5	1	1.7	4	6.9	4	6.9
	10^5 to $<10^6$	15	25.7	2	3.5	-	-	1	1.7	-	-
	10^6 to $<10^7$	9	15.5	-	-	-	-	-	-	-	-
	10^7 to $<10^8$	-	-	-	-	-	-	-	-	-	-
Sezar Salad (n=52)	$<1.0x10^2$	-	-	2	3.8	45	86.6	8	15.4	41	78.9
	10^2 to $<10^3$	-	-	4	7.7	-	-	-	-	2	3.8
	10^3 to $<10^4$	5	9.6	7	13.5	2	3.8	9	17.3	8	15.4
	10^4 to $<10^5$	3	5.8	7	13.5	4	7.7	15	28.9	1	1.9
	10^5 to $<10^6$	7	13.5	19	36.5	1	1.9	18	34.6	-	-
	10^6 to $<10^7$	26	50.0	13	25	-	-	2	3.8	-	-
	10^7 to $<10^8$	11	21.1	-	-	-	-	-	-	-	-
Tuna Fish Salad (n=45)	$<1.0x10^2$	-	-	5	11.1	38	84.4	6	13.4	38	84.4
	10^2 to $<10^3$	-	-	-	-	-	-	-	-	-	-
	10^3 to $<10^4$	-	-	1	2.2	3	6.7	3	6.7	5	11.1
	10^4 to $<10^5$	1	2.2	10	22.2	4	8.9	14	31.1	2	4.5
	10^5 to $<10^6$	9	20	23	51.1	-	-	14	31.1	-	-
	10^6 to $<10^7$	22	48.9	6	13.4	-	-	8	17.7	-	-
	10^7 to $<10^8$	13	28.9	-	-	-	-	-	-	-	-
Mediterranean Salad (n=57)	$<1.0x10^2$	-	-	-	-	53	93.0	-	-	57	100.0
	10^2 to $<10^3$	-	-	-	-	-	-	-	-	-	-
	10^3 to $<10^4$	-	-	-	-	4	7.0	5	8.8	-	-
	10^4 to $<10^5$	-	-	8	14.0	-	-	16	28.1	-	-
	10^5 to $<10^6$	-	-	28	49.1	-	-	25	43.9	-	-
	10^6 to $<10^7$	38	66.7	21	36.8	-	-	11	19.3	-	-
	10^7 to $<10^8$	19	33.3	-	-	-	-	-	-	-	-
Cig Kofte (n=49)	$<1.0x10^2$	-	-	37	75.5	39	79.6	48	98.0	49	100.0
	10^2 to $<10^3$	-	-	-	-	-	-	-	-	-	-
	10^3 to $<10^4$	2	4.1	10	20.4	7	14.3	1	2.0	-	-
	10^4 to $<10^5$	34	69.4	2	4.1	3	6.1	-	-	-	-
	10^5 to $<10^6$	7	14.3	-	-	-	-	-	-	-	-
	10^6 to $<10^7$	6	12.2	-	-	-	-	-	-	-	-
	10^7 to $<10^8$	-	-	-	-	-	-	-	-	-	-

calculated aerob plate counts for most of the RTE foods as $\geq 10^5$ cfu/unit. Researchers cited the reasons to explain higher aerob plate count values as: use of raw ingredients for the final product, temperature abuse during vending, inadequate cooking and use of leftovers.

In this study, overall *S. aureus* incidence >2 log cfu/g is found to be 13.02%. Among all RTE samples, cig kofte samples showed the highest incidence (20.4%) of *S. aureus* more than 2 log cfu/g possibly associated with the producing method of this product including increased manuel handling compared to other RTE salads analysed. The percentage of samples over contaminated with *S. aureus* following cig kofte samples were 15.55% (tuna fish salad), 13.46% (sezar salad) and 10.34% (Russian salad samples). Similar results were reported by Wei et al.[12] who reported 15.9%. 9.5% and 6.3% of seafood, meat products, and vegetarian food products were over contaminated

with *S. aureus* respectively. In this study 0.38% of samples were habouring more than 10^5 cfu/g *S. aureus* similar to the results of Fang et al.[16] who reported 0.7% of the 18°C ready to eat food samples contained more than 10^5 cfu/g *S. aureus*.

The European Scientific Committee [5] has reported that most the outbreaks linked to fresh produce have been associated with members of *Enterobacteriaceae*. *Enterobacteriaceae* were the second most common contaminating microorganisms for RTE foods in this study, as >4 log cfu/g of these bacteria were isolated from 54% of the RTE foods analysed. Our results are higher than that reported by Little et al.[22] who indicated 39.9% of the samples analysed presented *Enterobacteriaceae* counts higher than 4 log cfu/g but lower than that found by Tessi et al.[17] reporting 63% of analysed RTE samples were over contaminated with these agents.

In this study, higher percentage of *Enterobacteriaceae* >4 log cfu/g were detected in Mediterranean salad (100%) likely due to the use of highly contaminated raw material, lack of good hygienic practices during processing and inadequate storage temperature. Regarding *Enterobacteriaceae* contamination rate, Mediterranean salad were followed by tuna fish salad (86.6%) and sezar salad samples (75%). The microbiological safety and quality of the vegetables used for the preperation of RTE foods depend on the use of appropriate irrigation water and good practices during manipulation but inherent risks of contamination due to cultivation in close contact with soil and organic fertilizers make difficult the control of pathogenic and spoliage microorganisms. Thermal processing and holding temperatures of RTE foods also have critical importance to maintain the microbiological safety. The prevention of contamination and bacterial growth lies in the application of good hygiene practise during growing and processing, effective washing and decontamination, effective temperature control during storage and distribution and the selection of appropriate packaging.

High populations of yeasts and molds were found in this study. High incidence of these bacteria found in this study suggests a short shelf life for the product and poor hygienic quality probably due to highly contaminated raw material, lack of good hygienic practices during preperation and improper storage conditions or different combinations of these factors.

The results obtained within the frame of this study indicates the need of adoption of hygienic practices by food handlers to minimize the risks of transmission of foodborne pathogens through this kind of foods. The results of this investigation revealed that contamination of these foods presented a potential health hazard to consumers. Hygienic rules must be implemented to avoid contamination. Efforts must be employed to ensure that this kind of foods do not became contaminated before final packaging. The expiration dates of the products must accurately reflect the shelf life of product. Better control is needed. Besides some changes in manufacturing practices should be made to enhance the safety.

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RESEARCH ARTICLE

Litter Parameters after Newborn Piglets Peroral Treatment with "Hokovit" Immunomodulator Preparations [1]

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Summary

Litter parameters (piglets mortality, weaned piglets per litter, piglet daily gain and body weight at weaning), after peroral treatment of newborn piglets with natural immunomodulator preparations, were investigated at one commercial pig farm in Serbia. Each piglet in 30 litters were peroral treated with 2.5 mL of One Shot immunomodulator preparation within 2 h to 6 h after farrowing, and 1.2 mL Coloron Forte Plus immunomodulator preparation 24 h after One Shot treatment. Starting at day 7 after farrowing up to weaning, piglets were fed the diet for suckling piglets, supplemented with Piggy Guard Forte Plus immunomodulator preparation (15 kg of preparation/t of diet). In the control group (n=30), piglets were not treated with probiotics. Piglets mortality were significantly (P<0.05) lower in the probiotics treated group than in the untreated (control) group (av. 0.27 vs. 0.53 dead piglets per litter, resp.). Average number of weaned piglets per litter was significantly (P<0.05) higher in the treated group (9.16), compared with the control group (8.97). Piglet body weight (av. 8.60 kg vs. 7.53 kg) and daily gain (av. 228 g vs. 193.8 g) from farrowing to weaning, were significantly (P<0.01) higher in the treated group. The results showed that newborn piglets treatment with immunomodulator preparations can decrease piglets mortality rate and increase their growth performance during suckling period.

Keywords: *Immunomodulators, Mortality, Growth performance, Preweaning period, Newborn piglets*

Yeni Doğan Domuz Yavrularında Oral 'Hokovit' Immunomodulatör Uygulamasının, Sütten Kesim Öncesi Parametrelere Etkisi

Özet

Yeni doğan domuz yavrularında, doğal immunomodülatör uygulamasının, sütten kesim öncesi parametrelerine (mortalite oranı, sütten kesim oranı, canlı ağırlık artışı, sütten kesim ağırlığı) etkisi, Sırbistan'da ticari bir işletmede araştırıldı. Deneme grubundaki yavrulara (n=30), oral yolla, doğum sonrası 2 ila 6 saat içerisinde 2.5 mL One Shot immunomodulatör preparatı 24 saat sonrasında ise 1.2 mL Coloron Forte Plus immunomodulatör preparatı uygulandı. Doğum sonrası 7. günden, sütten kesime kadar olan dönemde yavrular, Piggy Guard Forte Plus immunomodulatör içeren başlangıç yemi (15 kgpreparat/ton) ile beslendiler. Kontrol grubundaki yavrulara (n=30) probiyotik uygulaması yapılmadı. Probiyotik uygulanan grupta, kontrol grubuna göre,yavrularda mortalite oranıdaha düşük seviyede tespit edildi (0.27 ve 0.53), (P<0.05).Sütten kesimdeki yavru sayısı, deneme grubunda (9.16) kontrol grubuna göre (8.97) istatistiksel olarak daha yüksek bulundu (P<0.05). Yavruların vücut ağırlığı (8.60 kg ve 7.53 kg) iledoğum sütten kesim arası günlük canlı ağırlık artışı (228 g ve 193.8 g) da muamele grubunda daha yüksek seviyede belirlendi (P<0.01).Bu araştırmanın bulgularına göre, yeni doğan domuz yavrularında, besin yoluyla alınan immunomodülatör katkı maddelerinin, mortalite oranını azaltabileceği ve sütten kesim öncesi büyüme performansını artırabileceği kanaatine varıldı.

Anahtar sözcükler: *İmmünomodülatörler, Mortalite, Büyüme performansı, Sütten kesim öncesi dönem, Yeni doğan domuz*

INTRODUCTION

Piglet mortality is a major factor that produce significant productive and economic losses in the pig industry. Preweaning piglets mortality rate ranges from 11 to 20% in UK, Japan, Denmark, US, Canada and Australia [1].

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In 47 Serbian pig herds, the live born preweaning mortality range from 10 to 12% (aver. 11.06%) [2]. The low viability, starvation and diarrhea have been reported as the major causes of preweaning piglets mortality [3]. Infectious diarrhea is the major infective ethiology cause of piglets mortality within first 7 days after farrowing [4,5]. As the pigs epitheliochorial placenta is impermeable to immunoglobulins (Ig) [6], piglets are born without immune protection [7]. Consequently, protection of neonatal piglets against systemic infection, within first days after birth, is totally dependent on the maternal immunoglobulins, taken by colostrum and milk [8]. However, these maternal immunoglobulins protection is significantly reduced or totally absent in the litters of sows with postparturient disorders, commonly categorized under the terms mastitis-metritis-agalactia (MMA) syndrome [9]. Significant reduction or totally absence of milk production results from inadequate colostrum intake and, consequently, leads to piglets death, primarily due to starvation and hypothermia or infectious diarrhea because of to inadequate transfer of maternal immunoglobulins to the piglets [10].

Given within medicated water, feed additives or injectable preparation, antibiotics are routinely used to reduce the risk for newborn piglets diarrhoea and mortality [11]. However, the resistance to antimicrobial agents [12] and transference of antibiotic resistance genes from animal to human microbiota [13], are the main reason for the limited use of probiotics for prophylaxis and therapy of infectious diseases, as well as for growth promote in newborn piglets [14,15].

The objectives of the present experiment were to determine the effects of oral administration of probiotic immunomodulators, on growth performance and mortality rate in suckling piglets.

MATERIAL and METHODS

The experiment was carried out in one pigs farm, on about 1.200 sows in reproductive herd, located in the AP Vojvodina, Serbia.

Experimental treatment: A total of 60 litters of healthy sows (no clinical signs of the reproductive organs or/and mammary gland diseases at farrowing), were divided into two groups (30 litters/group). Piglets in the first group were treated by orally, given probiotic immunomodulators (HU Hofmann AG-CU, 4922 Bützberg, Switzerland) and those in the second group were not given probiotic immunomodulator. "Hokovit" immunomodulator preparation was used. Each piglet was orally received 2.5 mL of One Shot immunomodulator preparation within 2 h to 6 h after farrowing, and 1.2 mL Coloron Forte Plus immunomodulator preparation 24 h after One Shot treatment. Starting at day 7 after farrowing up to weaning, piglets were fed by using the diet for suckling piglets, supplemented with Piggy Guard Forte Plus immunomodulator preparation (15 kg of preparation/t of diet). Lactation was ended on 32nd day. At the start of experiment (farrowing), litter parameters (average live born piglets per litter and body weight at birth) were not different between the treatment and control group *(Table 1)*.

Immunomodulator preparations: One Shot is a preparation for oral application for piglets at birth, including liquid premium colostrum immunoglobulins, as well as other essential immune substances (transferrin, lactoferrin). Effect of application is to increases the body's natural protective ability against infection. This enhances survival rate and stimulates growth of piglets in the first days after birth. Colorona Forte Plus is a liquid preparation for oral application in the first days of life. It contains natural colostrum, a hydrophobic mikrokapsulated lactic-acid bacteria, chelate form of iron (Fe) and copper (Cu), stabilized Micro GII, fructose and plant extracts. It is used instead of traditional iron supplements and vitamins. The preparation improves piglets health condition, stimulates body weight gain, increases appetite and prevents digestive disorders in newborn piglets. Piggy Guard Plus Forte is an immunomodulator which is applied to piglets via the food, on the 7th day, after birth up to weaning. It contains premium colostrum, amino acids in the chelate form, enzymes, yeast extracts and herbal extracts. It Increases natural immunity, piglets body weight gain and feed conversion ratio during lactation, as well as reduces stress of piglets during the transition to solid feed.

Analysis of data: Descriptive statistics, t-test, analysis of variance (ANOVA) were done in the software package Statistics 10th.

RESULTS

The parameters for probiotic treated and control litters, from farrowing to weaning, are shown in *Table 2*.

Dead piglets per litter, within lactation period, were significantly (P<0.05) higher in the untreated (control) group (0.53) than in the probiotics treated group (0.27). Significantly more (P<0.05) piglets were weaned in the treatment group (9.16), when compared to the control group (8.97).

Table 1. *Litter parameters at farrowing - start of experiment (X ± SD)*
Tablo 1. *Doğum sonrası yavru parametreleri-deneme başlangıcı (X ± SD)*

Parameters	Experimental Groups	
	Tretment	Control
Litters per group (n)	30	30
Total live born piglets (n)	283	285
Live born piglets per litter	9.43±0.94^{NS}	9.50±1.22^{NS}
Piglet body weight at birth, kg	1.31±0.98^{NS}	1.32±1.04^{NS}

^{NS} *Not significant (P>0.05)*

Table 2. *Litters parameters from farrowing to weaning (X ± SD)*
Tablo 2. *Doğum sütten kesim arası yavru parametreleri (X ± SD)*

Parameters	Experimental Group	
	Tretment	Control
Litters per group (n)	30	30
Lactation duration (days)	32	32
Total live born piglets (n)	283	285
Dead piglets per litter within lactation (n)	0.27 ± 0.58^{a}	0.53 ± 0.73^{b}
Total piglets weaned per group (n)	275	269
Weaned piglets per litter (n)	9.16 ± 0.83^{a}	8.97 ± 1.06^{b}
Piglet body weight at weaning (kg)	8.60 ± 0.5^{A}	7.53 ± 0.73^{B}
Piglet daily gain (g)	228.0 ± 16.73^{A}	193.8 ± 3.74^{B}

Values within rows with different superscripts differ: ^{A,B} (P<0.01), ^{a,b} (P<0.05)

Piglet body weight (8.60 kg vs. 7.53 kg) and piglet daily gain (228 g vs. 193.8 g), were significantly (P<0.01) higher in the treated, than the control group.

DISCUSSION

In the intensive pig production, the sows and their litters are confronted to many chronic stressors [16]. Stressors significantly reduce the immunity of animals [17], which significantly increases the susceptibility to infectious diseases [18,19]. Periparturient MAA-syndrome (metritis-mastitis-agalactia) in the sows [9,20], and diarrhea in the newborn piglets [1] are the main infectious diseases. Infectious diarrhea in the suckling piglets is the responsible from the high proportion of mortality and economic losses [21]. To prevent diarrhea and reduce the morbidity and mortality, as well as to promote the piglets growth, antibiotics were used [6]. *However,* long-term use of antibiotics creates resistant pathogenic microorganisms, and it has a negative impact on human health, due to antibiotic residues in animal products [22]. In the recent years, there are attempts to overcoming these negative effects by oraly treatment the piglets with the natural bioactive substances of plant and animal origin, by using immunogenic properties (immunomodulators) [23,24].

Our results demonstrated that individual treatment of newborn piglets by oraly administrated natural immunomodulator preparations can significantly improve the preweaning litter performances. Piglets preweaning mortality were reduced (av. 0.27 vs. 0.53 dead treated, and control group, resp.), resulting with a significant increase in the average weaned piglets per litter (9.16 vs. 8.97). Average piglet body weight at weaning (8.60 kg vs. 7.53 kg) and daily gain (228 g vs. 193.8 g) were also higher in the probiotic treated group, when compared with untreated litters. It has been demonstrated, that treatment with natural immunostimulator preparations [25,28], that contain mannan oligosaccarides [26,32], yeast culture [33] or yeast fermentation products [24,29], herbal extract [23], colostrum, chelate form of iron (Fe) and copper (Cu) [27,31], can prevent newborn piglets deseases and increase litter growth performances [30]. The results in the present study, as well as the results of other authors, indicated that probiotics can serve as a potential replacements of antibiotics and as a growth promoter in pigs. However, because the recent results are still inconsistent [34], more studies are needed to justify the use of probiotics in pig diets.

Based on the results on peroral newborn piglets treatment with natural immunomodulators, it can be concluded that: (1) The treatments significantly decrease piglets preweaning mortality, increase average weaned piglets per litter, piglets daily gain and body weight at weaning. (2) These findings suggest that the application of natural immunodulators can be a practical method to reduce suckling piglets mortality and increase the litter growth performances. The economic losses may reduce in the intensive pig production.

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RESEARCH ARTICLE

Effects of Thymoquinone on Plasma Leptin, Insulin, Thyroid Hormones and Lipid Profile in Rats Fed A Fatty Diet [1] [2] [3]

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[2] *This papaer was a part of PhD thesis titled 'effects of thymoquinone on plasma leptin, insulin, thyroid hormones and lipid profile in rats fed a fatty diet'*

[3] *One part of this study (effects of thymoquinone on plasma carnitine, serum paraoxanase and lipid profile in rats fed a fatty diet) was presented in "Vth National Veterinary Biochemistry and Clinical Biochemistry Congress (Adnan Menderes University, 6-8 September 2011) and the other part (effects of thymoquinone on plasma leptin, insulin, thyroid hormones in rats fed a fatty diet) "VIth National Veterinary Biochemistry and Clinical Biochemistry Congress (Kafkas University, 25-27 June 2013)*

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Summary

The purpose of this study was to investigate the effects of thymoquinone (TQ) on plasma hormone levels, which regulate energy metabolism, and on the lipid profile of rats fed a standard and fatty diets. The study was performed on four experimental groups for 6 weeks using Wistar Albino rats weighing 200-260 g, aged 5-6 months, with 10 rats in each group. 1) The control group: Rats were fed a standard diet and given 1 ml 0.9% saline /kg body weight/day by gastric gavage. 2) The TQ group: Rats were fed a standard diet and given 50 mg TQ/kg body weight/day dissolved in 1 ml 0.9% saline, by gastric gavage. 3) The fatty diet (FD) group: Rats were fed an experimental diet containing 50% animal fat and given 1 ml 0.9% saline/kg body weight/day by gastric gavage. 4) The FD+TQ group: Rats were fed an experimental diet containing 50% animal fat and given 50 mg TQ/kg body weight/day dissolved in 1 ml 0.9% saline by gastric gavage. At the end of the experimental period, plasma insulin, thyroxine (T_4), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels were significantly decreased in the TQ group compared with the control group while the plasma glucose level was increased ($P<0.01$). In the FD group compared to the control group, HDL and carnitine levels were significantly decreased while plasma leptin, glucose, total triiodothyronine (T_3), T_4, free triiodothyronine (FT_3), free thyroxine (FT_4), trigliseride (TG), LDL and very low-density lipoprotein (VLDL) levels were significantly increased. Compared to the FD group, in particular, leptin, T_3, FT_4, TG, LDL and VLDL levels were significantly decreased in the FD+TQ group except for increased glucose. The study concludes that TQ may be a natural source for the provention of the harmful effects of a fatty diet, due to the dropped levels of leptin, TG, LDL, VLDL and could be considered for use in the field of preventive medicine.

Keywords: *Fatty diet, Insulin, Leptin, Thymoquinone, Thyroid hormones*

Yağlı Diyet ile Beslenen Ratlarda Timokinonun Plazma Leptin, İnsulin, Tiroid Hormonları ve Lipid Profiline Etkileri

Özet

Bu çalışmanın amacı standart ve yağlı diyetle beslenen ratlarda Timokinon (TQ)'nun, enerji metabolizmasını düzenleyen plazma hormon seviyeleri ve lipid profiline etkilerini araştırmaktır. Çalışma 5-6 aylık, 200-260 g Wistar Albino rat kullanılarak 6 haftalık sürede 4 deneysel grupla ve her bir grupta 10 rat ile gerçekleştirildi. 1) Kontrol Grubu: Ratlar standart yem ile beslendi ve gastrik gavaj ile 1 ml 0.9% NaCl/kg canlı ağırlık/gün olarak verildi 2) TQ grubu: Ratlar standart yem ile beslendi ve gastrik gavaj ile 1 ml 0.9% NaCl'de çözülmüş 50 mg TQ/kg canlı ağırlık/gün olarak verildi 3) Yağlı Yem (YY) grubu: Ratlar kg'na %50 hayvansal yağ ilavesi yapılmış deneysel diyetle beslendi ve gastrik gavaj ile 1 ml 0.9% NaCl/kg canlı ağırlık/gün verildi 4) YY+TQ grubu: Ratlar kg'na %50 hayvansal yağ ilavesi yapılmış deneysel diyetle beslendi ve gastrik gavaj ile 1 ml 0.9% NaCl'de çözülmüş 50 mg TQ/kg canlı ağırlık/gün olarak verildi. Deneme süresi sonunda kontrol gruba göre TQ grupta plazma insulin, T4, HDL ve LDL seviyeleri azalırken, plazma glikoz seviyesi arttı ($P<0,01$). Kontrol grubu ile karşılaştırıldığında YY grubunda, plazma leptin, glikoz, T_3, T_4, FT_3, FT_4, TG, VLDL ve LDL seviyeleri önemli düzeyde artmasına rağmen, HDL ve karnitin düşüktü. Grup YY ile karşılaştırıldığına, glikozun artışı dışında özellikle leptin, T_3, FT_4, TG, VLDL ve LDL seviyeleri YY+TQ grubunda önemli düzeyde düşüktü. Çalışmada plazma leptin, TG, LDL ve VLDL düzeylerini düşürmesi nedeniyle, TQ'nun yağlı diyetin neden olduğu zararlı etkilerin önlenmesinde doğal bir kaynak olabileceği ve koruyucu hekimlik alanında kullanımının düşünülebileceği sonucuna varıldı.

Anahtar sözcükler: *İnsulin, Leptin, Timokinon, Tiroid hormonları, Yağlı diyet*

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INTRODUCTION

Nigella sativa L.(Ranunculaceae) has long been used in traditional herbal medicine as a natural remedy in many countries around the world, especially in the Middle East [1]. The seeds include 36%-38% fixed oils, proteins, carbohydrates, alkaloids, saponin and 0.4%-2.5% essential oils [2]. In research of recent years, several beneficial therapeutic effects have been attributed to thymoquinone (TQ). Thymoquinone, the main bioactive constituent of the volatile oil extracted from *Nigella sativa (NS)*, has many pharmaceutical uses, such as acting as an antioxidant, carminative, anticancerogenic, diuretic, lactagogue, anti-hyperlipidemic, hypocholestrolemic and anti-inflammatory agent [1,3]. Moreover, recent research has reported that TQ extracted from *NS* seeds and commercial TQ caused inhibition of the synthesis of cholesterol [4]. Currently, investigations have indicated that dietary fat intake can lead to obesity and lead to the development of many metabolic disorders, such as cardiovascular diseases, diabetes and hypertension [5,6]. In spite of widespread recognition of *NS* seeds in traditional medicines, the in vivo effects of its seeds or TQ on plasma leptin, insülin, thyroid hormones, carnitine, paraoxanase (PON) and lipid profile levels in standard and fatty diets have not been investigated until now.

Therefore, the present study was designed to investigate the effects of TQ on hormone levels which regulate energy metabolism, and on the lipid profile of rats fed standard and fatty diets, in order to reveal and elucidate the effect of TQ scientifically.

MATERIAL and METHODS

Thymoquinone

The TQ (2-isopropyl-5-methyl-1,4-benzoquinone, $C_{10}H_{12}O_2$) was purchased from Sigma-Aldrich Chemical Company. It was administered at a dose of 50 mg/kg body weight once daily by gastric gavages for 6 weeks. TQ was dissolved by the addition of a 0.9% serum physiologic (SP).

Animals, Diets and Experimental Protocols

Forty male Wistar Albino rats (5-6 months, 200-260 g body weight) were used. The study was approved by the Institutional Ethics Committee of Afyon Kocatepe University (No; 2008/09-08), and performed according to international standards for animal experiments. The animals were left for two days to acclimate to their room, where the researches maintained a 12 h light/12 h dark cycle at room temperature (22±2°C), and provides a standard pellet diet and water *ad libitum*. The fatty diet used for the groups was prepared to be enough for 2-3 days. The standard diet, which was in pellet form, was ground in a blender, and then mixed with minced animal fat, such that each kilogram of the diet contained 50% animal fat. This mixture was then converted to pellet form and kept at +4°C until it was given to the animals.

The study was performed on four experimental groups for 6 weeks using Wistar Albino rats weighting 200-260 g and aged 5-6 months. Ten animals were used in each group. The groups were as follows: 1) The control group: Rats were fed a standard diet and given 1 ml 0.9% saline/kg body weight/day by gastric gavage. 2) The TQ group: Rats were fed a standard diet and given 50 mg TQ/kg body weight/day dissolved in 1 ml 0.9% saline, by gastric gavage. 3) The fatty diet (FD) group: Rats were fed an experimental diet containing 50% animal fat and given 1 ml 0.9% saline/kg body weight/day by gastric gavage. 4) The FD+TQ group: Rats were fed an experimental diet containing 50% animal fat and given 50 mg TQ/kg body weight/day dissolved in 1 ml 0.9% saline by gastric gavage. The nutrient contents and metabolizable energy levels of the standard and fatty diets were determined by The Province Control Laboratory of Afyonkarahisar *(Table 1)*.

The body weight of all animals was recorded weekly during a 6- week period. At the end of the period, blood samples were collected after the animals were fasted overnight and killed under anaesthesia with a combination of ketamine and xylazine HCl. Their plasma and serum was obtained by blood centrifugation at 3.000 rpm (+4°C) for 10 min. and stored at -20°C until used.

Biochemical Analysis

Serum FT_3 (Roche/Cobas/code no: 03051986 190), FT_4 (Roche/ Cobas/ code no: 11731297 122), T_3 (Roche/Cobas/ code no: 11731360 122) and T_4 (Roche/Cobas/code no:12017709 122) concentrations were determined by the chemiluminance method on a Moduler E170 analyzer. Plasma insulin and leptin levels were measured using a commercial enzyme-linked immunosorbent assays kit (Biovender) according to the manufacturer's instructions (Trinitiy Biotech ELISA reader). Glucose, serum PON, plasma carnitine, total cholesterol (TC), TG, HDL and LDL concentrations were determined by using commercial kits (a Dimension RL Max, Hitachi Moduler analyzer and a Rel assay analyzer). The VLDL level was calculated using

Table 1. *Nutrient contents (%) and metabolizable energy levels (kcal/kg) of the standard and fatty diets (dry matter basis)*
Tablo 1. *Standard ve yağlı diyetin besin madde içeriği (%) ve metabolik enerji değeri (kcal/kg)*

Chemical Composition	Standard Diet	Fatty Diet
Metabolizable energy	2600	4345
Crude fat	1.52	40.68
Crude protein	15.22	11.15
Crude cellulose	8.0	8.80
Crude ash	9.10	5.06
Humidity	12.0	7.34

the Friedwald equation. The amount of saturated and unsaturated fatty acids in the beef tallow used in this study was determinated by TUBİTAK-MAM *(Table 2)*.

Statistical Analysis

Data obtained from experiment animals is expressed with mean standard error (±S.E.M.). The statistical differences between the control and experimental groups were evaluated by one-way ANOVA and Duncan post hoc tests [7]. A difference in the mean values with P<0.05 was considered to be significant.

RESULTS

At the end of the experimental period, an analysis of the fatty acids in the animal fat, the rats body weights and the rats' biochemical parameters was conducted. The results are shown *Table 2, 3* and *4*, respectively.

Table 2. *Saturated and unsaturated fatty acid compounds in the animal fat*
Tablo 2. *Hayvansal yağdaki doymuş ve doymamış yağ asitleri kompozisyonu*

Analysis	Fatty Acids (%)	Method
Myristic acid (C14:0)	2.46	IUPAC II D19
Arachidic acid (C20:0)	0.24	IUPAC II D19
Gadoleic/ekiekosenoic acid (C20:1)	0.39	IUPAC II D19
Palmitic acid (C16:0)	22.50	IUPAC II D19
Palmitoleic acid (C16:1)	0.37	IUPAC II D19
Heptadekanoic/margaric acid (C17:0)	2.95	IUPAC II D19
Heptadesenoic/margoleic acid (C17:1)	0.70	IUPAC II D19
Stearic acid (C18:0)	32.10	IUPAC II D19
Oleic acid (C18:1)	29.65	IUPAC II D19
Linoleic acid (C18:2)	3.12	IUPAC II D19
Linolenic acid (C18:3)	0.20	IUPAC II D19
Saturated fatty acids	60.25	
Unsaturated fatty acids *	34.43	
Unidentified compounds	5.32	

** The unsaturated fatty acids in the animal fat were calculated as 36.36%; this percantage includes unidentified compounds in the animal fat*

At the end of the experimental period, body weight had decreased (P<0.05) in the TQ group compared to the control group. In the FD group, as comparared with the control group, body weight had insignificantly increased. Body weight had significantly decreased (P<0.05) in the FD+TQ group, compared to the FD group.

Plasma insulin (P<0.05), T_4 (P<0.01), HDL (P<0.05) and LDL (P<0.01) was significantly decreased in the TQ group compared to the control group, while the plasma glucose level was increased (P<0.01). In the FD group, compared to the control group, HDL (P<0.05) and carnitine (P<0.01) were significantly decreased while plasma leptin, glucose, T_3, T_4, FT_3, FT_4, TG, LDL and VLDL levels were significantly increased (P<0.01). Compared to the FD group, in particular, leptin, T_3, FT_4, TG, LDL and VLDL were significantly decreased (P<0.01) in the FD+TQ group except for increased (P<0.01) glucose. The changes in PON levels were found insignificant in all groups compared to their control groups respective. Carnitine levels were significantly lowered (P<0.01) in the FD group in compared to the control group *(Table 4)*.

DISCUSSION

As shown in *Table 3*, body weight decreased (P<0.05) in the TQ group compared to the control group. Previous studies on rats reported that rats which received a daily administration of 1 mg/kg of *NS* oil had significantly lower body weight than a control group [8]. This finding shows that reduced body weight may be associated with TQ-induced body weight loss in this group in the present study.

A high fat diet leads to increases in body weight, mainly due to excessive energy intake compared to a standard diet [9]. In this study, however, the numerical increases in body weight were found to be statistically insignificant in the FD group compared to the control group at the end of the 6th week. Several studies reported that body weights in high fat diet groups were heavier than standard diet groups during 6-12 week periods [9,10]. The reason for this result may be due to the short experimental period of

Table 3. *Mean body weight (g) of the groups during the experimental period (X ± SEM)*
Tablo 3. *Deneme süresi boyunca grupların ortalama canlı ağırlıkları (g) (X ± SEM)*

Period	Control Group	Group TQ	Group FD	Group FD+TQ	P
0 day	247.00±6.80	256.50±4.17	258.00±5.81	242.40±5.81	0.201
1st week	275.70±6.05^{b}	242.50±7.04^{a}	271.40±5.72^{b}	261.60±7.09^{b}	0.005
2nd week	277.00±6.49^{b}	249.30±5.96^{a}	286.20±6.05^{b}	262.70±8.70^{ab}	0.004
3rd week	283.50±6.18^{bc}	252.50±6.20^{a}	292.60±6.13^{c}	270.90±8.33^{ab}	0.001
4 th week	282.80±5.94^{bc}	261.60±7.40^{a}	299.20±5.22^{c}	276.10±8.18^{ab}	0.004
5th week	292.70±6.05^{ab}	274.00±6.80^{a}	297.60±4.69^{b}	280.00±7.27^{ab}	0.041
6 th week	297.30±6.13^{bc}	278.90±6.50^{a}	305.80±3.81^{c}	287.70±6.76^{ab}	0.017

S.E.M: *mean standard error, Different superscripts $^{a, b, c}$ in the same line indicate a significant difference between groups (P<0.05)*

Table 4. *Results of the analysis of biochemical parameters in all groups (X ± SEM)*
Tablo 4. *Tüm gruplardaki biyokimyasal parametrelerin analiz sonuçları (X ± SEM)*

Parameters	Group Control	Group TQ	Group FD	Group FD +TQ	P
Leptin (pg/ml)	434.99±52.83^{ab}	283.19±32.53^{a}	995.51±163.56^{c}	623.86±111.92^{b}	0.000
Insulin (ng/ml)	3.23±0.25^{b}	2.38±0.32^{a}	3.75±0.32^{b}	3.36±0.27^{b}	0.015
Glucose (mg/dl)	143.50±5.27^{a}	170.60±6.74^{b}	171.40±8.48^{b}	197.10±7.31^{c}	0.000
T_3(ng/ml)	1.02±0.87^{ab}	0.98±0.87^{a}	1.40±1.24^{c}	1.15±1.08^{b}	0.000
T_4(µg/dl)	5.40±4.92^{b}	4.76±4.39^{a}	6.39±5.60^{c}	5.62±4.90^{bc}	0.002
FT_3(pg/ml)	2.85±2.58^{a}	2.55±2.18^{a}	4.53±3.97^{b}	4.04±3.76^{b}	0.000
FT_4(ng/dl)	2.52±2.26^{a}	2.32±2.02^{a}	3.01±2.66^{b}	2.61±2.34^{a}	0.006
PON(U/L)	130.61±32.20^{ab}	174.61±16.54^{b}	93.18±18.38^{a}	114.64±14.68^{ab}	0.071
Carnitine (µmol/L)	35.33±2.06^{bc}	42.83±3.39^{c}	23.00±2.55^{a}	29.00±2.38^{ab}	0.000
TC(mg/dl)	72.60±4.16^{ab}	66.90±1.93^{a}	79.70±3.03^{b}	77.70±2.71^{b}	0.025
TG(mg/dl)	131.30±13.55^{a}	127.50±10.72^{a}	254.50±31.04^{b}	176.60±14.01^{a}	0.000
HDL(mg/dl)	51.60±3.86^{b}	40.70±2.34^{a}	38.50±3.29^{a}	39.60±2.04^{a}	0.013
LDL(mg/dl)	41.40±1.81^{b}	29.17±2.19^{a}	79.89±4.84^{d}	56.62±3.23^{c}	0.000
VLDL(mg/dl)	26.26±2.71^{a}	25.50±2.14^{a}	50.90±6.21^{b}	35.32±2.80^{a}	0.000

Different superscripts $^{a, b, c}$ in the same line indicate a significant difference between groups (P<0.05)

this study. Body weight significantly decreased (P<0.05) in both the TQ group and the FD+ TQ groups compared to the control and FD groups, respectively. This result is in agreement with previous studies [4,11]. There isn't any meaningful difference when the FD+TQ group compared to the control group. According to the literature [8], this result may be related to the effect of TQ which prevents weight gain.

Previous studies reported that reduced body weight results in dramatically decrease in plasma leptin concentration [12,13]. Le et al.[11] stated that *NS* treated rats had lower fasting plasma levels of insulin at the end of a 4-week period. Despite intensive research on TQ, its effects on leptin or insulin are still not very clear. As shown in *Table 4*, plasma leptin (though not statistically significant) and insulin levels decreased (P<0.05) in the TQ group. This finding shown that reduced plasma insulin levels may be associated with TQ-induced body weight loss as TQ reduced food intake [8]. Additionally, other research has stated that during food deprivation, levels of insulin are decreased by low plasma leptin [14], which is consistent with the report of Seeley and Schwartz [13]. Research has recently indicated that leptin, insulin and thyroid hormones are the most conspicious hormones in the regulation of energy metabolism [15]. Leptin, an adipocyte-derived hormone, stimulates signals from adipose tissue for energy expenditure and increases in the basal metabolic rate and energy expenditure [13]. Insulin regulates lipid metabolism by stimulating lipogenesis and so inhibiting fatty acid oxidation. Plasma leptin and insulin levels are increased by a positive energy balance, and thereby activate catabolic pathways and inhibit anabolic pathways. Thus, these changes in the central nervous system changes lead to decreased food intake and increased energy expenditure, resulting in the loss of excess calories stored in the form of adipose tissue [16].

In this study, when the plasma leptin level significantly increased (P<0.01), an enhanced insulin level was not found statistically significant in the FD group with respect to control group. Woods et al.[10] indicated that plasma leptin levels increased in rats with fed a high fat diet, due to increasing fat mass. Additionally, the 36.36% unsaturated fatty acids in the animal fat used in this study could increase the plasma leptin level, consistent with the report of Yıldız et al.[17]. In this study, however, parallel to the increase in the plasma leptin level in the other study, variations in the insulin of the group eating a fatty diet were found insignificant compared to the control group, which was not consistent with the findings of Woods et al.[10]. This difference may be attributed to the unchanged body weight of the rats or the short experimental period of this study. As seen in *Table 4*, the plasma leptin level significantly decreased (P<0.01) in the FD+TQ group compared to the FD group while the insulin level was not significantly changed. These results show that decreased plasma levels of leptin may be related to reduced body weight caused by TQ in the same group.

Despite intensive study of the effects of TQ on diabetes, the effects of neither *NS* oil nor TQ on insulin or leptin levels in animals fed with a fatty diet have been investigated until now. However, several studies reported that TQ increased insulin levels while decreasing blood glucose levels in diabetic rats [18]. Additionally, Kanter et al.[19] demonstrated that *NS* treatment caused a sharp decrease in the elevated serum glucose and a slight increase in the lowered serum insulin concentrations in diabetic rats. In this study,

however, plasma glucose levels increased (P<0.01) in all experimental groups compared to the control group. The increased glucose, however was not at hyperglycemic levels (300 mg/dl) [20]. This study thus did not replicate the hypoglycemia obtained by administering the fixed oil of *NS* [8] to normal rats. However, recent studies have demonstrated that the plasma glucose level remained stable [11] or slightly increased [21] in normal rats treated with *NS* or TQ. This study's result is thus in line with the findings of previous research. The results show that increased plasma glucose may be due to a decreased insulin level in the TQ group. Furthermore, there was a statistically significant increase (P<0.01) in the plasma glucose level in both the FD group and the FD+TQ group as compared with their control groups respective. The reason for this may be that the high fat diet induced hyperglycemia, hyperinsulinemia and insulin resistance [9]. However, the short experimental period of this study may not have been long enough to observe hyperinsulinemia and insulin resistance.

Thyroid hormones are potent stimulants of basal metabolism, leading to loos of body weight and reduced circulating leptin and insulin [15]. Several studies have investigated the relationship between leptin and thyroid hormones; some found a negative correlation [22], while others found a positive correlation [23]. The effects of TQ on thyroid hormones in rats have not yet been sufficiently investigated. In this study, T_4 concentration and insulin levels significantly decreased (P<0.05) in the TQ group as compared to the control group, while the other thyroid hormones did not significantly change. Given the restrictive effect of *NS* or TQ-induced food intake reduction [11], these results may be associated with TQ-induced body weight loss and/or a reduced leptin level in the same group. In addition, thyroid hormones change during the transition from the fed to the starved state, since starvation rapidly inhibits T_4 and T_3 levels.

Leptin is involved to maintaining TRH (Thyrotrophin-releasing hormone), which is necessary for the synthesis of TSH (thyroid stimulation hormone) and other thyroid hormones [22]. Brito et al.[24] reported that a high fat and low-protein diet could have a direct effect on TSH secretion or influence pituitary T_4 uptake or deiodination. In this study, all thyroid hormones, in parallel with leptin levels, significantly increased in the FD group as compared to the control group. Additionally, Avcı et al.[25] reported that plasma leptin, insulin and FT_3 levels increased in mice fed a high fat diet while the FT_4 level was decreased. Similar findings were reported elsewhere [24,26]. Compared with the FD group, in particular, T_3 and FT_4 levels in parallel with leptin levels significantly decreased (P<0.01) in the FD+TQ group. Given that leptin stimulates the conversion of T_4 to T_3 [24], changes in thyroid hormones may be associated with a reduced leptin level in this group.

Paraoxonases (PON_1, PON_2, PON_3) are a group of enzymes involved in the hydrolysis of several organophosphates and aromatic carboxylic acid esters of fatty acids. In addition, PON_1 is an esterase closely associated with HDL, and may contribute to HDL's antiatherogenicity by preventing the oxidation of LDL [27]. In this study, serum PON levels did not change in any of the experimental groups compared to the control group. Previous studies have shown that the PON level decreased in rats and mice fed a high fat diet for several weeks, due to a reduction of PON_1 mRNA levels in males and to a reduced stability and/or number of HDL particles responsible for PON_1 transport in females [27,28]. In the TQ group compared with the control group, HDL and LDL levels were significantly lowered, while the remaining lipid parameters did not decrease significantly. In the FD group, compared to the control group, a decreased HDL (P<0.05) level and increased TG, LDL and VLDL (P<0.01) levels were found significant. However, TG, LDL and VLDL levels were significantly decreased (P<0.01) in the FD+TQ group as compared with the FD group. These results may be associated with a TQ-induced hypocholesterolemic effect by decreasing the LDL level significantly. Al-Naqeep et al.[4], state that TQ and TQ-rich fractions extracted from *NS* seeds caused a hypocholesterolemic effect by inhibiting hepatic HMG-CoAR (3-hydroxy-3-methylglutaryl-coenzyme A reductase) and reducing the expression mRNA level of LDL receptors. Ahmad and Beg [29], reported that thymoquinone phenolic compound common to the methanolic extract (2.64% contained TQ) and volatile oil extracted (13.53% contained TQ) from *NS* seed oil extracts ameliorated all the cardiovascular risk parameters via reduction in hepatic HMG-CoAR activity, levels of LDL receptor and antioxidant mechanisms in atherogenic suspension fed rats.

Carnitine is an important bio-molecule for the transport of long-chain fatty acids from the cytosol into the mitochondria during beta-oxidation [9]. Normal plasma carnitine concentration may be regulated by dietary sources, excretion from the kidney and endogeneous bio-synthesis. A few studies have reported that the plasma carnitine level was neither reduced [30] or increased [31,32] in rats or mice fed a high fat diet. In the TQ and the FD+TQ groups, compared with the control group, carnitine levels did not change while they significantly lowered (P<0.01) in the FD group in comparison with control group. The decreace of plasma carnitine level in the FD group seems consistent with the results of the previous study [30]. There is no information to explain the relationship between TQ and carnitine. Thus, further research on this subject is needed.

In conclusion, the observed leptin, TG, LDL, VLDL-reducer effects of TQ may have medicinal value and explain its ethnomedical use. This study suggests that TQ may be a natural source for the preventation of the harmful effects of a fatty diet, but more advanced research is needed to explain the relationship between TQ and energy metabolism.

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RESEARCH ARTICLE

Development of Indirect ELISA for the Diagnosis of Bovine Hypodermosis *(Hypoderma lineatum)* in the Cattle of Subtropical Region of Pakistan

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Summary

The aim of this study was to develop an indirect ELISA for the detection of *Hypoderma lineatum* antibodies and to determine the influence of seroepidemiological factors on the seroprevalence of bovine hypodermosis in cattle of subtropical region of Pakistan. For this purpose a total of 1000 blood samples were taken from twenty eight villages of Rawalpindi, Attock, Chakwal and Jhelum districts. First instar larvae (L_1) of the warble fly were collected from the surrounding abattoirs to obtain the antigen (HyC) used for development of indirect ELISA. The seroprevalence was 17.4% (174/1000). The highest seroprevalence was recorded in the Rawalpindi district (28.81%), followed by Attock (21.51%), Jhelum (10%) and Chakwal (6.66%). In present study the sensitivity and specificity was 97.28% and 96.44%, respectively. The epidemiological factors showed that the village having hilly locations, local breed, young animals, water bodies, extensive grazing, primary exposed and non medicated were more seropositive as compare to others ($P<0.05$). In conclusion, indirect ELISA developed during this study is very useful tool for early detection of bovine hypodermosis in cattle grazing in subtropical region of Pakistan.

Keywords: *Hypodermosis, Hypoderma lineatum, Cattle, Indirect ELISA, Subtropical Region, Seroprevalence, Pakistan*

Pakistan'ın Subtropikal Bölgelerinde Sığır Hypodermozisinin *(Hypoderma lineatum)* Tespit Edilmesinde İndirek ELİSA Yönteminin Kullanılması

Özet

Bu çalışmanın amacı *Hypoderma lineatum*'a karşı üretilen antikorları tespit etmek amacıyla bir indirek ELİSA yöntemi geliştirmek ve Pakistan'ın subtropikal bölgelerinde sığır hypodermozisinin seroprevalansı üzerine seroepidemiyolojik faktörlerin etkisini belirlemektir. Bu amaçla Rawalpindi, Attock, Chakwal ve Jhelum bölgelerinde yirmi sekiz köyden toplam 1000 adet kan örneği alındı. Nokra sineğinin ilk instar larvaları (L_1) indirek ELİSA yöntemini geliştirmek maksadıyla kullanılacak antijeni (HyC) elde etmek amacıyla çevre kesimhanelerden toplandı. Seroprevalans %17/4 (174/1000) olarak belirlendi. En yüksek prevalans Rawalpindi (%28.81) bölgesinde tespit edilirken bunu Attock (21.51), Jhelum (%10) ve Chakwal (%6.66) takip etti. Bu çalışmada duyarlılık ve özgüllük sırasıyla %97.28 ve %96.44 olarak tespit edildi. Epidemiyoojik faktörlerden tepelik yerleşimli köyler, lokal ırklar, genç hayvanlar, su kaynakları, geniş meralar, primer maruz kalanlar ile ilaçlanmamış hayvanlarda diğerlerine göre seroprevalanslık daha yüksek olarak belirlendi ($P<0.05$). Sonuç olarak, bu çalışmada geliştirilen indirek ELİSA Pakistan'ın subtropikal bölgelerinde sığır hypodermozisinin erken tespitinde oldukça yararlıdır.

Anahtar sözcükler: *Hypodermozis, Hypoderma lineatum, Sığır, İndirek ELİSA, Subtropikal Bölge, Seroprevalans; Pakistan*

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INTRODUCTION

Hypodermosis is caused by a parasite which belongs to genus *Hypoderma*. Bovine hypodermosis has a worldwide distribution pattern in the northern hemisphere of the world, where its larvae induce myiasis [1]. Previous studies showed that the animals were mostly infested by *Hypoderma bovis* and *H. lineatum* on worldwide basis [2]. In USA the economic losses attributed to hypodermosis were more than $ 600 million [3,4]. Hypodermosis causes economic losses due to meat trim at slaughter and also cause detrimental effects on hides [5].

Hypodermosis is an important endoparasitic infection of cattle and goats prevalent in hilly and semi-hilly areas of Pakistan [6]. In Pakistan, *H. lineatum* causes one of the most common parasitic problems reported in large ruminants in Potowar region [7]. The incidence of hypodermosis is reported to be 29% in cattle in Dera Ghazi Khan Hypodermosis, in cattle is resulted in more economic burden especially for the leather industry by lowering down the market value of hides. On the basis of warbled and warble-free hides, the economic losses were estimated as 22.8 million Pakistani rupees per annum. Economic losses due to warble fly infestation were reported to be 20.6 and 2.2 million Pakistani rupees in cattle and buffaloes in Dera Ghazi Khan and Rajan Pur districts, respectively [8].

There are number of diagnostic tools designed to diagnose the disease including grub monitoring procedure. By using traditional method of grub monitoring (back palpation), the hypodermosis is diagnosed at very late stages of infestation when a great extent of damage has already been done, so there dire need to diagnose the infestation at proper time to minimize the economic loss. So there should be a need of reliable diagnostic technique that would help us because the detection of L_1 at the beginning of the migratory phase allows systemic treatment, avoiding the damage provoked by the parasite on the host tissues [9]. The serodiagnosis is another method that is used for early diagnosis of hypodermosis. For this purpose, Enzyme Linked Immunosorbent Assay (ELISA) can be used for diagnosis of hypodermosis at early stage. This serological technique has been used to monitor levels of hypodermosis in Britain for a number of years [10]. In France hypodermosis was diagnosed by direct examinations of animals and also by using the ELISA test in milk samples with a highly satisfactory degree of sensitivity (92%) and specificity (98.1%) [11]. In Pakistan the best time to collect blood samples to perform an immunodiagnosis is from July to September.

Keeping in view the importance of ELISA for early detection of hypodermosis in cattle, the present study was designed to study the seroprevalence of hypodermosis *(Hypoderma lineatum)* in the cattle from a subtropical region (Pakistan) by using indirect (HyC) ELISA.

MATERIAL and METHODS

Study Area

The present study was carried out in subtropical region of Punjab Province, Pakistan.

Study Plan

The present study comprises into the following two phases; Firstly, the development of an indirect ELISA test and to determine the seroprevalence of *Hypoderma* spp. *(Hypoderma lineatum)* based on different epidemiological factors.

ELISA Development

The following steps were carried out in the ELISA development and validation:

- Larvae Collection and Storage

In present study the L_1 were collected in fresh state from esophagus of naturally infected cattle from local abattoirs. The first instar larvae of *H. lineatum* were dissected from the oesophageal tissue of affected animals from August to October and all visible tissue adhesions were removed. They were washed several times in 0.01M Phosphate buffer saline solution (PBS), pH 7.4 to remove mucosal debris and stored in PBS and were freezed at -20°C.

- Isolation of Antigen

In present study the raw or total extract antigen (crude extract) was used for ELISA, the larvae were homogenized with a tissue grinder Polytron (Kinematica AG) in Tris-HCl 0.1 M, pH 7.5, at the rate of 10 larvae per 5 ml buffer, using 5 cycles of 1 min at 14.000 rpm. To avoid temperature rises that could activate the enzymatic processes or denature the proteins, between each cycle, the mixture was kept in an ice bath. Subsequently, the homogenates were centrifuged during 5 min at 10.000 rpm to facilitate separation of the soluble fraction. The supernatant (crude extract) was distributed in aliquots and frozen at -30°C until use. The supernatant obtained in was fractionated by an ion-exchange chromatography, using as DEAE anion exchanger (Di-Ethyl-Amino-Ethyl) bound to cellulose (DEAE cellulose, Whatman) as described by Panadero [12]. The protein concentration of each aliquot by using the Pierce BCA technique method. The protein fraction called hypodermin C (HyC) was separated similar as previously made several authors. In the present study the confirmation of purity of the HyC was analyzed by Polyacrylamide Gel Electrophoresis under reducing conditions (SDS-PAGE) and non reducing (PAGE), following the classical discontinuous system described by Laemmli [13].

- Indirect ELISA Protocol

Simple ELISA plates (Delta Lab, Hot Bottom) were coated with natural hypodermin C (1:500): 20 µl HC + 10

ml PBS was added in 100µl/well and incubated at 37°C for 30 min. Wells were blocked for 30 min at 37°C with 200 µl of dilution buffer PTL (60 ml PBS-Tween +1.2 g skim milk powder) in each well. Then they were washed one time with 200 µl and three times with 100 µl PT. Serum samples were diluted in duplicate (1:10) (and 100 µl were added in each well. Both positive and negative control sera were prepared at the same dilution and were used as standards in each plate. Following the addition of sera, plates were incubated for 1 h at 37°C. Then they were washed once with 200 µl of PT and 3 times with 100 µl of PT. After incubation, Immuno-Conjugate (Horseradishperoxidase conjugated rabbit Antibovine Ig G (H + L) Geneway: GWB-C158CO) was used 50 µl in each well by dilution (1:800). The plate was incubated for 30 minutes at 37°C and them was washed once with 200 µl and twice with 100 ul PT and finally twice with 100 µl PBS. Finally, 100 µl of substrate consisting of 11 ml citrate buffer (pH 5) and 10 mg (0.01 g) of OPD (Ortho Phenylene Diamine), 4 µl of 30% H_2O_2 was added to each well. After 5-20 min in the dark the reaction was stopped by the addition of 100 µl of 3N sulfuric acid in each well. After 2-5 min the absorbance was measured at 492 nm read using a spectrophotometer Labsytem Multiskan™ spectrophotometer. The samples having greater value than the cut off value were considered as positive while below that value were considered as negative. In the present study positive and negative control sera were obtained from a clinically infested animal and a non-infested cow from a warble free area and these standards were provided from abroad [14]. In the present study the true positive animals were confirmed after the clinical examination of animals by Palpation Method and cutoff point to define a positive or negative serum sample was established using ELISA with a positive sera population obtained from cows that afterwards had grubs in their back and with negative sera from native animals that had never grazed.

The diagnostic sensitivity (DSN) and diagnostic specificity (DSP) was determined by the following formula:

Diagnostic Sero-negativity = True Positive/(True Positive + False Negative)

Diagnostic Sero-positivity = True Negative/(True Negative + False Positive)

The cutoff values were calculated by using the following formula:

Cut off value = Mean Negative Control Panel ± 2SD

Seroepidemiological Factors

One thousand random blood samples were collected from different herds in August to September from Attock (n=172), Chawkal (n=225), Rawalpindi (n=354) and Jhelum (n=249) districts. Different factors (district, village, age, sex, location, breed, management, presence of water bodies, medication, previous exposure and grazing pattern) were recorded in a questionnaire in order to study their influence on the warble fly seroprevalence. The animal's clinical examination was carried out on monthly basis.

Statistical Analysis

The statistical analysis was performed by using SPSS software (18 Version) and Chi square Analysis.

RESULTS

In the present study the L_1 larvae were used for antigen preparation and protein estimation was carried by BCA method and HyC was purified from crude protein by ion exchange chromatography *(Fig. 1)*. The HyC was used in development of indirect ELISA. In the present study, the sensitivity and specificity of HyC antigen based ELISA were 97.28% and 96.44%, respectively. The indirect ELISA was first time developed in this region. In Pakistan the life cycle of *Hypoderma* starts from February to December. So during this time the disease can be diagnosed by ELISA method, because the animals having higher titre of antibodies in their blood during the migration of L_1 larvae.

The results of present study were summarized in *Table 1*. The overall seroprevalence was 17.4% (174/1000). The results showed that the conditions in hilly and semihilly areas were more favorable for the life cycle of *Hypoderma* as compared to the plane locations. The type of management (extensive and intensive) was considered. The animals were in extensive system were significantly more infested (P<0.05) then intensive system. The presence of water

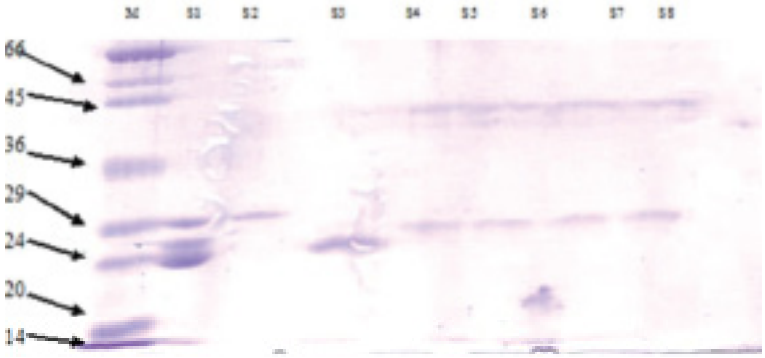

Fig 1. SDS-PAGE (12%) showing the different proteins of H. lineatum {HyC (24) kDa, HyA (31) kDa and HyB (23) kDa. lane 1 molecular weight markers}

Şekil 1. SDS-PAGE (%12) H. lineatum'un farklı proteinlerinin görünümü {HYC (24) kDa Hya (31) kDa ve Hyb (23) kD. Sütun 1 moleküler ağırlık işaretleyicisi}

Table 1. *Potential risk/indicator factors for animal-level bovine hypodermosis sero-positivity. The p-values were determined based on a Chi square (χ^2 Test) analysis with infested and non infested animals of each factor values and levels*

Tablo 1. *Hayvan düzey sığır hypodermozis sero-pozitifliği için potansiyel risk/göstergesi faktörler. P-değerleri enfekte olan ve olmayan hayvanlarda her bir değer faktörleri ve düzeyleri için ki-kare (χ^2 Testi) kullanılarak belirlendi*

S. No	Factor Values	Levels	Examined Animal	Indirect (HyC) ELISA test			
				Negative	Positive	Seroprevalence (%)	P-value
1	Districts	Jhelum	249	225	25	10	F = 60, df = 3, P<0.05)
		Rawalpindi	354	252	102	28.81	
		Chakwal	225	210	15	6.66	
		Attock	172	135	37	21.51	
2	Water Bodies	Water bodies present	437	324	113	25.85	F=43.16 df = 1, P<0.05
		Water bodies Absent	563	506	57	10.12	
3	Grazing Pattern	Tired at Home	378	374	4	1.05	F=109 df = 1 P<0.05
		Field	622	456	166	26.68	
4	Locations	Plane	484	445	39	8.05	F=61.16 df = 2 P<0.05
		Hilly	175	142	33	18.85	
		Semi hilly	341	243	98	28.73	
5	Management	Intensive	313	300	13	4.15	F =53.48 df = 1 P<0.05
		Extensive	687	530	157	22.85	
6	Breed	Local Breed	651	495	156	23.96	F = 69.91 df = 6 P<0.05
		Cross Breed	181	180	1	0.55	
		Sahiwal	59	57	2	3.38	
		Australian	29	28	1	3.44	
		Neelibar	3	2	1	33.3	
		Jarsi	6	5	1	16.66	
		Lohani	71	63	8	11.26	
7	Age	1-3 Years	380	289	91	23.94	F = 58 df = 3 P<0.05
		4-6 Years	324	272	52	16.04	
		7-9 Years	198	179	19	9.59	
		10< Years	98	90	8	8.16	
8	Sex	Male	133	121	12	9.02	F =6.91 df = 1 P<0.05
		Female	867	709	158	18.22	
9	Medication	Medicated	191	182	9	4.71	F =65.57 df = 1
		Non Medicated	809	648	161	19.9	P<0.05
10	Previous Exposure	Reinfested	22	13	9	40.9	F = 9.11 df = 1 P<0.05
		Prim Infested Exposed	978	817	161	16.46	

bodies (present, 25.85% and absent, 10.12%) is considered as an important seroepidemiological factor having a significant difference (P<0.05). The seroprevalence in the animal having previous infestation was significantly higher (P<0.05) than those without previous infestation. It is evident from the results that the older animals have lower prevalence rate then the younger one. The medication (ivermectin) based sero-epidemiological information's were taken in this study. The results showed a significant difference (P<0.05) between non-medicated (19.9%) as compared to medicated (4.71%) animals. The seroprevalence in cattle raised in extensive system was higher than in those kept at intensive system. The Chi square analysis showed that there is significant difference (P<0.05) between these two groups. The seroprevalence in the female (18.22%) was higher as compared to male (9.02%) and having a significant difference (P<0.05) *(Table 1)*.

DISCUSSION

The present study shows that hypodermosis is moderately distributed in Pakistan. Similarly, the prevalence of bovine hypodermosis in district Chakwal was 35.50% in field area [6], 21.62 to 23.8% in cattle [15], 29% in Dera Ghazi Khan, 26% in Rajanpur districts [8], in D. I. Khan, Kohat, Malakand and Abbotabad districts of NWFP [15] and D. G. Khan & Barkhan districts [7,16]. Warble fly infestation ranged from 25 to 90% in goats and 21 to 26% in cattle [15]. The infestation rate of hypodermosis varies within Pakistan [15-18] and many parts of globe [19-21] it might be due to the differences in climatic conditions. In Pakistan all the studies in the past were based on traditional method of grub monitoring that has very low sensitivity and specificity. Similarly, the prevalence of bovine hypo-dermosis is regularly observed in Canada, Europe and in Africa [21], Czech and Solvac Republic (80%), Italy (85%), Greece (49.2%), United Kingdom (40%) at the time of eradication and in Romania (32-43%) [18]. In the east and southeast region of Turkey seroprevalence was 22.1%, 22.3%, 26.3% from Diyarbakir, Malatya and Elazig provinces, respectively. Similarly, it was 47.8%, 38.9%, 33%, 41.6% and 41.9% in Southeast Anatolia, Central Anatolia, Mediterranean, Agean and East Anatolia [23]. In Spain the seroprevalence was 26-42% [20]. In Turkey, the seroprevalence in Thrace region was 3.56% [24] and 4% in Elazig province [23], while 43.3% in Vicenza province of North eastern Italy [25]. In north China, from Xinjiang, Inner Mongolia, Heilongjiang, Jiling and Gansu provinces obtaining percentages of 51.77%, 27.02%, 13%, 6.03% and 44.41% respectively in yaks and cattle by ELISA [26]. In Pakistan, so far there is no previous study on the seroprevalence of hypodermosis. In the present study, the seroepidemiological information was taken in the form of questionnaire based on the district, village, age, sex, breed, location, herd size, previous exposure, grazing pattern and medication. This variability might be due to the differences in the climatic factors that affects on the developmental stages of the larvae [27,28].

Palpation method is used for the monitoring the intensity of infestation on regular basis throughout the warble emergence period, the emergence period of fly lasts from 3-4 months [29]. The ELISA test is used to detect the circulating antibodies in the sera of infested animals and diagnose the cattle grub infestation [30-32]. The antibody detection ELISA test is based on the hypodermin C [14]. In France, Charbon reported the higher degree of sensitivity and specificity 92.2% and 98.1%, respectively in ELISA [11]. Our results are similar to hypodermin C ELISA having sensitivity and specificity 96.4% and 95.6%, respectively [12]. Webster *et al.*[4] reported a competitive ELISA for the detection of antibodies to *Hypoderma* species in cattle sera with a sensitivity and specificity 100% and 92%, respectively. During the last years, immunodiagnostic techniques for cattle hypodermosis have been improved so that their increased diagnostic sensitivity allows investigators to avoid: visual examinations, palpation of warbles or postmortem examination of the oesophagus *(H. lineatum)* [32]. In the present study, we isolate the HyC from the local *Hypoderma* strain and it is used for the development of indirect ELISA. The sensitivity and specificity of the ELISA test developed in this study was 97.28% and 96.44%, respectively.

The hypodermosis is endemic and reported in hilly region of Pakistan [33]. The young animals were more infested due to their softer skin, that's makes easier for 1st instar in to their penetration as reported earlier [34]. On the contrary, the destruction of the developing larvae by internal regulatory systems of the host and development of resistance by continuous exposure of animals to larvae could also explain the low prevalence in aged animals. The age-wise trend was determined [35]. The younger animals have higher infestation then the mature milking cows. Because the older animals has develop the immunity against larvae [36]. Previous insecticide treatments had also influence in the prevalence, but the grazing pattern was the most influencing factor, so that the prevalence of WFI was higher in those animals maintained in an extensive management system than in those kept at home. Otranto reported that free grazing practice is one of the major risk factor for hypodermosis herd's positivity [32]. On contrary, the prevalence was higher in males (26.50%) than in females (20.50%) in Chakwal districts [6]. The seroprevalence was 31% in female and 14.1% in male [37]. The desi breed (27.7%) has higher seroprevalence, crossbreed (26.8%) and pure breed (19.7%), respectively [23].

The present study concludes that the bovine *H. lineatum* infestation is a serious threat and it is moderately distributed in subtropical region, Pakistan. Because there might be a possibility of increasing its prevalence rate in future. So, there is a need of further studies on immunodiagnosis of WFI. Eradication strategies should be implemented because Pakistan is an agricultural country and livestock contributes a major part of the economy.

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RESEARCH ARTICLE

Effects of Boiling on Nitrofuran AOZ Residues in Commercial Eggs

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Summary

The aim of this study was to determine the effects of boiling on nitrofuran 3-amino-2-oxazolidinone (AOZ) residues in eggs. The use of furazolidone in food-producing animals is banned within the EU and Turkey. The nitrofuran AOZ residues in raw and boiled eggs were analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in a chromotographic run of 20 min. The method validation was done according to the criteria laid down in Commission Decision No. 2002/657 EC. Linearity was proved between 0 to 1.5 µg/kg, decision limit (CCα) was 0.70 µg/kg, detection capability (CCβ) was 0.77 µg/kg, recovery values ranged between 88-97.9% and repeatability (CV) was 3-4.3%. The detected avarage nitrofuran AOZ residue level in 13 uncooked eggs by LC-MS/MS was 0.86±0.017 µg/kg which was increased to 2.42±0.037 µg/kg after boiling. In this study, it was surprisingly found that protein-bound side-chain metabolite, nitrofuran AOZ levels in eggs were significantly increased after boiling. This finding runs counter to the claim that heat process in general should decrease various antibiotic levels in food. The observed increase ($P<0.001$) in nitrofuran AOZ levels in boiled eggs relative to uncooked eggs may be due to enhanced efficiency of extraction in boiled samples. Therefore boiled eggs should be used for analysis of nitrofuran AOZ levels in order to obtain more reliable and more predictive results.

Keywords: *Nitrofuran AOZ, Residue, Egg, LC-MS/MS, Validation, Heat process*

Ticari Yumurtalarda Nitrofuran AOZ Kalıntıları Üzerine Kaynamanın Etkisi

Özet

Bu çalışmanın amacı, yumurtalarda nitrofuran 3-amino-2-okzazolidinon (AOZ) kalıntıları üzerine kaynamanın etkisini belirlemektir. Furazolidonun gıda değeri taşıyan hayvanlarda kullanımı Avrupa Birliği'nde ve Türkiye'de yasaklanmıştır. Çiğ ve haşlanmış yumurtalarda nitrofuran AOZ kalıntıları sıvı kromatografi-tandem kütle spektrometrisi (LC-MS/MS) kullanılarak 20 dakikalık süre içerisinde analiz edilmiştir. Uygulanan yöntemin validasyonu Avrupa Birliği Komisyon Kararı No 2002/657 EC 'de belirtilen kriterlere göre yapıldı. Doğrusallık 0-1.5 µg/kg, tayin limiti (CCα) 0.70 µg/kg, tayin kapasitesi (CCβ) 0.77 µg/kg, geri kazanım değerleri %88-97.9, tekrarlanabilirlik (CV) %3-4.3 arasında gerçekleşti. LC-MS/MS ile 13 adet çiğ yumurtada 0.86±0.017 µg/kg miktarında tespit edilen ortalama değer haşlama sonucu bakılan aynı yumurta örneklerinde istatistiki olarak önemli derecede artış göstererek 2.42±0.037 µg/kg ortalama değerlere yükselmiştir. Şaşırtıcı bir şekilde bu çalışmada, kaynamadan sonra yumurta içinde protein-bağlı yan-zincir metaboliti nitrofuran AOZ düzeylerinin arttığı tespit edildi. Bu bulgular genel olarak ısıl işlemin gıdalarda çeşitli antibiyotik düzeylerini azaltması gerektiği iddiası ile ters düşmektedir. Pişmemiş yumurtalara göre haşlanmış yumurtalarda nitrofuran AOZ düzeylerinde gözlenen artış ($P<0.001$) haşlanmış örneklerde ekstraksiyon etkinliğinin artmasına bağlı olabilir. Bu nedenle nitrofuran AOZ seviyelerinin analizinde daha güvenilir ve anlamlı sonuçlar elde etmek amacıyla haşlanmış yumurtalar kullanılmalıdır.

Anahtar sözcükler: *Nitrofuran AOZ, Kalıntı, Yumurta, LC-MS/MS, Validasyon, Isıl işlem*

INTRODUCTION

Veterinary drugs are used in layer flocks by addition to feed or drinking water in order to prevent and treat diseases, promote egg productions, assist in converting stress due to environmental changes, beak trimming,

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vaccination and other management practices. Improperly and illegal administration of these drugs can cause residue problems in eggs and these residues can cause severe harmful effects on consumers. In addition, trace amounts of antibiotic compounds in eggs favor the development of antibiotic-resistant bacteria [1-3].

Nitrofurans have been widely used in the treatment of gastrointestinal infections such as fowl cholera, coccidiosis and blackheads in laying hens. Nitrofurans, including furazolidone, in veterinary practice are banned in many countries, including Turkey, because of their mutagenic, carcinogenic and genatotoxic effects [4-7].

Parent compound furazolidone is rapidly and extensively metabolized *in vivo* (*in vivo* half life is less than a few hours) [8,9]. The side chain metabolites (3-amino-2 oxazolidinone, AOZ) can bind to proteins to form bound residues. These residues are extensively formed in liver, kidney, muscle tissues and also eggs [10-12]. They have a long residence time and their extraction from the tissues is very difficult [12-14].

Short half-life of nitrofurans has made screening for parent drugs difficult in food products, but the development of assays that can detect highly stable nitrofuran metabolites have been used to demonstrate the persistence of tissue-bound residues in egg and the other animal origin foods [9-11,15,16].

Several liquid chromatography-tandem mass spectrometry (LC–MS/MS) methods have been developed for determination of nitrofuran metabolites in different tissues [17-21] and eggs [18]. The detection of AOZ in poultry eggs, was carried out by Mccracken *et al.*[11] using LC-MS.

Several studies have generally described drug residues in uncooked tissues and the other various of food product [18,20,22]. However, the effects of cooking on nitrofuran 3-amino-2-oxazolidinone (AOZ) in eggs have not been studied. It is important to determine if residues of nitrofuran AOZ are reduced or not by boiling procedure. Since egg is usually cooked before consumption, more findings about the effects of boiling on residues of nitrofuran AOZ are needed to determine the risks to the consumer from dietary exposure to these substances. We carried out this study to examine whether boiling has an effect on residues of nitrofuran AOZ in eggs.

MATERIAL and METHODS

Reagents and Water

2-NP-AOZ-D4 internal standard was purchased from Sigma–Aldrich (St. Louis, MO, USA). The other chemicals such as dimethyl sulfoxide (DMSO), hydrochloric acid (HCl), 2-nitrobenzaldehyde, sodium hydroxide (NaOH), dipotassium hydrogen phospate (K_2HPO_4), ethyl acetate, methanol (MeOH) and acetic acid were HPLC grade and were purchased from Merck (KGaA, Darmstadt, Germany). The water used was purified with a Milli-Q water purification system from Millipore (Millex GV, 0.45 µm).

Standard Solutions

The standard stock solution and internel standart were stored at -20°C in the dark and warmed up to room temperature before use. The working standard solution was stored at +4°C in the dark. DMSO was stored at room temperature and the other chemicals were stored at room temperature (22 to 25°C).

Samples

Samples of eggs were collected from different layer flocks as part of the "National Program of Residue Control", between 1 Jan 2010 - 31 December 2010. Nitrofuran AOZ was found positive from one poultry farm's layer flocks by LC-MS/MS. In this study we used nitrofuran AOZ positive 13 egg samples from these layer flocks.

Sample Preparation

The extraction was performed as described previously [23,24] with some minor modifications. Before boiling process, each egg (liquid) was scrambled, split and 2±0.03 g of that was put into 50 ml centrifuge tubes. The remaining amount of each egg (yolk and albumen) were immersed in 100°C water for 10 min, removed and cooled immediately in a chilled water bath. We used the same extraction method for 10 min boiled (solid) eggs. 100 µl 2-NP-AOZ-D4 internal standard and 5 ml 0.1 M HCl were added. They were vortexed for 60 sec and homogenized. Then 300 µl 50 mM 2-nitrobenzaldehyde, which was dissolved in dimethylsulfoxide (DMSO), was added and mixed for 1 min by multireax vortex. They were incubated in the shaker incubator at 37°C for 16 h at 50 rpm. After incubation, samples were cooled to the room temperature. 700 µl neutralization solution was added [1 M NaOH/0.1 M K_2HPO_4 + $3H_2O$ (2:1, v/v)] [25]. They were vortexed for 1 min. 5 ml ethyl acetate was added onto it and they were mixed in the shaker incubator at room temperature for 10 min at 300 rpm, then to prevent soap forming between lipid and ethyl acetat, 3 ml n-hexane was added. Then they were centrifuged at 15°C for 10 min at 4000 x *g*. 6 ml of upper phase were taken and transfered into 15 ml glass tube, and dried under nitrogen flow in 5.0 psi pressure at 42°C. After the drying operation, 1 mL n-hexane was added into the tube and vortexed for 1 min. 750 µl MeOH/deionized water (1:4, v/v) was added. They were mixed with the multireax vortex for 5 min. They were centrifuged at 15°C for 10 min at 2500 x *g* and the sample was taken from the lower phase by the help of an injector. Then this liquid part was filtered onto a 0.45 mm filter and 50 µl filtrate is transferred to a microvial capped and adapted to the LC autosampler [23,24].

LC–MS/MS Analysis

This study was carried out by Tandem Gold Triple Quadrupole LC-MS/MS (ZIVAK, Kocaeli, Turkey) system. LC separation was achieved by using a Synergi™ Max-RP (4 µm, 150 x 2 mm) column. The mass spectrometer operated in selective reaction monitoring mode (SRM) with positive electro-spray interface (ESI). The mobile phase A and B used were 0.2% acetic acid and methanol, respectively. The column was thermostated at 40°C. The flow rate was 0.2 ml/min, with an injection volume of 50 µl. The linear gradient was: 0-3 min 80% A, 3-7min 50% A, 7-9 min 30% A, 9-9:50 min 25% A, 9:50-13 min 0% A, 13-20 min 90% A. The electrospray capillary voltage was 5000V. Nitrogen was used as curtain and collision gas. Auto sampler, MS/MS and screening parameters are shown in *Table 1, Table 2* and *Table 3*, respectively.

Table 1. *Auto sampler parameters*
Tablo 1. *Otomatik örnekleyici parametreleri*

Parameter	Value
Injection volume	50 µl
Syringe speed	8.0 µl/sn
Washing volume	1000 µl
Washing speed	250 µl/sn
Auto sampler temperature	10°C
Column temperature	40°C

Table 2. *MS/MS parameters*
Tablo 2. *MS/MS parametreleri*

Parameter	Value	
Ionization mode	ESI +	
Screening	SRM	
Spray voltage	5023	
API nebulizing gas pressure	55	
Drying gas pressure	2.0	
Aux gas pressure	15	
Drying gas temperature	400°C	
Quad MS/MS bias	-1.0	
Screening time	0.01 sec	
SIM width	0.7 amu	
Neddle	+ 5000 V	
Shield	+ 500 V	
Dedector	+ 1700 V	
CID gas pressure	1.30 mTorr	
Spray chamber temperature	65°C	
Mass peak width in Amu	Q1=0.7	Q3=0.7

Table 3. *MS/MS screening parameters*
Tablo 3. *MS/MS tarama parametreleri*

Analyte	MS MH+ (m/z)	MS-MS (m/z)	Fragmentation Energy
2-NP-SEM	209.00	166.00	13
2-NP-SEM	209.00	192.00	11
2-NP-SEM-13$C_{15}N_2$	212.00	168.00	12
2-NP-AHD	249.00	134.00	14
2-NP-AHD	249.00	104.00	19
2-NP-AOZ	236.00	134.00	15
2-NP-AOZ	236.00	104.00	18
2-NP-AOZ-D4	240.00	134.00	15
2-NP-AMOZ-D4	340.00	296.00	11
2-NP-AMOZ	335.00	262.00	19
2-NP-AMOZ	335.00	291.00	12
I. Segment	335		
II. Segment	209.00-249.00-236.00-240.00		
Screening mode	SRM		
Segmentation energy	10		

Preparation of Matrix Standard and Validation Procedure

Five pieces of 2 g blank tissue samples were taken into 50 ml centrifuge tubes. The external standard solution was added on these samples (50, 100 and 150 µl for 0.5, 1 and 1.5 µg/kg, respectively). Then they were extracted like the other samples which are described in the sample extraction section extracted like the other samples that was described in the part of sample extraction. The method validation was done according to the criteria laid down in Commission Decision No. 2002/657 EC [26].

Calibration Curves and Results

We used blank egg samples from nitrofuran AOZ negative layer flocks for the calibration process and calculation value of CCα and CCβ. Calibration curves, used in validation process, were based upon responses (analyte/internal standard peak area ratio versus concentration of analyte) from four concentration levels ranging from 0 to 1.5 µg/kg (0, 0.5, 1 and 1.5 µg/kg), were linear. The recovery values (Rec) ranged between 88-97.9%, and repeatability (CV) ranged between 3 and 4.3%. Calibration curves, used in calculation process, were made by spiking blank matrix samples of egg with nitrofuran AOZ corresponding to concentrations of 0.5, 1 and 1.5 µg/kg.

Statistical Analysis

SPSS 17.0 programme was used for calculations. Results are expressed as mean (x) and standard error (S.E.). Differences between groups were evaluated using Wilcoxon test.

RESULTS

During the validation process, different egg samples were analysed by the same instrument and the same operator. No significant differences were found between the curves. The calibration curves were linear, with the corresponding correlation coefficients (R^2) values higher than 0.998. The decision limit (CCα) and detection capability (CCβ) were calculated for m/z 236→134, ion transition for AOZ. These values were calculated using eighteen calibration curves (at four levels 0.0, 0.5, 1.0 and 1.5 µg/kg) from six different experiments in a day, on different egg samples and 3 different days. All curves, in validation and in calculation of residues, were constructed using analyte/internal standard peak area ratio versus concentration of analyte. Linearity was proved between 0.5-1.5 µg/kg, CCα was 0.70, CCβ was 0.77 and the recovery values ranged between 88-97.9% and CV was between 3-4.3%.

The within-laboratory reproducibility was calculated in different spiked egg samples at concentration of 1 µg/kg (MRPL level). They were analysed for three different days, with the same instrument and different operators. The within-laboratory reproducibility was found satisfactory for the nitrofuran AOZ. For the calculation of the residue levels we used one calibration curve *(Fig. 1)* with four spike concentrations (0.0, 0.5, 1.0 and 1.5 µg/kg). The validation results were in accordance with the performance method of the European Commission Decision 2002/657/EC and the method was successfully applied to confirm and quantify nitrofuran AOZ in eggs [26].

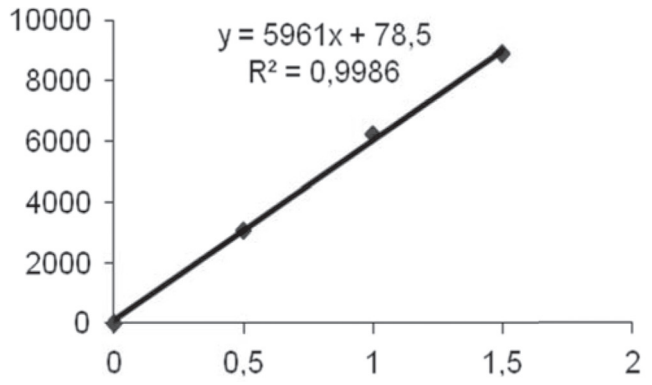

Fig 1. Calibration curve and R^2, spiked with 0.5, 1 and 1.5 µg/kg concentrations for uncooked eggs

Şekil 1. 0.5, 1 ve 1.5 µg/kg düzeyinde yüklenmiş pişmemiş yumurtaların kalibrasyon eğrisi ve R^2 değeri

The detected avarage nitrofuran AOZ residue level in 13 uncooked eggs was 0.86±0.017 µg/kg. The range of detections was between 0.76-0.97 µg/kg for uncooked eggs. After boiling, residue level was increased to 2.42±0.037 µg/kg *(Table 4)*. The range of detections in cooked eggs was between 2.04-2.55 µg/kg. Thus it was observed that the residue level of nitrofuran AOZ was higher (P<0.001) in boiled eggs than in uncooked eggs (*Fig. 2* and *3*).

DISCUSSION

Improperly and illegal administration of nitrofurans may produce residues in various foodstuffs such as eggs. The presence of these residues arises as a serious public health issue including mutagenic, carcinogenic and genatotoxic effects, induces allergic reactions in humans

Table 4. *Nitrofuran AOZ residue levels before and after boiling in eggs*
Tablo 4. *Yumurtalarda kaynamadan önce ve sonra Nitrofuran AOZ kalıntı düzeyleri*

Sample No	Before Boiling (µg/kg)	After Boiling (µg/kg)
01	0.82	2.44
02	0.81	2.04
03	0.97	2.54
04	0.76	2.31
05	0.8	2.39
06	0.87	2.49
07	0.93	2.55
08	0.88	2.49
09	0.94	2.53
10	0.85	2.42
11	0.83	2.41
12	0.82	2.46
13	0.9	2.42

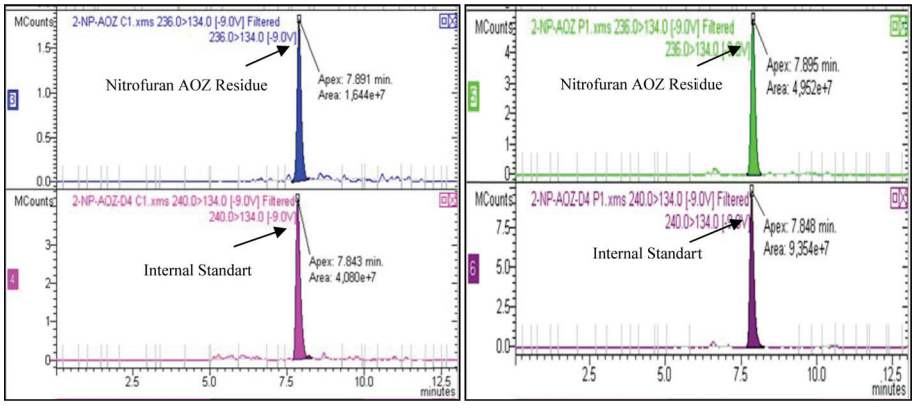

Fig 2. LC-MS/MS analysis of Nitrofuran AOZ residues and internal standart concentrations of first sample before (0.82 µg/kg) and after boiling (2.44 µg/kg) respectively

Şekil 2. Birinci örneğin haşlama öncesi (0.82 µg/kg) ve sonrası (2.44 µg/kg) LC-MS/MS analizi ile ölçülen Nitrofuran AOZ kalıntı değeri ve iç standart konsantrasyonları

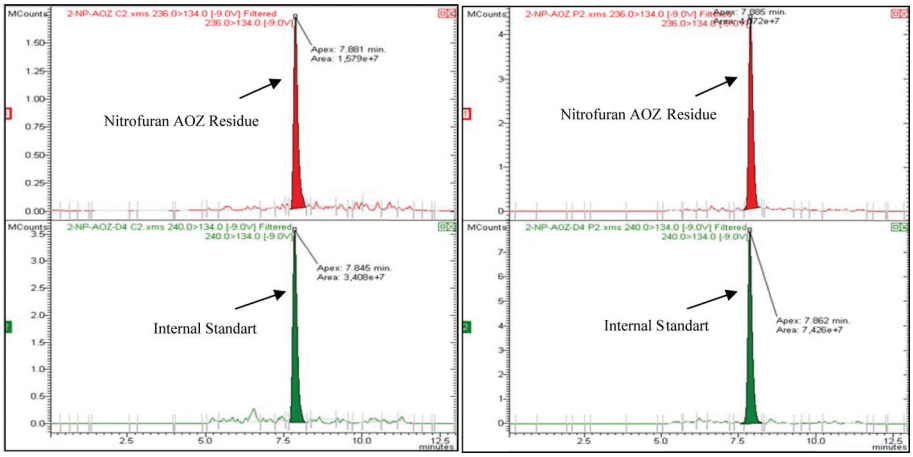

Fig 3. LC-MS/MS analysis of Nitrofuran AOZ residues and internal standart concentrations of second sample before boiling (0.81 µg/kg) and after boiling (2.04 µg/kg) respectively

Şekil 3. İkinci örneğin haşlama öncesi (0.81 µg/kg) ve sonrası (2.04 µg/kg) LC-MS/MS analizi ile ölçülen Nitrofuran AOZ kalıntı değeri ve iç standart konsantrasyonları

and give rise to an increase in the antibiotic resistance of pathogenic bacteria. Due to all these hazardous and severe problems, application of efficient detection and quantification methods and elimination of these drug residues from food matrices are important issues for consumers.

Our results run counter to the claim that heat process in general should decrease various antibiotic levels in food. In general, antibiotics in various tissues such as amphenicols residues in chicken meat [1], sulfanomides residues in chicken meat [27], ormetoprim (OMP) and sulfadimethoxine (SDM) in channel catfish muscle [28], malachite green (MG) and its major metabolite, leucomalachite green (LMG) in carp muscles [29], OTC in black tiger shrimp [30] and Japanese eel [31], chlorotetracycline and oxytetracycline in canned pork [32] are reduced by several heat process. On the contrary, some residues, such as clenbuterol in food [33] and AOZ in liver kidney and muscle [10] are stable during several heat process. Similarly, Cooper and Kennedy [34] also reported that Nitrofuran AOZ, 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ), 1-aminohydantoin (AHD) and semi-carbazide (SEM) residues remained after cooking techniques in muscle and liver of pigs and continue to pose a health risk. Furthermore, Mccracken and Kennedy [10] also reported that grilling increased the extractable AOZ content, at the expense of bound AOZ residues in both liver and kidney. The present study also showed that nitrofuran AOZ levels in eggs were significantly increased after boiling agreement with that reported by Mccracken and Kennedy [10]. These studies have also shown that the heat stability of drug residues in foodstuffs depend on type of drugs.

In conclusion, the occurrence of nitrofuran AOZ residues in eggs in Turkey was determined. Results of this study showed that nitrofuran AOZ residues were still present in the boiled eggs. In this study, the boiling procedure could not degrade residual nitrofuran AOZ in eggs. Moreover, nitrofuran AOZ residues increased with boiling. Therefore, boiling process might increase possible pharmacological and/or toxic effects of these compounds. The observed increase ($P<0.001$) in nitrofuran AOZ levels in boiled eggs relative to uncooked eggs may be due to enhanced efficiency of extraction in boiled samples. For this reason, boiled eggs should be used for analysis of nitrofuran AOZ levels in order to obtain more reliable and more predictive results.

So, it may not be assumed that heating of nitofuran residues in food will result in a safe product for human consumption. Alternative management options such as vaccinations should be applied that could reduce the frequency of antibiotics usage in laying hens, emergance of drug residues in eggs and spread of antibiotic-resistant bacteria. To ensure efficient food safety and accurate monitoring of compliance with the ban, periodic analysis to monitor the drug residues in different foodstuffs should be made more frequently and more effectively by national legal authority.

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RESEARCH ARTICLE

Serum ß-carotene and Vitamin A Levels in Spontaneous Premature Calves with Respiratory Distress Syndrome

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Summary

The aim of this study was to investigate serum ß-carotene and vitamin A levels in spontaneous premature calves with respiratory distress syndrome. As study material, 25 spontaneously newborn premature calves with respiratory distress syndrome brought to Fırat University, Faculty of Veterinary Internal Medicine Clinic for examination and treatment were used. After clinical examination, blood samples received from calves anaylzed spectrometrically for serum ß-carotene and vitamin A levels. Average values of serum β-carotene and vitamin A were found as 5.22±0.72 µg/dL and 16.63±0.96 µg/dL respectively. As a result; serum ß-carotene levels in the animals examined here were found to be lower when compared to normal values thus, lack of ß-carotene was considered to increase incidence of premature birth of calf with a multi-faceted etiology.

Keywords: *Premature, Calves, ß-carotene, Vitamin A*

Respiratorik Distres Sendromlu Spontan Prematüre Buzağılarda Serum β-Karoten ve A Vitamini Düzeyleri

Özet

Bu çalışmada, respiratorik distres sendromlu spontan prematüre buzağılarda serum β-karoten ve A vitamini düzeylerinin araştırılması amaçlanmıştır. Çalışma materyali olarak, Fırat Üniversitesi Veteriner Fakültesi İç Hastalıkları Kliniğine muayene ve tedavi için getirilen spontan olarak doğmuş 25 respiratorik distres sendromlu spontan prematüre buzağı kullanılmıştır. Buzağıların klinik muayeneleri yapıldıktan sonra alınan kan örneklerinde serum β-karoten ve A vitamini analizleri spektrometrik olarak yapılmıştır. Kan serumu ortalama β-karoten değeri 5.22±0.72 µg/dLve A vitamini değeri ise 16.63±0.96 µg /dL olarak bulunmuştur. Sonuç olarak; çalışmaya alınan hayvanlarda β-Karoten düzeylerinin sağlıklı buzağılar için bildirilen normal değerlerin altında olması nedeniyle çok yönlü etiyolojiye sahip prematüre buzağı doğum insidansının β-karoten yetersizliği ile artabileceği kanısına varılmıştır.

Anahtar sözcükler: *Prematüre, Buzağı, β-Karoten, A Vitamini*

INTRODUCTION

Term of born-dead or removal of offspring from uterus that no longer live in a state in external envorinment due to underdevelopment takes its name as immaturus abortion (abortion). The term of birth of underdeveloped or can-continue-to-live-in-community-with-special-care offsprings defined as abortus prematurus (premature birth) [1-3] and offspring called as premature (immature) [2,3].

Mean duration of normal pregnancy reported as 285 (284-286 days) days in culture bred cows and in general, discarded fetuses and offspring within 42 and 260 days of pregnancy were considered as abortion [4] and born offspring after 260 days of pregnancy but those born before normal time were considered as premature birth [5-11].

Causes of abortions in the animals may be infectious or non-infectious. Non-infectious causes may have nutritional, hormonal, genetic or congenital origin, as well as traumas, poisoning, vaccines and pharmaceutical applications, allergic and anaphylactic reactions, maternal febrile diseases, stress and psychic events, twin pregnancies also can be effective [1,4,5,11,12].

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Altough, Vitamin A defiency (lack of ß-carotene), ioide and selenium defiency, dietary rations poor from protein and energy are nutritional factors that cause premature births [1,5], none of them referred as effective as Vitamin A defiency [13].

Especially, Vitamin A defiency can cause fetal deaths, stillbirths and premature births [4,5,13-16].

Different values of ß-carotene and vitamin A have been reported in various sources for healthy calves [11,16-19].

In this study, we aimed to investigate serum ß-carotene and vitamin A levels at spontaneous premature calves with respiratory distress syndrome.

MATERIAL and METHODS

Study material includes a total of 25 premature calves from both sexes (11 female, 14 male) and different species (16 Simmental, 5 Holstein, 4 Montofon) brought to Fırat University, Faculty of Veterinary Internal Medicine clinic for examination and treatment at 1-5 days age and approximately at 30-40 kg weights and born spontaneously. In the study, due to risk of infection, materials here selected from enterprises with no abortion history. Also, the mothers of premature calves were said to be healthy before the events.

Information about the insemination date of mothers of premature calves was obtained from owners and pregnancy times were calculated. Calves that were born between 260-275 days during period of pregnancy were defined as premature.

During general clinical examination of calves [20], general status, birth weight, hair cover, incisive teeth, ears, nails, and sucking reflex examinations were especially performed. Also presence of several symptoms such as difficulty of breathing and cyanosis were noted.

After systematic clinical examination [20] of all the calves, for analysis of ß-carotene and Vitamin A, blood samples collected from V. jugularis were transferred into glass tubes. Then, blood sera were tested by using Schimadzu UV-1208, UV-VIS 240 spectrophometer as described Suzuki and Katoh [21].

The statistical evaluation of data was carried out by using SSPS MS Windows Release 10.0 software.

RESULTS

According to the anamnesis, the premature calves were brought to clinic 1-5 after parturition with the complaints to reluctance rise, difficulty in sucking or with no sucking ability in some of them. The owners expressed that some colostrum was given to the calves which do not suck from the mother with feeding bottle. Also, it was said that none of these calves received Vitamin A injection, but their mothers ussually received vitamin A injection once a month, and in general mothers of the calves were given straw, bran, masoor (Lathyrus cicera), barley (broken), dry sugar beet pulp with molasses (some of them was humid), hay, dairy farming (industrial food) and rock salt kept in front of animals, but no additional food additives were added to ration.

Average duration of pregnancy of premature calves included in the study were determined as 268.56±0.95 days (260-275 days).

In general examination of premature calves, body temperature (T), heart (P), and respiratory (R) average (min-max) values determined as 38.31±0.24ºC (35.3 to 41.0), 136±6.46 pcs/min (100 to 164), and 55.20±2.75 pcs/min (36 to 100) respectively.

Premature calves that brought to clinic were observed to show general weaknesses, most of them were distressed and some were comatose and in lateral decubitus position. Calves in lateral decubitus position, were determined to have difficulty to rise, can not cope up, most of them could not lie in sterno-abdominal position, and their sucking reflex was weak or not-exist.

Several findings, such as inadequate incisive teeth development, hyperemia of gums, short and glossy appearance of hair (fur appearance), easily bending ears and soft tabs were found in all premature calves.

Premature calves with respiratory problem history, were observed to have significant difficulty in breathing (very significantly type of intercostal and abdominal breathing, pulling their nose wings backward during inspiration), developing tachypnea and cyanosis (at nasal mucosa), hyperemic conjunctivae and evident episcleral veins during clinical examination.

In most patients, harsh vesicular sounds were heard during lung auscultation. Also diarrhea in six calves, inflammation of umbilical cord in four calves and both diarrhea and inflammation of umbilical cord in two calves were noted.

Mean serum levels of ß-carotene and vitamin A of animals included in the study were presented in *Table 1*.

Table 1. *Mean serum levels of ß-carotene and vitamin A in premature calves (n = 25)*
Tablo 1. *Prematüre buzağılarda ortalama serum β-karoten ve A vitamini düzeyleri (n = 25)*

Parameter	X±Sx	Min. - Max.
ß-carotene (µg/dL)	5.22±0.72	2.71-19.77
Vitamin A (µg/dL)	16.63±0.96	6.78-23.24

DISCUSSION

Fetal death, stillbirth, premature birth, and calve births with poor chance of survival, which cause should be seen as a complex. The causes of these are not only infectious. In most cases, metabolic disorders which causes weak immunity and defensive power, care and feeding problems should also be considered. In general, there is a combination indicated between microorganisms, immunity weakness and lack of maintenance and nutrition [5].

Average 268 days gestation period of premature calves included in this study was found to be lower when compared to normal gestation periods (286 days) of normal calves and average birth weights of these calves were also found to be lower than normal Holstein, Simmental and Montofon calves that have average of 38, 38, 40 kg live weight, respectively [4]. In addition to lower gestation period and lower birth weight of premature calves, findings such as, short and glossy appearance of hair (fur appearance), soft tabs, inadequate incisive teeth development, easily bending ears, general weakness, weak or non-existent sucking reflex, could not cope up and grogginess shows full compliance to premature calves reported in literature [6-10,22,23].

Respiratory distress syndrome (RDS) is a progressive disorder characterized by abnormal respiration (tachypnea, apnea) and cyanosis at premature calves due to lack of surfactant [23,24]. Abnormal respiration and cyanosis symptoms determined at premature calves shows relevance that reported in RDS [7,23,24].

Although average values of overall clinical findings detected in premature calves (T, P, R) shows compliance with healthy calves [10,11,20,25] and premature calves reported in literature [10,23] in some cases higher body temperature, and higher cardiac and respiratory frequency can be explained possible complications due to infections detected in calves that were studied here (enteritis, omphalitis, pneumonia) [10,12,23] and respiratory distress syndrome [23]. Because of immune system weakness and problems with colostrum intake of premature calves, susceptibility to infectious agents such as, bacteria, protozoa and viruses also increased [11,23]. Premature calves have more susceptibility to respiratoric, metabolic and infectious agents compared to calves that were born in time [23].

In some cases, reported in literature [10,22,23], lower body temperatures were determined. Initially, there was no heat loss in premature newborn calves and body temperature was found around 39ºC [10], but then premature calves were determined to have difficulty in maintaining body temperature [22] and reducing body temperatures [10,22,23].

Low body temperatures found in some calves, may be explained by premature calves born with lack of adipose tissue, that develops towards to the end of fetal life and provide thermoregulation, exposed to cold [10], energy sources can not be used economically due to respiratory difficulty and organism that is unable to produce heat [10,23].

Although, sucking reflex stimulated by finger occurs in 2 to 20 min in healthy calves, weakness or absence of this reflex at premature calves are compatible with literature notifications [6-10,22,23].

Serum levels of vitamin A and ß-carotene, which are one of precursors of this vitamin, considered as the most important and utilized parameters in diagnosis of vitamin A deficiency [12,14,16,27-34]. Also serum carotene levels were considered as sensitive indicators of carotene supply [13].

In a study conducted by Stöber [16], blood vitamin A levels were considered as normal, critical and pathological over 12 µg/dL, between 7-12 µg/dL and below 7 µg/dL, respectively, and blood ß-carotene levels were considered as normal, critical and pathological over 30 µg/dL, between 10-30 µg/dL and below 10 µg/dL, respectively.

Mean serum vitamin A levels of animals in this study [16.63 µg/dL) were found to be compatible with expressed as normal values by other researchers [16-19]. These results were reported as close to mean 18.25 µg/dL [12.60-31.40 µg/dL) of serum vitamin A levels of healthy calves from the region in a study conducted by Gazioglu and Gül [27]. But, average values of serum vitamin A were found to be significantly lower than 25 to 85 µg/dL levels for healthy cows reported by Smith [11], also found to be lower than levels of 25 µg/dL, which determined as complete security against hypovitaminosis [16,35].

Alcohol form of vitamin A does not exceed placental barrier, thus liver reserve do not occur in fetus of cows that graze in green grass, in contrast ester form of vitamin A (found as in fish oil) can exceed placental barrier [12,14,36]. In that case, because mothers that feed with green pasture during pregnancy, vitamin A levels in liver of calves are not affected [14] so vitamin A supply of fetuses and calves depends on blood ester vitamin A levels of mother [12,14,36]. At beginning, calves provide their vitamin A needs from colostrums [14]. Vitamin A levels in colostrum also depend on provision of vitamin A of mother during late period pregnancy and because it depends largely on liver reserves [14,16], vitamin A and carotene provision to mother before birth increases vitamin A and carotene levels of colostrums [12,36].

Reddy and Ganapathy [18], reported that vitamin A levels of calves were between 12-24 µg/dL (mean 19 µg/dL) after birth and determined that these values increased within 3 days after supply of colostrums.

Burgstaller et al.[17], reported that blood vitamin A levels of hungry calves (10.3 µg/dL) were up to one-third of the mother's blood values, and it increases significantly within

5 days because of high levels of vitamin A in colostrums (22.4 µg/dL).

Levels of vitamin A and ß-carotene in blood and especially in liver of newborn offspring are very low [37,38]. It has been reported that these levels increase with colostrums intake [17-19,38,39] and especially there is a positive correlation between mother, colostrums and calf for ß-carotene [40].

Because premature calves have weak or no sucking or swallowing reflex [6-10,23], their colostrums intake is low or zero. Therefore normal vitamin A levels of the animals studied here, may be explained by vitamin A injection to mother (placental absorption) and vitamin A supply via low or mandatory colostrum intake.

In this study, the values of serum β-carotene were not seen below 0.8 µg/dL which was reported by Stöber [16] the time of birth for healthy calves. This can be explained slight sucking or colostrum intake necessarily by the owners to calves.

Although, serum ß-carotene levels of premature calves (mean 5.22±0.72 µg/dL) found to be up to border levels reported by Stöber [16] for newly-born offspring (6.5 µg/dL), due to it is lower than reported values for calves which supplied with green fodder or colostrum (30-100 µg/dL) and it is compatible with values reported by Gül and İssi [31] for calves with amaurosis condition, this shows mothers of calves, which has not been feeded yet, has been feeded with rations without ß-carotene and their colostrum has lower ß-carotene levels [12,27,32].

Serum ß-carotene levels of study offspring (mean 5.22±0.72 µg/dL) found to be lower than values that accepted [16] values for defiency (10 µg/dL), and maximum measured serum ß-carotene levels between study animals (19.77 µg/dL) also found to be between values that accepted values for critical level [16] (10-30 µg/dL). Mean ß-carotene levels identified for study offspring found to be significantly lower than reported values for healthy calves by Reddy and Ganapathy [18] and Surynek et al.[19] which is 43 µg/dL and 87.5 µg/dL, respectively. Although these differences may be the result of different nutritional conditions, no or poor sucking reflex of premature calves believed to be effective enough for this results.

Due to ruminants only taking precursor ß-carotene for vitamin A supply, vitamin A deficiency in cattle always shows a primary ß-carotene deficiency. This also expressed that embyronic deaths and aborts occurred due to advanced degree ß-carotene deficiency (despite vitamin A supply is high). Bostedt and Klein [5] determined that in 2003 to 2005, 30% of 38 enterprise with stillbirth complexity has ß-carotene supply deficiency.

Serum ß-carotene levels of premature calves in this study (mean 5.22±0.72 µg/dL), suggest that these calves were affected from severe ß-carotene deficiency.

In accordance with the literature notifications about catarrhal and haemmorrhagic bowel inflammation can be seen due to vitamin A deficiency [15,29,41,42], diarrhea has been observed in some premature calves. It is thought that this might have occured due to ß-carotene deficiency [31] because compliance of reported serum vitamin A levels for healthy animals and in serum vitamin A levels of premature calves with observed diarrhea [16-18]. Because it has been reported as low levels of ß-carotene results in breakage of intestinal epithelium resistance and helps formation of intestinal infections [15,41]. Also contrary to conventional opinion, it has been expressed as ß-carotene deficiency is predisposing factor for colibacillosis rather than vitamin A deficiency [29]. Prohaszka [42] has determined that a significant relationship between frequency of diarrhea due to collibacillosis and seasonal patterns of ß-carotene levels of cows.

Gazioglu ve Gül [27], reported that injection of vitamin A preparations may be useful due to the lower serum ß-carotene and vitamin A levels of premature calves with diarrhea.

Gül [29] expressed that feeding cows with ß-carotene-rich rations reduces morbidity and mortality of calf diseases (enteritis, neonatal, septicemia).

Lotthammer [43] investigated effects of ß-carotene deficiency on health, and reported that despite being granted sufficient vitamin A, calves of cows that has undernourisment of ß-carotene, have more cases observed diarrhea and died few weeks after birth.

Clinic observations, other studies conducted in region [27-34] and the results of this study showed that in general, cattle has been undernourished with ß-carotene in this region.

As a result; serum ß-carotene levels in animals examined here were found to be lower when compared to normal values thus, lack of ß-carotene was considered to increase incidence of premature birth of calf with a multi-faceted etiology.

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RESEARCH ARTICLE

Survey on the Presence of Nematodes and Associated with Pathology in Marine Mammals from Turkish Waters

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Summary

Presence of marine mammal nematodes in Turkey is poorly known. One bottlenose dolphin *(Tursiops truncatus)* and four harbour porpoises *(Phocoena phocoena)* stranded on the Turkish coast were examined at necropsy between 2009 and 2012. Harbour porpoise stranded from Marmara Sea were only infected by three species of lungworms (Metastrongyloidea): *Stenurus minor, Halocercus invaginatus* and *H. taurica*. Anisakid, pseudaliid, filarioid and spirurid nematodes were not found in the investigated organs and alimentary canal from harbour porpoises and bottlenose dolphin from Black Sea. The present study represents the first geographic record of *H. taurica* from Turkish waters. Moreover, *S. minor* is the first and new host record of harbour porpoise from the coast of Black Sea. Additionally, pathological findings associated with lungworms are also presente.

Keywords: *Metastrongyloid nematodes, Marine mammals, Pathological findings, Turkish waters*

Türkiye Suları'ndaki Deniz Memelilerinde Nematodların Varlığının Araştırılması ve Patolojisi

Özet

Türkiye'deki deniz memelilerinin nematodlarının varlığı yeterince bilinmemektedir. 2009-2012 yılları arasında sahile vuran bir şişe burunlu yunus *(Tursiops truncatus)* ile dört liman yunusunun *(Phocoena phocoena)* nekropsileri yapıldı. Marmara Denizinde sahile vuran liman yunusu sadece üç tür akciğer kurdu (Metastrongyloidea): *Stenurus minor, Halocercus invaginatus* ve *H. taurica* ile enfekte idi. Karadeniz'deki şişe burunlu yunus ile liman yunuslarının sindirim kanalı ve incelenen organlarında anisakid, filarioid, pseudaliid ve spirurid nematodlar bulunamadı. Bu araştırma Türkiye sularında *H. taurica*'nın ilk coğrafik kaydını göstermektedir. Bununla birlikte *S. minor* Karadeniz kıyılarındaki liman yunuslarında ilk ve yeni konak olarak kaydedilmiştir. Ayrıca akciğer kurtları ile ilgili patolojik bulgular da sunulmuştur.

Anahtar sözcükler: *Metastrongyloid nematodlar, Deniz memelileri, Patolojik bulgular, Türkiye suları*

INTRODUCTION

Cetaceans are parasitized by a wide diversity of endo and ectoparasites in various tissues, organs and cavities [1]. Damage to and mortality of individuals and populations caused by parasitic infections are dependent upon several factors, including the parasite species, its abundance, the health status of the host and competition with other pathogens. Parasites of cetaceans can influence the behavior of their hosts, population size, the dynamics of the food chain and community structure [2]. Nematodes represent the broadest group of parasites in the marine mammals. The Crassicaudinae are the largest nematodes in cetaceans. Pseudaliids often infect the respiratory system, causing sufficient damage to affect survival. Filarioids are highly pathogenic in pinnipeds and are probably responsible for significant mortality, especially in young animals. Anisakid nematodes in the stomach are of little

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consequence to the host [3]. The parasitic fauna of cetaceans in Turkey is still little known. Parasites have been reported in a few species of cetaceans in Turkey, including striped dolphins and harbour porpoises [4,5]. Therefore, this is the first geographic record of *Halocercus taurica* from Turkish waters. Moreover, *Stenurus minor* was the first record of harbour porpoise from the Black Sea. Additionally, pathological findings associated with lungworms are also presented.

MATERIAL and METHODS

Parasites were collected from stranded or dead one bottlenose dolphin *(Tursiops truncatus)* and four harbour porpoises *(Phocoena phocoena)* in Turkish waters, during the period 2009 and 2012. One harbour porpoise were obtained from the Marmara Sea, while the others were collected from the Black Sea. Postmortem examinations were performed according to standardized European necropsy protocol for cetaceans [6]. Lung nematodes were detected by visual observation of light-colored nodules on the surface, by palpation, or by searching major airways. Nematodes were collected during the necropsy. After collection, nematodes were fixed and preserved in 70% ethanol. Nematodes were cleared in phenol from 5 to 60 min, depending on the size and thickness of the parasite. After clarification, the parasites were mounted on slides. The internal structures were visualized under a Nikon Eclipse 80i light microscope equipped with differential interference contrast (Nomarski DIC) optics and morphological identification was based on identification keys [7-9]. Accurate estimates of intensity of parasites were generally not possible. The nematodes were photographed the digital image analysis system (Nikon Digital Sight DS-L1). After necropsy of porpoises, lung tissue samples were fixed in 10% neutral formaldehyde solution for pathological examination. Tissue samples were routinely taken processed and embedded in paraffin. Tissue sections 4–6 µ in width were stained with haematoxyline-eosin (HE) and examined under light microscope (Nikon Eclipse 80i).

RESULTS

Parasitological investigations were carried out on bottlenose dolphin and harbour porpoises originating from the Turkish waters. *S. minor*, *H. invaginatus* and *H. taurica* were only found in the lungs of porpoise from Gemlik Bay (40°26'N, 29°09'E), Marmara Sea, Turkey *(Fig. 1e-g)*. This is the first geographic record of *H. taurica* from the Turkish waters. Moreover, *S. minor* is the first and new host record of harbour porpoise from the coast of Black Sea. Filarioids, pseudaliids, spirurids and anisakids, especially *Anisakis* spp., were not found in the investigated organs and alimentary canal from porpoises and the bottlenose dolphin in Black Sea. In the macroscopic examination, whitish to grey nodules that were raised above the pleural surface, pulmonary parenchyma, and in the bronchi, and bronchioles, varying between pin point to 0.5 cm diameter over the surfaces and cross sections were observed. The cross sections of these nodules revealed a thick cystic capsule formation with parasitic remnants inside. There were also congestion and emphysema observed in the lung tissue samples *(Fig. 1a)*. In the microscopic examination, different maturation stages according to three species of parasites were observed in bronchus, bronchioles, alveolar lumens and interstitial tissues covered with fibrous connective tissue. There was also concomitant bronchointerstitial pneumonitis composed of mononuclear and necrotic cells. In addition, dystrophic calcification and bacterial colonies were seen in the areas close to the parasites *(Fig. 1b-d)*.

DISCUSSION

There was little evidence on the presence of parasitic infections in marine mammals in Turkey. Numerous parasitological studies have been carried out on marine mammals [10-14] but few were reported on parasites of marine mammals from Turkish waters [4,5]. *Anisakis* spp., *Contraceacum* spp. and *Pseudoterranova* spp. were collected from striped dolphins (*Stenella coeruleoalba*) in Eastern Mediterranean coast [4]; while ascaridoids were not found in dolphin and porpoises from Black Sea in the current study. In concordance to these findings, larval ascaridoids, especially *Anisakis* spp. larvae, were not found in more than ten fish species (mackerel, whirling, anchovy, etc.) from Black Sea [15]. A molecular study should be performed for the identification of ascaridoids from Turkish cetaceans. Parasitism of the respiratory system is a relatively common finding in stranded cetaceans, resulting in mild chronic lesions. Metastrongyloid nematodes usually associated with respiratory system, cranial sinuses, middle ear, and circulatory system of their odontocete hosts. Lungworms can be quite pathogenic, but the infections are not the primary cause of death. Lungworm (Metastrongyloidea: Pseudaliidae) are known to occur in the lungs of harbour porpoises [9,12,16]. Lungworms of the family Pseudaliidae, genera *Halocercus*, *Pseudalius* and *Stenurus* are the most common parasites found in cetaceans [16]. Lungworms have been implicated as a common and important contributing factor in the mortality of harbour porpoise [17]. Infections with *Halocercus* spp. are usually confined to the lungs, but other pseudaliids (e.g. *Stenurus* spp.) invade the pulmonary and mesenteric arteries, cranial sinuses, brain, middle and inner ears, eustachian tube and oral cavity [18]. Pseudaliid nematodes have been found in the respiratory and alimentary tract and auditory/cranial sinuses on *P. phocoena* originating from different areas: the German North Sea, the German Baltic, southern Baltic and Norwegian and Icelandic waters [6,19-21]. *S. minor* has been reported in harbour porpoises from different geographical localities [6,12,17,20,22,23].

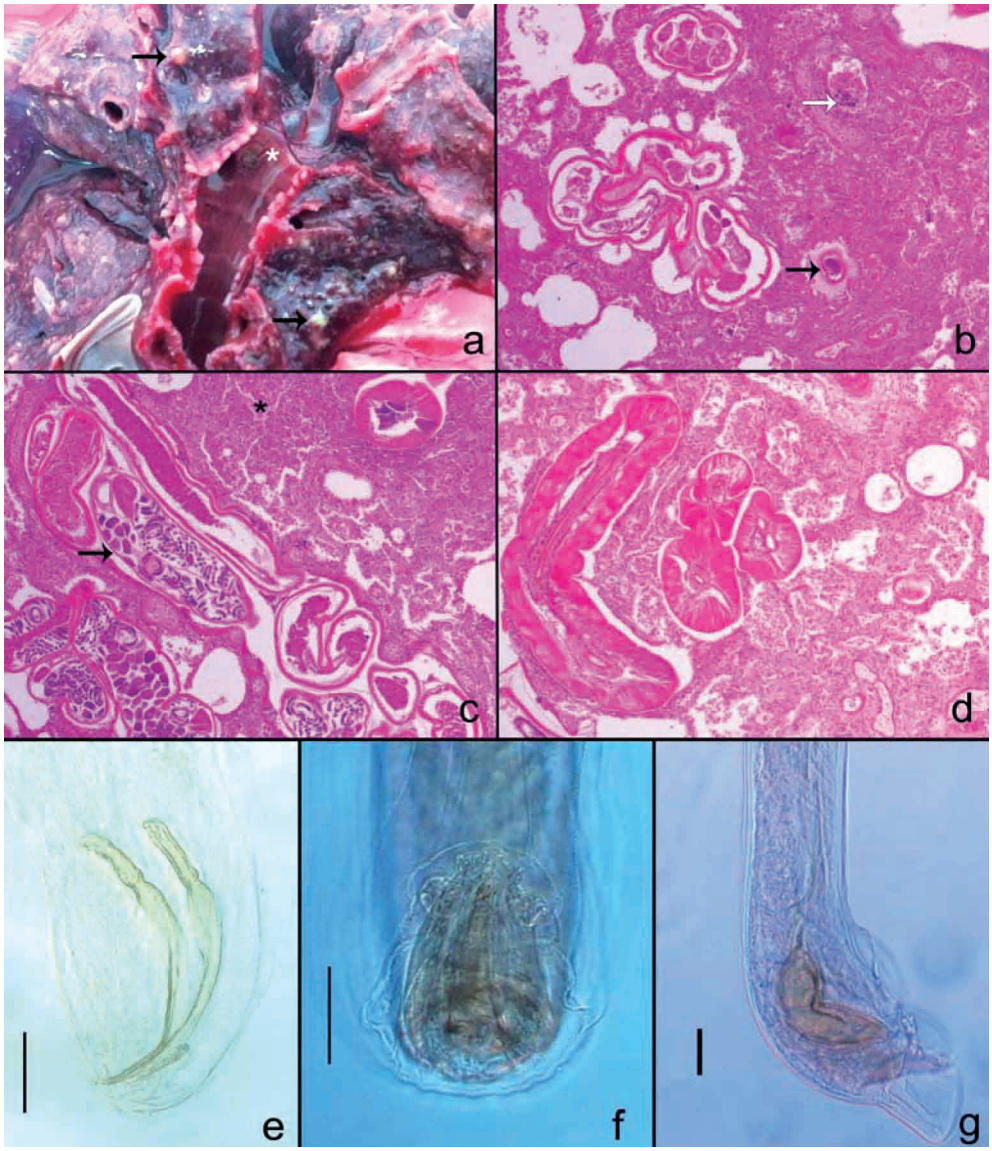

Fig 1. a- Appearance of whitish to grey nodules of parasite *(black arrow)* in the lung and parasite cluster *(asterisk)* in the bronchus lumen, **b-** General appearance of parasite, calcification *(black arrow)* and basophilic bacteria cluster *(white arrow)* in the lung, **c-** Female Metastrongyloid, with developing larvae, within the bronchus lumen *(black arrow)* and interstitial inflammatory reaction *(asterisk)*, **d-** Transverse and longitudinal cross section of parasites, **e-** *H. invaginatus*, caudal end male, lateral view, original, **f-** *H. taurica*, caudal end male, ventral view, original, **g-** *S. minor*, caudal end male, lateral view, original. Bar: 50 µm

Şekil 1. a- Akciğerdeki beyazımsı-grimsi parazit nodülleri (*siyah ok)* ve bronş lümenindeki parazit yığınlarının *(yıldız)* görünümü, **b-** Akciğerde parazit, kalsifikasyon *(siyah ok)* ve basofilik bakteri kümesinin *(beyaz ok)* genel görünümü, **c-** Bronş lümenindeki dişi Metastrongyloid, gelişmiş larvalı, *(siyah ok)* ve interstitial yangı reaksiyonu *(yıldız)*, **d-** Parazitlerin uzunlamasına ve enine kesitleri, **e-** *H. invaginatus*, erkek arka uç, lateral görünüm, original, **f-** *H. taurica*, erkek arka uç, ventral görünüm, original, **g-** *S. minor*, erkek arka uç, lateral görünüm, original. Bar: 50 µm

S. minor is the first geographic report of from harbour porpoises in Black Sea. However, this species was found from striped dolphins in Turkish Eastern Mediterranean Sea coast [4]. Pulmonary nematodes belonging to the genus *Halocercus* were one of the most prevalent [13]. Three species of *Halocercus* have been reported in harbour porpoises, *H. invaginatus, H. taurica* and *H. kirbyi* [15]. *H. invaginatus* has been reported from Dutch waters, Spanish Atlantic coast, German and Norwegian waters [11,20,24]. Veryeri [5] reported *H. invaginatus* from a stranded harbour porpoise from Black Sea. *H. taurica* was previously reported from the Northwest Atlantic, Northwest Pacific, Black and Azov Seas [16]. Moreover, this is the first geographic report of *H. taurica* from in Turkish coast of the Black Sea. The macroscopic and microscopic changes according to the parasites and the other pneumonia findings of the lungs resembled the results of the studies of dolphin and other marine mammals performed by Parsons et al.[25] and Gonzales-Viera et al.[26]. The bacterial mixed infection case was showed concurrency with previous studies [25,26]. The identification of the parasitic fauna of cetaceans not only contributes to a better understanding of the causes

of stranding and mortality, but also provides valuable information on the biology and ecology of cetaceans [13]. Additionally, parasites could be used as biological tags of marine mammal populations, as indicators of migration and feeding, as a means of separating intraspecific variation of inshore and off-shore populations, as indicators of general state of health, and as an aid in assessing mortality [27]. For this reason, additional data from other cetaceans are required to establish the parasitological information in Turkish waters.

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RESEARCH ARTICLE

Analysis of Electrocardiographic Parameters in the Conscious Common Pheasants *(Phasianus colchicus)*

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Summary

The aim of this study was to establish normal electrocardiographic values and patterns in pheasants *(Phasianus colchicus)*. The electrocardiograms were recorded in 24 conscious pheasants from both genders. All electrocardiograms were standardized at 1 mV=20 mm , with a paper speed of 50 mm/sec. Morphological patterns of P, QRS and T deflections were evaluated in the six limb leads (I, II, III, aVR, aVL, aVF). The amplitude and duration of waves and intervals were determined by using lead II. A regular sinus rhythm was observed in all birds. There was no significant difference between male and female pheasants except for amplitudes of P and T waves and duration of T wave. In all leads P waves were mainly positive in both sexes except for lead aVR. The dominant waveforms of the QRS complexes were rS in lead II, III and aVF, and qR in lead aVR and aVL in both sexes. Generally, T waves were positive in lead I, II, III and aVF, and negative in aVL, aVF. The majority of birds showed a slightly elevated ST segment in lead II. Heart rates were 193.5±45.8 and 235.7±59.9 beats per minute and mean electrical axes were -91±10° and -92±18° in male and female birds, respectively. This study provides electrocardiographic data for pheasants, which can be used for clinical purposes.

Keywords: *Electrocardiography, Pheasant, Phasianus colchicus, Heart rate, Mean electrical axis*

Uyanık Haldeki Adi Sülünlerde *(Phasianus colchicus)* Elektrokardiyografik Parametrelerin Analizi

Özet

Bu çalışmanın amacı adi sülünlere *(Phasianus colchicus)* ait elektrokardiyografik parametrelerin belirlenmesiydi. Bu amaçla her iki cinsiyetten 24 adet uyanık sülünün elektrokardiyogramları 50 mm/sn hızda ve 20 mV/mm amplitüdte kaydedildi. Bipolar ve unipolar ekstremite derivasyonlarında (I, II, III, aVR, aVL, aVF) P, QRS ve T dalga morfolojileri değerlendirildi. Dalgaların süre, yükseklik ve aralıklarının hesaplanmasında derivasyon II kullanıldı. Çalışmada tüm kuşlarda düzenli sinüs ritmi izlendi. Erkek ve dişi sülünler arasında P ve T dalgalarının amplitüdü ve T dalgalarının süresi dışında önemli fark saptanmadı. P dalgası aVR dışında tüm derivasyonlarda her iki cinsiyette de pozitifti. Her iki cinsiyette de QRS kompleksi II, III ve aVF derivasyonlarında rS formunda, aVR ve aVL derivasyonlarında qR formunda gözlendi. T dalgası I, II, III ve aVF derivasyonlarında pozitif iken aVL ve aVF derivasyonlarında negatif olarak belirlendi. Derivasyon II'de ST segmentinde hafif bir yükselme saptandı. Kalp atım sayısı erkeklerde 193.5±45.8 atım/dk ve dişilerde 235.7±59.9 atım/dk olarak hesaplanırken, ortalama elektriksel eksen erkeklerde -91±10° ve dişilerde -92±18° olarak hesaplandı. Bu çalışma ile klinik amaçlar için kullanılabilecek sülünlere ait elektrokardiyografik veriler elde edilmiştir.

Anahtar sözcükler: *Elektrokardiyografi, Sülün, Phasianus colchicus, Kalp hızı, Ortalama elektriksel eksen*

INTRODUCTION

An electrocardiogram is the record of the electrical activity generated by the heart. Electrocardiography (ECG) has been extensively used for diagnostic or research purposes in both human and animal species for more than a century. The first avian ECG study is dating back to the beginning of 20th century [1]. Despite this early start in avian ECG studies, it is not possible to find published reference values for some of the common avian species (e.g. pheasants). With the increasing popularity of bird species as a pet animal, the veterinarians more often

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are confronted with cardiology cases in these species. The findings of ECG are commonly used in the accurate cardiac diagnosis in various bird species such as chicken [2-5], turkey [6-8], quail [4,9,10], duck [2,11], pigeon [2,12,13], parrot [14], and other species [15-17]. Electrocardiography is also used in monitoring cardiac and vital status during the anesthesia in birds [18-21]. However, ECGs obtained from anesthetized birds may not be applicable in un-anesthetized birds [8,22]. Therefore, obtaining the reference ECG patterns and values of unanesthetized birds would be useful.

The pheasant, originally native to Georgia, has been introduced all across the world as a game bird. The word pheasant is derived from the ancient river name *Phasis*, which flows from the Caucasus Mountains into the Black Sea [23]. The most well known is the "Common pheasant", which is widespread throughout the world. In the United States and Europe, especially in the United Kingdom, there are many commercial pheasant breeding farms. In some parts of the world where pheasant population has dropped, efforts are being made to reintroduce farm-raised pheasants to wild. At Samsun, Turkey, a city located at the southern part of Black Sea, pheasants are raised at Gelemen Pheasant Production Station and dispatched to nature in an effort to support the pheasant population in this area.

In this study we wanted to create an ECG reference value from healthy conscious pheasants for the first time, to help the veterinarians in evaluating the heart disease profile of these birds.

MATERIAL and METHODS

Animals

In this study, a total of 24 mature and healthy pheasants *(Phasianus colchicus)* of both sexes (12 males, 12 females) were used. Body weights (BW) of male and female pheasants were 1257±117 g and 1021±181 g, respectively. Pheasants were 8-12 months old ages and were obtained from the Gelemen Pheasant Production Station, Samsun. Pheasants were brought from the station to the physiology laboratory within plastic transport cages. To acclimate to the laboratory environment, pheasants were kept in semi-darkness zone for 3 days. Pheasants received commercial feed mixtures and water *ad libitum*. This study was approved by the Local Ethics Committee of Samsun Veterinary Control Institute (Approval no: 30). It was not applied any invasive treatment to the animals.

ECG Recording

ECG recordings were performed at the same time of the day to eliminate changes derived from biorhythms. To minimize stress, all the procedures were carried out in a quiet and dim room and birds` heads was covered with a thin cloth during the manipulation. The pheasants were gently placed on a rubber surface in a dorsal recumbent position. Throughout the recordings neither sedation nor anesthesia were used. Electrocardiograms were recorded with a portable electrocardiograph (Cardiette, ar600adv, Italy). Blinded alligator clip electrodes were attached after gel application to the skin at the base of the wings and to the skin overlying the stifle on the right and left sides. When optimal immobilization and good electrode contacts were obtained, ECG recordings were started (approximately within 5 min after the electrode placement). If a pheasant showed any movement, ECG recordings was discontinued until the movement ceased. The standard bipolar (I, II and III) and augmented unipolar (aVR, aVL and aVF) limb leads were recorded with a paper speed of 50 mm/sec. The lead II recordings run for at least 60 sec and all the other leads for 30 sec. Lead II was selected for the measurement of amplitude and duration of waves. Because of the small waveforms a double standard was used (20 mm equals to 1 mV). The mean heart rate (HR) was calculated by using the average of 10 consecutive RR intervals. The QRS waveforms were labeled according to the standard nomenclature: the major deflection was indicated by a capital letter and the minor deflections by lower case letters. Mean electrical axis (MEA) was calculated by using lead II and III as described by Edwards [24], since the QRS complex was not represented in Lead I. The morphologic patterns of P, QRS and T waves were evaluated for each lead. Amplitudes and durations of the waves, PQ/PR and QT intervals, and duration of ST segments were determined by the inspection of lead II.

Statistical Analysis

Mean, standard deviation (SD), minimum and maximum values were calculated by using the MINITAB statistical package (MINITAB Inc., State College, PA, USA). Data are presented as mean±SD. The amplitudes of waves and the duration of intervals, BW, HR and MEA were analyzed by one-way analysis of variance. The relationship BW and amplitudes of waves and, BW and durations of waves were determined by correlation analyses.

RESULTS

The vast majority of lead recordings were obtained without major artifacts. A regular sinus rhythm was observed in all birds. A representative ECG of a pheasant is shown in *Fig. 1*. P and T waves could not be evaluated in some leads in both genders because the waves were isoelectric *(Table 1)*. In two female pheasants, the measurement of duration and amplitude of P and T waves in lead I, III, aVL, and in one bird only in lead I were almost impossible because of respiratory artifacts; therefore quantitative data for these leads were not evaluated. The remaining leads were regular and had assessable wave morphologies.

There was a significant difference between male (0.18

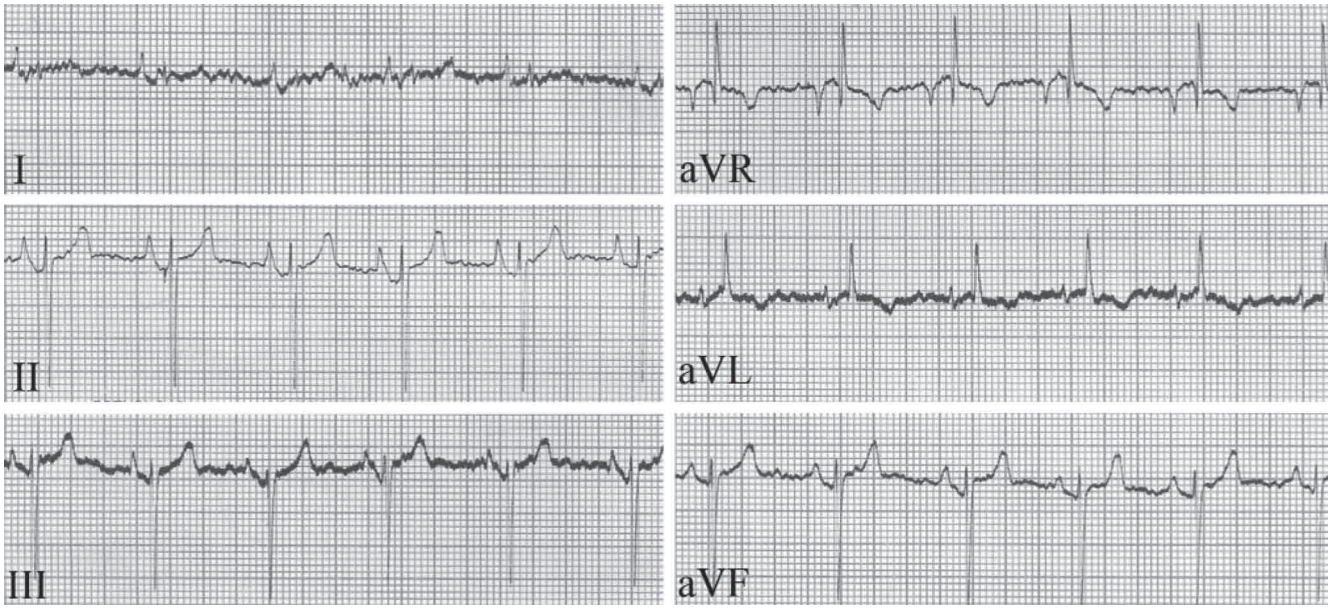

Fig 1. Representative electrocardiogram of a male pheasant in six leads. Standardization: 20 mm=1 mV; chart speed, 50 mm/sec

Şekil 1. Bir erkek sülüne ait altı ekstremite derivasyonunda kaydedilmiş örnek elektrokardiyogram. Standardizasyon: 20 mm=1 mV; kâğıt hızı: 50 mm/sn

Table 1. *Morphologic patterns of P and T waves and QRS complex in standard bipolar and augmented unipolar limb leads in male (n=12) and female (n=12) pheasants (Values are given number of 12)*

Tablo 1. *Dişi (n=12) ve erkek (n=12) sülünlerde standart bipolar ve artırılmış unipolar ekstremite derivasyonlarında P ve T dalgaları ile QRS kompleksinin morfolojik biçimleri (Değerler 12'de adet olarak verilmiştir)*

Sex	P Wave					QRS Complex							T Wave		
	Leads	Pos	Neg	Iso	Bip	Iso	rS	qR	Rs	QS	Qr	R	Pos	Neg	Iso
Male	I	7	1	4	0	7	2	1	0	1	1	0	7	1	4
	II	10	0	0	2	0	12	0	0	0	0	0	12	0	0
	III	12	0	0	0	0	12	0	0	0	0	0	12	0	0
	aVR	0	10	1	1	0	0	11	1	0	0	0	0	11	1
	aVL	1	3	0	8	0	0	12	0	0	0	0	0	12	0
	aVF	12	0	0	0	0	12	0	0	0	0	0	12	0	0
Female	I	6	1	3	0	5	3	0	2	1	0	0	6	1	3
	II	9	1	0	2	0	9	0	0	3	0	0	11	0	1
	III	6	1	2	2	1	7	0	0	3	0	1	8	2	1
	aVR	1	9	2	0	1	0	9	0	0	0	2	1	9	2
	aVL	4	2	4	1	1	0	5	1	1	1	0	3	4	4
	aVF	7	0	2	3	2	6	1	0	3	0	0	8	2	2

Pos: *positive,* ***Neg:*** *negative,* ***Iso:*** *isoelectric,* ***Bip:*** *biphasic (+/- or -/+)*

mV) and female pheasants (0.10 mV) in amplitudes of P waves (P=0.01) but durations were similar *(Table 2)*. As shown in the *Table 1*, there were a total of five isoelectric P waves in the male pheasants and thirteen isoelectric P waves in the female pheasants. In all leads, P waves were mainly positive in both sexes except for lead aVR in which P waves were negative. The majority of P waves were biphasic in lead aVL in male pheasants at ratio of 8:12. There were four notched P waves in male (two waves in each lead II and III) and four waves in female birds (three waves in lead II and one wave in lead aVF). P waves were tall and pointed in shape in one male pheasant in lead II, III and aVF, and in another one in lead I and aVF.

The duration of PQ/PR and QT intervals were similar in both sexes *(Table 2)*.

There was no significant difference between male and female birds in amplitude and duration of QRS complexes although both parameters were high in males than females *(Table 2)*. The dominant waveforms of the QRS complexes were rS in lead II, III and aVF, and qR in lead aVR and aVL in both sexes. However, Rs, QS, Qr and R forms of

Table 2. *Amplitudes and durations of P, QRS and T waves and durations of P-Q/P-R, Q-T intervals and S-T segment, and heart rates and electrical axis in male and female pheasants*

Tablo 2. *Dişi ve erkek sülünlerde P, QRS ve T dalgalarına ait amplitüd ve süreler ile P-Q/P-R, Q-T aralıklarına ve S-T segmentine ait süreler ile kalp atım sayıları ve elektriksel eksen değerleri*

Sex	Variation	P wave		P-Q/P-R Interval	QRS Complex		T Wave		Q-T Interval	S-T Segment	BPM	MEA
		Amp	Dur		Amp	Dur	Amp	Dur				
Male	Mean	0.183	0.033	0.071	-0.600	0.033	0.276	0.072	0.140	0.037	193.5	-91
	SD	0.101	0.008	0.012	0.247	0.006	0.113	0.017	0.016	0.008	45.8	10
	Min	0.000	0.020	0.050	-1.100	0.020	0.080	0.040	0.120	0.020	100.0	-104
	Max	0.330	0.040	0.090	-0.250	0.040	0.450	0.100	0.180	0.050	254.0	-68
Female	Mean	0.096	0.030	0.063	-0.450	0.028	0.137	0.051	0.134	0.046	235.7	-92
	SD	0.037	0.013	0.011	0.171	0.010	0.058	0.014	0.019	0.014	59.9	18
	Min	0.000	0.010	0.050	-0.700	0.020	0.050	0.020	0.100	0.020	160.0	-127
	Max	0.150	0.060	0.080	-0.250	0.050	0.250	0.060	0.160	0.060	341.0	-60
P value		0.010	NS	NS	NS	NS	0.002	0.004	NS	NS	NS	NS

The amplitudes and durations are given in millivolt and second, respectively, ***SD:*** *standard deviation,* ***Min:*** *minimum value,* ***Max:*** *maximum value,* ***Amp:*** *amplitude,* ***Dur:*** *duration,* ***BPM:*** *beats per minute,* ***MEA:*** *mean electrical axis (°),* ***NS:*** *no significance*

QRS complexes were observed in a small number of male and female pheasants in various leads *(Table 1)*.

Both amplitude (*P*=0.002) and duration (*P*=0.004) of T waves were higher in male pheasants than females *(Table 2)*. There were a total of five isoelectric T waves in male and 13 isoelectric T waves in female pheasants. Generally, T waves were positive in lead I, II, III, aVF and negative aVR and aVL in both sexes *(Table 1)*. In five male pheasants T waves were notched in lead II and aVF, while in one male bird all the leads had notched T waves. In females, notched T waves were observed in only one bird on lead II and aVF.

Durations of ST segments were similar in both sexes. It ranged from 0.02 to 0.05 sec and 0.02 to 0.06 sec in male and females, respectively *(Table 2)*. In the majority of the birds a slightly elevated ST segment was observed in lead II.

Average heart rate and MEA values were similar in male and female pheasants *(Table 2)*.

DISCUSSION

This study was conducted at the Faculty of Veterinary Medicine in Samsun, Turkey (latitude 41° 13` N, longitude 36° 29' E). Anesthetics can alter ECG values by changing heart rate and QT interval [14,25]; hence no anesthetic or tranquilizing agents were applied in this study. Instead, ECGs were recorded in conscious pheasants by covering birds` heads with a thin and dark-colored cloth. The recordings were performed approximately 5 min after the attachment of clips. This calming period allowed us to obtain considerably proper ECG wave patterns in pheasants.

ECG values were similar in both sexes except for amplitudes of P and T waves, and duration of T waves. The amplitudes of P and T in male birds were higher than those of females (*P*=0.01 and *P*=0.002, respectively). Similar to our findings, Lopez-Murcia et al.[13] reported sex related differences in amplitudes of P, R and T waves in competition pigeon with the males having higher amplitudes than females. The author explains these differences with higher cardiac tissue mass of males than females due to the competition. But in our study, we could not attribute these differences to heart weight directly since the hearts of the birds were not weighed. However, in previous studies [26,27] a positive correlation between BW and heart weight were reported. From this point of view it can be expected that the heart weights of males would be greater than those of females since in this study, the BWs of males were significantly (1257 vs. 1020, *P*=0.001) higher than those of females. Therefore, the explanation of Lopez-Murcia et al.[13] about the amplitude differences in male and female pigeons could be applied to our result. However, there was no relationship between BW and amplitudes of P and T wave in correlation analysis (*P*=0.831 and *P*=0.299, respectively). Similar findings were reported in raptors [17] and in macaws [15] earlier by other authors. They pointed out that there is no significant relationship between BW and wave amplitudes in conscious raptors and macaws.

In this study, a total of 18 isoelectric P waves were observed in different leads and derivations, looking at the ECGs of all the pheasants. Uzun et al.[28] reported a similar finding where they detected five and eleven silent P waves in rock and chukar partridges, respectively. The P waves were dominantly positive in all leads except for lead aVR and only in male pheasants it was biphasic in lead aVL. In this respect, there is compatibility between our results and those findings reported by many authors [16,17,28-30]. On the other hand, Hassanpour et al. found that the P waves were mainly positive in all leads in green peafowl [31]

and in helmeted guinea fowl [32]. Machida and Aohagi [33] reported that the P waves are generally biphasic in lead I and III, and positive in lead II. The amplitude of P wave in our study was higher than the value described by Uzun et al.[28] and Hassanpour et al.[31]; lower than the value notified by Casares et al.[15] and Lopez-Murcia et al.[13] and, similar to the value reported by Espino et al.[30] and Talavera et al.[17]. The mean P wave durations in lead II were 0.03 and 0.03 sec in male and female pheasants, respectively, which was fairly similar to the values reported by many authors [13,15,17,28,30]. We observed a total of eight notched P waves in birds. According to author's knowledge, there was not any report on this phenomenon in avian species.

In this study, the QRS polarity was always negative in lead II, III and aVF, and positive in lead aVR and aVL. The QRS complex in lead I was either very low or isoelectric, and both negative and positive deflections were observed. The deflection of QRS complex in leads II, III and aVF was dominantly identified as the rS type. Likewise, in lead aVR and aVL the most frequent morphologies were qR. Our findings were almost similar to results obtained in many studies [15-17,28,30-32].

There was no Q wave in lead II and III, whereas Q wave was present in lead aVR and aVL in 22 of 24 pheasants. These results are confirmed by other ECG studies in birds [16,17,28,31-33]. The mean values of duration and amplitude of QRS complexes were similar to those reported previously in buzzards [30] but higher than those reported in pigeons [13].

T waves were always positive in lead I, II, III and aVF, and always negative in the other two leads. These findings of T wave morphology were similar to those reported previously in various birds [16,17,28-31,33].

The amplitudes and durations of T waves in males were higher than those of females. The amplitudes in our study were lower than those reported earlier in turkeys [29], macaws [15], in pigeons [13], in kestrels [17] and in peafowl [31], but were similar to those in buzzards [30] and some raptor species [17]. The mean duration of T wave was 0.07 and 0.05 sec in male and female, respectively in our study. The our result for duration of the T wave in males (0.072 sec) was similar to the finding in vultures of Talavera et al.[17] who studied in four raptor species without distinction of sex. Similarly, this parameter in kestrels, owls and eagle owls was consisted with that of females (0.05 sec) in our study. Otherwise, Espino et al.[30] mentioned that T wave durations in buzzards were 0.03 sec which was lower than our values. T wave values obtained in an ECG study in macaws [15] were fairly similar to our results. In a pigeon study, Uzun et al.[28] reported that the duration of T wave was approximately 0.05 sec, which is similar to those of females in our study, and Lopez-Murcia et al.[13], mentioned that this duration was an average of 0.03 sec, which is lower than our values. The duration of T waves in turkeys was reported by McKenzie et al.[29] as 0.08 sec, which was higher than those of these pheasants.

A normal sinus rhythm was assessed in all pheasants and no remarkable arrhythmia was found in our study in contrast with other studies on anesthetized birds [14,15]. The mean heart rates of male and female pheasants were similar and were 194 bpm and 236 bpm, respectively. The mean HR in this study was much lower than previously studied species: macaws [15]; buzzards [30]; peregrine falcon [16]; partridges [28]; pigeons [13]; Eurasian kestrel, little owl and Eurasian eagle owl [17] and green peafowl [31]. However, our values were fairly similar to those reported for streaked shearwater, night heron, grey heron, little egret, intermediated egret, large egret, Ural owl [33] and were higher than those mentioned for turkeys [29]; whooper swan, mallard, teal, spot-billed duck, great crested grebe [33] and griffon vulture [17].

The MEA was negative in all birds and there was no sex-related difference. The MEA values were reported before for avian species as +95° to -165° for Pekin ducks [34], -95° to -101° for macaws [15], -99.2° for buzzard [30], -64° to -120° for various free-living birds [33], -99.9° for peregrine falcon [16], -95° for rock partridges and -99.5° for chukar partridges [28], -88° for pigeons [13], -78° to -98° for raptors [17] and -96.8° for green peafowl [31]. As can be seen from the *Table 2*, the average MEA values in this study were negatives and were consistent with those reported in earlier studies mentioned above.

The ST segment represents the end of ventricular depolarization and the beginning of ventricular re-polarization. The ST segment extends from the end of the QRS complex to the beginning of the T wave, and it is normally isoelectric. The normal ST segment may be slightly elevated above or depressed below the baseline. The amount of the elevation or depression is considered as abnormal when it is greater than 0.2 mV for dogs and 0.1 mV for cats [24], but there is no reported criterion for ST segment elevation and depression in any avian species. As another abnormal situation, ST segment slurring may be seen when the ST segment goes directly into the T wave without first straightening out even with the baseline, which ST slurring is frequently described with an undetermined cause in healthy birds [13,35]. In our study, the ST segment was almost identifiable in all tracings, and ST slurring was found in only one tracing. In agreement with previous ECG studies on other bird species [14,16,33,35], the ST segment mainly appeared elevated over baseline.

Some authors reported the fusion of P and T waves (P on T phenomenon) in various avian species possibly due to high heart rates [14,25,33]. We did not observe this phenomenon most likely because compared to the birds in the previously mentioned studies the pheasants in this study had lower HRs.

In conclusion, morphology, duration and amplitude of P, QRS and T waves, and heart rates and mean electrical axis of conscious pheasants show similarities and differences with the other avian species studied previously. These variations in avian species explain the need for the specific electrocardiographic patterns and reference values for all wild bird species. Hopefully, The ECG values and patterns derived from these clinically normal pheasants will provide a reference value data to help the diagnosis of cardiovascular abnormalities in this species.

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SHORT COMMUNICATION

Allergen Determination in Thoroughbred Stallions via Detecting Serum Specific IgE

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Summary

In horses, allergic disease has been implicated in the pathophysiology of atopic dermatitis, recurrent urticaria, and recurrent airway obstruction. With the present study, it is aimed to give information about allergens which determined in Thoroughbred stallions with detecting serum IgE. 18 Thoroughbred stallions averaged 11 years old were included in the study. Fifteen single and five mix allergens were checked for allergen-specific IgE. English plantain *(Plantago lanceolata)*, Birch/Alder/Hazel and *Micropolyspora faeni/Thermoactinomyces* were detected as quite allergic to six Thoroughbred stallions at total. Consequently, assays detect equine IgE, can be an effortless method in determining allergens in horses.

Keywords: *Allergen, IgE, Stallion, Thoroughbred, Urticaria*

İngiliz Aygırlarında Serum Spesifik IgE Tespiti ile Alerjen Tayini

Özet

Atlarda alerjik hastalıklar atopik dermatitis, tekrarlayan ürtiker ve tekrarlayan havayolu hastalıklarının patofizyolojisinde rol oynamaktadır. Bu çalışma ile İngiliz aygırlarında serum spesifik IgE tespiti ile alerjenlerin tayini hakkında bilgi verilmesi amaçlanmıştır. Yaşları ortalama 11 olan, 18 İngiliz aygır çalışma materyalini oluşturmuştur. On beş tek ve beş karışık alerjene serum spesifik IgE yönünden bakılmıştır. Toplam altı İngiliz aygırda English plantain *(Plantago lanceolata)*, Birch/Alder/Hazel ve *Micropolyspora faeni/Thermoactinomyces* oldukça alerjik olarak tespit edilmiştir. Sonuç olarak, atlara özgü IgE'yi belirleyen serolojik testler alerjenlerin tespiti bakımından zahmetsiz bir yöntem olarak değerlendirilebilir.

Anahtar sözcükler: *Alerjen, Aygır, IgE, İngiliz atı, Ürtiker*

INTRODUCTION

In horses, allergic disease (hypersensitivity to insect and environmental allergens) has been implicated in the pathophysiology of atopic dermatitis (AD), recurrent urticaria (RU), and recurrent airway obstruction (RAO) [1]. Diseases in horses such as AD, RU, and RAO [2] have long been thought to have an allergic etiology due to similarities with human allergies which have been shown to be mediated by IgE [3]. The clinical signs in AD, RU cases can become so severe that the animals injure their skin and become unable to be worked [4] or predisposing to RAO, which leads unable to race. Therefore, it is important to determine whether a horse is allergic or not or the allergens have to be detected. The molecular mechanisms of allergy and the recent proof of the integral role of IgE in mediating equine skin hypersensitivities [5] suggests that serological assays using detection of allergen-specific IgE could be an alternative method in horses. The recent development of highly specific monoclonal antibodies to detect equine IgE [3] allowed us to show the causal role of IgE in skin hypersensitivity and allergies. With the present

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study, it is aimed to give information about allergens detected in Thoroughbred stallions via detecting serum specific IgE and take attention the importance of detecting allergens before revealing the symptoms.

MATERIAL and METHODS

Horses

18 Thoroughbred stallions which ranged from 5 to 17 years (mean 11 years) were included in the study. The stallions were kept in The Jockey Club. Four of them showed signs of urticaria with different levels of pruritis, and fourteen of them were free of urticaria. There were several wheals and plaques that elevated, round, flat-topped, and 0.5-4 cm in diameter, mainly presented on the back, flanks, and legs in these four urticaria positive stallions. Based on the skin scraping and culture tests any bacterial or mycotic infections were detected in the uricaria positive stallions in their routine clinical examinations. All stallions had no history of respiratory diseases or any urticaria symptoms before on their medical records. They were stabled and fed hay and had a regular deworming protocol at 3 month intervals with a standard vaccination program. The stallions had received no antihistamine, corticosteroid or other medications including vaccines by any route for at least eight weeks prior to the test. The health statuses of the stallions were also checked routinely by the veterinary surgeons of the Jockey Club.

Test Procedure

Venous blood (10 ml) was obtained from the jugular vein and was centrifuged at 3.000 *g* for 10 min at room temperature. Serum were separated and stored at -25°C until the analysis. Blood samples were collected by the veterinary surgeons of the Jockey Club within their routine clinical examinations in the summer season. The assay procedure was performed according to the manufacturer's recommendations and directions (Polycheck Equss®, Germany). In comparison to the standard curve the amount of allergen-specific IgE for each allergen is given as relative kilo units per liter (kU/l) and results determined based on their concentration of IgE as negative (0), mild (1), marked (2) and strong (3).

Allergy Panels

Fifteen single allergens and five mix (pollens: grass, trees, weed; house dust mites; storage mites, moulds; insects) were checked for allergen-specific IgE. These included with their numbers used in the study *(Table 1)*.

Statistical Analyzes

Zero-inflated Poisson Regression described by Famoye and Singh [6] is used to model count data that has an excess of zero counts. Furthermore, structure of present data as well as many count datasets is the joint presence of excess zero observations and the long right tails, both relative to the Poisson assumption [7]. An odds ratio of 1 indicates that the condition or event under study is equally likely to occur in both groups. An odds ratio greater than 1 indicates that the condition or event is more likely to occur in the first group. An odds ratio less than 1 indicates that the condition or event is less likely to occur in the first group. The odds ratio must be nonnegative if it is defined.

RESULTS

Thoroughbred stallions were tested for specific serum IgE for 20 allergens and the test results with the degrees of reactions were given in *Table 1*. Only 4 stallions were classified as urticaria positive based on their clinical findings while others were described as negative. Contrary to the urticaria positive stallions, number 1 and 13 stallions gave positive reactions to *P. lanceolata* and Birch/Alder/Hazel allergens even they were urticaria negative *(Table 1)*. According to the odds ratios Black fly *(Simulium equinum)* was determined 1.77 times much more allergic in the presence of the other eight allergens in urticaria positive stallions and 1.75 times more allergic in urticaria negative stallions compared to the other eight allergens *(Table 2)*. Biting midges *(Culicoides nubeculosus)*, Horsefly *(Tabanus* spp.), Rape *(Brassica napus*), Mugwort *(Artemisia vulgaris)* and Ragweed *(Ambrosia)* also have the risk of giving positive reactions as 3.57 times greater in the presence of the other eight allergens in urticaria positive stallions while, these allergens have the risk of giving positive reactions as 3.52 times greater in urticaria negative stallions when compared to the other eight allergens *(Table 3)*. On the other hand, *P. lanceolata*, Birch/Alder/Hazel and *Micropolyspora faeni/Thermoactinomyces* were found the most allergic compounds among all the allergens. *P. lanceolata* was determined 7.75 times more allergic in the presence of the other eight allergens in urticaria positive stallions and 7 times more allergic in urticaria negative stallions contrasted with the other eight allergens. With the presence of other six allergens *P. lanceolata*, Birch/Alder/Hazel and *M. faeni/Thermoactinomyces* were detected as quiet allergic substances in stallions even they were urticaria negative when compared to others *(Table 3)*.

DISCUSSION

Allergic disease (hypersensitivity to insect and environmental allergens) has been implicated in the pathophysiology of AD, RU, and RAO [1] and may be associated with IgE-mediated hypersensitivity. They often result in pruritus and self excoriation and on rare occasions may affect athletic performance and even prompt euthanasia [8]. Therefore, determining the allergens in the horses even with different protocols is indisputably very important. Based on a specific monoclonal antibody test

Table 1. *Allergic reactions with varying degrees and urticaria conditions of the stallions (Urticaria positive stallions are marked with upper asterisk*)*
Tablo 1. *Aygırların değişen derecelerdeki alerjik reaksiyonları ile ürtiker olan aygırlar (Ürtiker pozitif aygırlar yukarı asteriks ile işaretlenmişlerdir*)*

Allergens	Stallions																	
	1	2*	3*	4*	5	6*	7	8	9	10	11	12	13	14	15	16	17	18
Tyrophagus spp.																		
Meal mite *(Acarus siro)*																		
Stable fly *(Stomaxys calcitrans)*																		
Black fly *(Simulium equinum)*		1																
Mosquito																		
Biting midges *(Culicoides nubeculosus)*			1															
Horsefly *(Tabanus* spp.)			1															
Rape *(Brassica napus)*			2															
Mugwort *(Artemisia vulgaris)*			2															
English plantain *(Plantago lanceolata)*	2	2	2	1		2							2					
6-grass mix																		
Rye, common *(Lolium perenne)*																		
Platane/Willow *(Salix viminalis)*/Poplar																		
Birch/Alder/Hazel	1	2		1		1							1					
Ragweed *(Ambrosia)*			2															
Micropolyspora faeni/Thermoactinomyces		2		1														
Aspergillus fumigatus/Penicillium notatum																		
Lepidoglyphus destructor																		
Dermatophagoides pteronyssinus																		
D. farinae																		

Table 2. *Likelihoods of nine allergens as percentages*
Tablo 2. *Yüzde olarak dokuz alerjenin olasılıkları*

Allergens No	Allergy Positive	Allergy Negative	Among 9 Allergens		With All Allergens	
			Allergy Positive	Allergy Negative	Odds	Relative Risk
4	0.33	0	0.027	0	1.77	1.75
6	0.33	0	0.027	0	3.57	3.52
7	0.33	0	0.027	0	3.57	3.52
8	0.33	0	0.027	0	3.57	3.52
9	0.33	0	0.027	0	3.57	3.52
10	100	0.14	0.111	0.015	7.75	7
14	0.75	0.14	0.083	0.015	5.63	5.25
15	0.33	0	0.027	0	3.57	3.52
16	0.50	0	0.055	0	7.05	7.41

Table 3. *Risk ratios, odds values and their log.ods in 9 allergens (¹ indicates 95% confidence limits for Lower and Upper)*
Tablo 3. *9 Alerjende risk oranları, odds değerleri ve bunların log.ods değerleri (¹ %95 güven aralığı için Alt ve Üst sınırları ifade eder)*

Sample		Risk Ratio[1]	Odds	Odds Ratio[1]	Log Odds	F. exact
4	1 0	3.5 (0.27-44.35)	0.33 0.07	4.33 (0.206-90.852)	1.46	0.03
6	1 0	3.5 (0.27-44.35)	0.33 0.07	4.33 (0.206-90.852)	1.46	0.03
7	1 0	3.5 (0.27-44.35)	0.33 0.07	4.33 (0.206-90.852)	1.46	0.03
8	1 0	3.5 (0.27-44.35)	0.33 0.07	4.33 (0.206-90.852)	1.46	0.03
9	1 0	3.5 (0.27-44.35)	0.33 0.07	4.33 (0.206-90.852)	1.46	0.03
10	1 0	0.233 (0.105-0.514)	0.25 0.07	0.041 (0.012-0.136)	-3.17	<.0001
14	1 0	0.2917 (0.135-0.626)	0.33 0.07	0.055 (0.016-0.18)	-2.89	<0.001
15	1 0	3.5 (0.27-44.35)	0.33 0.07	4.33 (0.206-90.852)	1.46	0.03
16	1 0	0.5 (0.187-1.332)	1 0.07	0.0714	-2.63	0.03

to detect equine IgE will be useful to get reliable results in horse allergy with its easy and effortless application. Our Thoroughbred horses in the study were all stallions, though Scott and Miller [9] have been reported there is no convincing gender predilection to atopy or urticaria in horses. However, the influence of seasonal factors on serum IgE level is poorly understood, the tests for allergen specific IgE quantification in all eighteen stallions in the present study were made during the summer season. Wilson et al.[4] reported that horses generally, do not develop clinical signs of skin hypersensitivities before the age of 3-4 years of ages. In addition, our stallions were all aged than this pointed years. In current study, surprisingly, *P. lanceolata* was detected as the most allergen rather than the others. Birch/Alder/Hazel and *M. faeni/Thermoactinomyces* were followed as the second and third most important allergens. One of the most common species is *P. lanceolata*, which is distributed in the temperate zones of Europe, Australia and North America [10]. Besides, *P. lanceolata* pollen has already been associated with hay fever since the beginning of this century and this weed has been considered as one of the most important dicotyledons that cause allergic diseases. Our results are also verifies that this plant is also an important allergen in horses. Birch/Alder/Hazel was the second most important allergen even they gave positive reactions in urticaria negative stallions. Over 50 species of fungi and actinomycetes have been identified in stable air, the highest challenge from stable materials and feed being thermophilic and thermotolerant mould species [11]. However, the most important organisms, in terms of their role in chronic RAO, are *Faenia rectivirgula (M. faeni), Thermoactinomyces vulgaris* and *Aspergillus fumigatus* [12]. Exposure to fungal spores is associated with allergic IgE related and non-specific neutrophilic inflammation in lower airways of horses [13], which may result directly from inhalation of fungi or from inhalation of volatile chemicals produced by fungi. As mentioned *F. rectivirgula, T. vulgaris* and *A. fumigatus* could also be allergic in horses. Results of the present study also indicate that these allergens are quite allergic to Thoroughbred horses. Interestingly, beside the summer season our stallions were less allergic to flies especially Stable fly *(Stomaxys calcitrans)*, Black fly *(Simulium equinum)*, Mosquito, Biting midges *(Culicoides nubeculosus)* and Horsefly *(Tabanus* spp.). On other hand, *Culicoides* spp. are the most important inducers of skin hypersensitivities such as summer eczemas, especially in horses [13] which the prevalence of the disease varies between 3-72%. There is only one stallion all over eighteen gave positive reaction to this allergen in the present study, even the tests were performed during summer season.

Consequently, blood sampling is a simpler procedure and provides a convenient diagnostic tool for identifying causal allergens in skin hypersensitivities even in horses. Such assays highly specific monoclonal antibodies to detect equine IgE, can be an alternate method in detecting allergens in horses. Accordingly the results of current study, *P. lanceolata,* Birch/Alder/Hazel and *M. faeni/ Thermoactinomyces* were found quite allergic sources to Thoroughbred stallions even they were urticaria negative. That is why some precautions such as keeping away of these allergens from horses should be taken. For example, the constituents of commercial feeds such as alfalfa cubes and pellets generally contain fewer mites and their faeces, fungi, actinomycetes and fungal spores particularly those incriminated in inducing chronic airway inflammation or RAO than hay and haylage. Horses housed on deep litter wood shavings bedding, or on bedding that is inconsistently changed and cleaned of excretions (as may occur in some poorly managed racetrack stables), may be exposed to higher concentrations of fungi and actinomycetes, which multiply in degrading wood shavings, (particularly pinewood) and stable dung. Further studies evaluating larger numbers of both normal horses and horses with allergic diseases (AD, RU, and ROA) in Thoroughbred horses are required. Therefore, it is important to determine whether a horse is allergic or not or the allergens have to be detected.

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CASE REPORT

Congenital Segmental Hypoplasia of the Spinal Cord in A Holstein Calf

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Summary

A case of congenital segmental spinal hypoplasia was described in a 40-day-old Holstein calf. The calf was submitted to our clinic (Istanbul University, Faculty of Veterinary Medicine, Education and Research Hospital, Surgery Clinic) with the complaint of not being able to rise to feet after birth. After clinical and radiologic inspection an anomaly was detected in spinal cord at the level of 3rd and 4th lumbar vertebrae. As the prognosis of the animal deteriorates, the calf was euthanized and systemic necropsy of the animal was performed. There was no malformation at the vertebrae around lumbar region, but a constriction was found at the third and fourth lumbar region of the spinal cord in macroscopy. It was detected that the sections prepared from the narrowest part of the medulla spinalis revealed the entire disappearance of the canalis centralis, microscopically. Furthermore syringomyelic cavities with different dimensions were observed in dorsal and ventral funiculi.

Keywords: *Calf, Congenital malformation, Spinal cord, Hypoplasia*

Holstein Irkı Bir Buzağıda Omurilikte Kongenital Segmental Hipoplazi Olgusu

Özet

Bu olguda 40 günlük Holstein ırkı bir buzağıda segmental hipoplazi tanımlanmıştır. Buzağı doğum sonrası ayağa kalkamama şikâyeti ile kliniklerimize (İstanbul Üniversitesi, Veteriner Fakültesi, Eğitim ve Araştırma Hastanesi, Cerrahi Kliniği) getirildi. Klinik ve radyolojik muayene sonrasında 3. ve 4. lumbal vertebralar hizasında omurilikte anormallik saptandı. Prognozun kötüleşmesi üzerine buzağıya ötenazi ve sistemik nekropsi uygulandı. Makroskopide lumbal bölge vertebralarında herhangi bir malformasyon izlenmedi ancak 3. ve 4.lumbal segmentte omuriliğin çapında daralma tespit edildi. Omurilik çapının en dar kesiminden hazırlanan kesitlerde, mikroskopik olarak, kanalis sentralisin tamamen gözden silindiği izlendi. Ayrıca dorsal ve ventral funikuluslarda siringomiyelik kavitasyonlar şekillendiği belirlendi.

Anahtar sözcükler: *Buzağı, Kongenital malformasyon, Omurilik, Hipoplazi*

INTRODUCTION

The terms of myelodysplasia or dysraphic states describe congenital anomalies of the spinal cord. Spina bifida, neural tube closure defect and spinal duplication are most common congenital malformations occurred in cattle, concerning myelodysplasia [1]. Segmental hypoplasia of the spinal cord is a rare form of myelodysplasia which is described as a developmental defect of one or more segments of the medulla spinalis [1]. Congenital malformations of the spinal cord usually occur in calves and especially encountered in the thoraco-lumbar region [2]. In cattle, those hypoplasia have been previously reported in Angus and other breeds [3,4]. Genetic and nutritional factors, environmental factors like infections and toxins together with fertilization techniques have been shown as common reasons of congenital malformations [5,6]. Especially some viral diseases, such as Akabane, Bovine viral diarrhea, Blue-

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tongue and Cache valley infections have been reported among the reasons of congenital anomalies [4,6]. It is strike out that, such malformed animals cannot rise to their feet and their clinic aspects get worse after their birth. In this study, we aimed to describe a rare anomaly in a calf; congenital segmental hypoplasia of the spinal cord that was not associated with vertebral abnormalities.

CASE HISTORY

A 40-old-day Holstein calf with the complaint of not being able to rise to feet after birth was submitted to the Surgery Clinic of Istanbul University, Faculty of Veterinary Medicine, Education and Research Hospital. In clinical analyzes it is established that the animal had paraplegia. In neurologic inspection it is determined that the animal had neither tendon reflex nor deep pain sense in its rear legs. Because of ancylosis in the front legs which has occurred as a result of perpetual laying the front leg tendon reflex inspection was concluded as insignificant, however the animal had pain sense in the front legs. Although there was no pathologic alteration in the lumbar region radiography of the vertebrae, in cisternal myelography, a constriction was determined in spinal cord at the level of 3rd (L3) and 4th (L4) lumbar vertebras *(Fig. 1)*.

In order to achieve decompression of spinal cord, hemilaminectomie was performed to the calf. As the prognosis of the animal deteriorates after a week calf was euthanized with the approval of the animal's owner and systemic necropsy of the animal was performed. Spinal canal of the calf was dissected transversally. After macroscopic examination tissue samples were collected into 10% formalin, processed routinely, stained with Heamatoxyline & Eosine and examined under light microscope.

Macroscopically, there was no malformation at the vertebrae around lumbar region *(Fig. 2A)*. But a constriction was found at the third and fourth lumbar region of the spinal cord. It was observed that the diameter of spinal cord was starting to get narrower at the cranial level of L3 and then it was starting to get broaden and reached its normal size at caudal level of L4 *(Fig. 2B, 2C)*. It was determined that gray matter had an irregular and atrophic appearance in transversal sections. The anomaly observed at the lumbar spinal cord involved an area of 4 cm and at its narrowest point the diameter of medulla spinalis was 0.4 cm. The thickness of the cranial and the caudal parts of the narrowest point were 1.1 cm and 1.3 cm respectively. In the cross sections of the cranial part of the narrowest point, there were 1-2 mm dorsally located cavitation *(Fig. 2D)*.

Tissue samples prepared from cerebrum, cerebellum and spinal cord were evaluated microscopically. There was no inflammatory reaction in the any of tissue sections. Neuronal degeneration and atrophy in gray matter and evident demyelinisation in white matter were observed in sections prepared from hypoplastic part of medulla spinalis. Canalis centralis got broaden and became irregular. What is more, it was transformed to hydromyelic cavities in some areas *(Fig. 3A)*. The section prepared from the narrowest part of the medulla spinalis revealed the entire disappearance of the canalis centralis *(Fig. 3B)*. Furthermore, syringomyelic cavities with different dimensions were observed in dorsal and ventral funiculi. Then it was detected that the canalis centralis reappeared in the consecutive sections of lumbar spinal cord *(Fig. 3C, 3D)*.

DISCUSSION

Generally vertebral defects are detected in cows with segmental hypoplasia defect of spinal cord [5]. In cases like

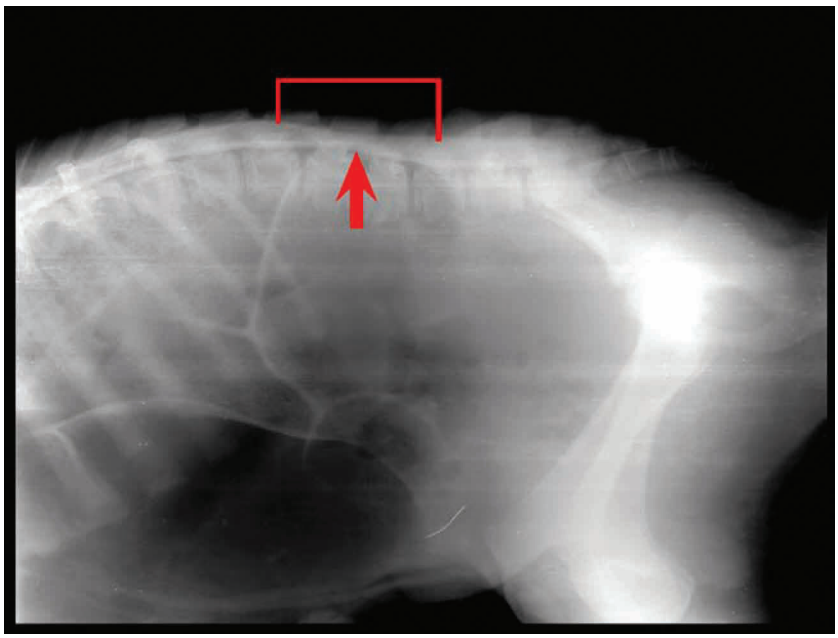

Fig 1. Myelography. The constriction at the 3rd and 4th lumbar spinal cord

Şekil 1. Miyelografi. Omuriliğin 3. ve 4. lumbal vertebral segmentinde daralma

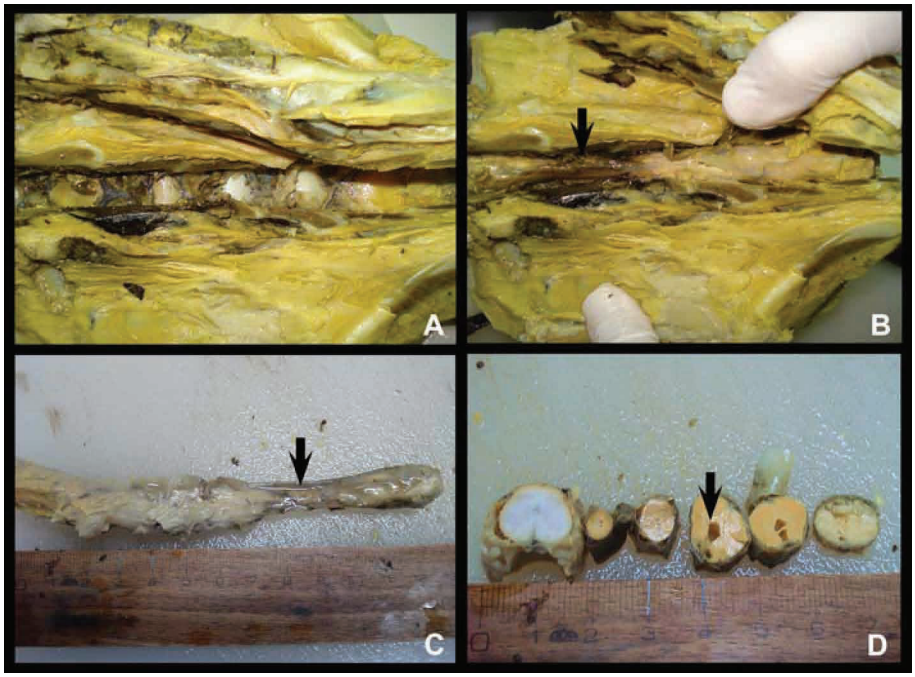

Fig 2. The appearance of the lumbar vertebral channel (A), The constriction at the lumbar spinal cord *(arrow)* (B), The appearance of the lumbar spinal cord, constriction of the diameter *(arrow)* (C), Syringomyelic cavities on the cross sectional surface of the spinal cord *(arrow)* (D)

Şekil 2. Lumbal vertebral kanalın görünümü (A), Lumbal omurilikte daralma *(ok)* (B), Lumbal omuriliğin görünümü, çapında daralma *(ok)* (C), Omuriliğin kesit yüzünde siringomiyelik kavitasyonlar *(ok)* (D)

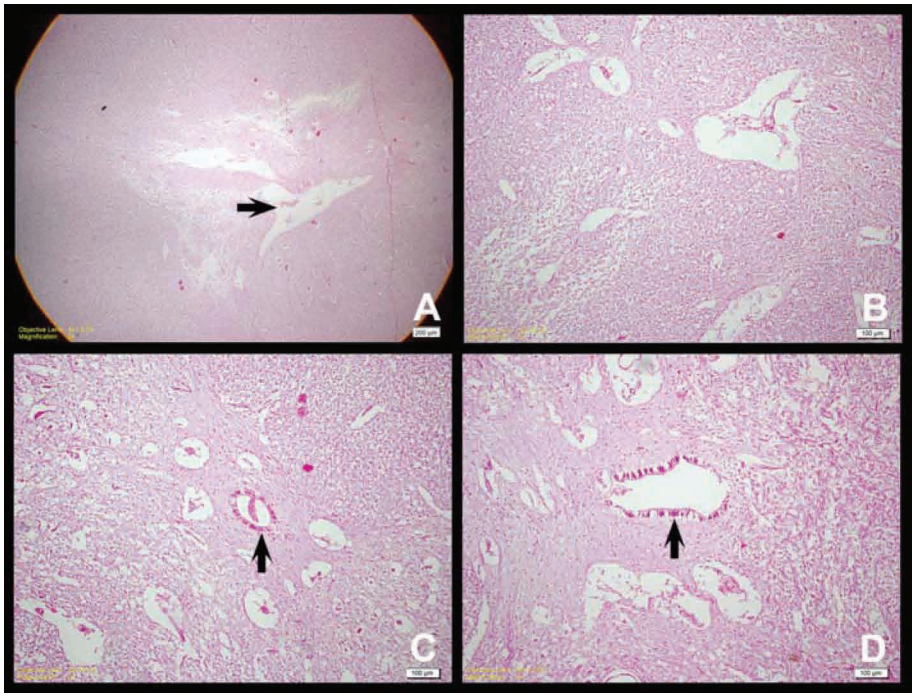

Fig 3. Hydromyelic cavitiy *(arrow)*, H&E, Bar=200 µm (A), The lack of central canal, H&E, Bar=100 µm (B), The reappearance of central canal *(arrow)*, H&E, Bar=100 µm (C and D)

Şekil 3. Hidromiyelik kavite *(ok)*, H&E, Bar=200 µm (A), Sentral kanalın yokluğu, H&E, Bar=100 µm (B), Sentral kanalın tekrar ortaya çıkışı *(ok)*, H&E, Bar=100 µm (C ve D)

Perosomus elumbis is characterized by the aplasia of the lumbosacral spinal cord and vertebrae [3,8]. However, in our case there was neither defect nor anomaly in vertebras, but there was only segmental hypoplasia in the lumbar spinal cord.

Previously, cavitations of the spinal cord without vertebral defect were reported in spinal dysraphisms of cervical spinal cord [9,10] and in syringomyelia of thoracic spinal cord [5,10]. Likewise in the cranial part of hypoplastic medulla spinalis we detected a cavitation approximately 1-2 mm without any defect in vertebras. Besides, no links between microscopic syringomyelic cavitations were observed. In spinal cord's segmental hypoplasia of human, generally constriction of spinal channel and focal vertebral anomalies develops and it is acknowledged that they generate in relation with spinal dysgenesis [11,12]. Also in our case the spinal cord was significantly narrower in the designated location, but the thicknesses of spinal cord in the cranial and caudal of that location were normal. As there were no vertebral neural arch or bone/skin lesions like the gashing of skin the segmental spinal cord hypoplasia in this case was not considered as neural channel defect. It is known that congenital anomalies were related with

infectious agents as well as genetic and nutritional factors. It has been reported that Fetal Akabane or Bluetongue virus infections were determined in some cases with hypoplasia and aplasia of spinal cord [4,6]. Because isolation and identification for infectious agents was not performed, the aetiology of the present case could not be detected. On the other hand, as there was no inflammatory reaction in the microscopic evaluations of cerebrum, cerebellum and medulla spinalis, we considered that the determined hypoplasia might be related to an anomaly occurred during organogenesis depending on a genetic aberration.

The ancylosis observed in the front leg joints reminds 'Arthrogryposis congenita' which is also called as 'Crooked Calf Syndrome', known as *a malformation characterized by arthrogryposis, torticollis and scoliosis and sometimes palatoschisis seen among cows* [13]. Generally, some deformities such as fracture, luxation, hyperextension, flexion or joint skew in the lower extremity joints are seen in the arthrogrypotic animals [14]. However, in our case, except ancylosis, there was no finding mentioned above. Besides, the authors considered that ancylosis occurred secondarily as a result of perpetual laying after birth and immobility.

As the clinical findings were observed after birth and there were no pathological changes detected in the bone structure of the spinal channel and according to the histopathological findings we described the case as congenital segmental spinal cord hypoplasia. Since this was the first report in our country, the present case was considered to be worth of being presented due to its contributory value to veterinary literature.

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CASE REPORT

Immunohistochemical and Histopathological Findings of Chondrosarcoma in Mammary Gland of A Dog: A Case Report

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Summary

Chondrosarcoma is a malignant tumor of chondrocytes and occurs most commonly on the flat bones and the nasal cavity. Mammary chondrosarcoma is an uncommon tumor in canine practice. The aim of present paper is to report a case of mammary chondrosarcom in a 10-year-old German shepherd bitch. The dog with a mass in her right caudal thoracic mammary gland which grown gradually in 8 months, was referred to the veterinary teaching hospital. The mass and local lymph nodes were resected by complete mastectomy and were sent to department of veterinary pathology for histopathological examination. Macroscopically, the mass was fluctuant containing firm mases in it, totally measuring 5×4×5 cm in diameter. The cross section was yellow and there were many cavernous structures which were filled with grayish fluid. Microscopic examination revealed a mass of large mature cartilage structures with lacunae and chondrocytes and chondroblastes with new forming structures of cartilage. Immonohistochemical investigation revealed that the tumor is negative for Estrogen receptor (ER) expression. According to macroscopic and microscopic investigations chondrosarcoma was diagnosed.

Keywords: *Chondrosarcoma, Dog, Mammary tumor, Histopathology, Immunohistochemistry*

Bir Köpeğin Meme Bezinde Gözlenen Kondrosarkoma Olgusunda İmmunohistokimyasal ve Histopatolojik İncelemeler: Olgu Sunumu

Özet

Kondrosarkoma kondrositlerin malignant bir tumörüdür ve en yaygın olarak yassı kemiklerde ve nazal boşlukta oluşur. Meme kondrosarkomu köpeklerde yaygın değildir. Bu makalenin amacı 10 yaşlı dişi bir Alman çoban köpeğinde meme kondrosarkoma olgusunu tanımlamaktır. Sağ torasik meme bezinde 8 ay süresince büyüme gösteren bir kitleye sahip olan köpek Veteriner Hastanesine getirildi. Kitle ile beraber bölgesel lenf yumrusu total mastektomi ile uzaklaştırıldı ve histopatolojik incelemeler için patoloji anabilim dalına gönderildi. Makroskopik olarak kitle 5x4x5 cm boyutunda, içerisinde sert yapılar ihtiva eden fluktuan bir yapıya sahipti. Kesit yüzü sarı renkli olup içerisi grimsi sıvı ile dolu olan pekçok boşluksu yapılar içermekteydi. Mikroskopik incelemede kitlenin lakuna, kondrosit ve kondroblastlardan oluşan geniş olgun kıkırdak yapısında olduğu belirlendi. İmmunohistokimyasal incelemede kitlenin Östrojen reseptör için negative olduğu gözlemlendi. Makroskopik ve mikroskopik bulgular doğrultusunda kitle kondrosarkoma olarak tanımlandı.

Anahtar sözcükler: *Kondrosarkoma, Köpek, Meme tümörü, Histopatoloji, İmmunohistokimya*

INTRODUCTION

Chondrosarcoma is malignant tumor of chondrocytes and the flat bones and the nasal cavity is the most common site [1]. The cause of chondrosarcoma is unknown, and probably it originates from populations of primitive multi potent mesenchymal cells [2]. In canine oncology, sarcomas comprise less than 5% of all mammary tumors and are much less common than carcinomas [3,4]. The rate of metastasis of the tumor rangins from

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0-20.5% [1,5]. Primary chondrosarcoma has been reported in appendicular skeleton, synovium, trachea, larynx, lung, liver, subcutaneous, digit, tongue, kidney, abdominal wall, pulmonary artery, omentum, mammary gland, heart and urethra [2-4,6-11]. In veterinary literature, canine skeletal chondrosarcoma accounts for 1-13% of all chondrosarcomas [1,5]. In a retrospective study in 537 dogs with mammary gland malignant neoplasms only one case of mammary chondrosarcoma (0.18%) was reported [4].

The present paper describes immunohistochemical and histopathological findings of mammary chondrosarcoma in a bitch. This is the first report of mammary chondrosarcoma in Iran, to the best knowledge of authors.

CASE HISTORY

A 10 year-old female German shepherd dog was referred to veterinary teaching hospital at the university of Tehran with history of a mass in her right caudal thoracic mammary gland in July 2011. The mass and local lymph nodes were resected by complete mastectomy and were sent to department of veterinary pathology at the university of Tehran for histopathlogical examination. The mass was fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin (H&E) method. For immunohistochemical investigation monoclonal antibodies raised against human ER-α was used using the streptavidin-biotin-peroxidase technique. 4 µm -thick section on poly-L-lysine coated glass slide was deparaffinised in xylene and rehydrated in alcohol. Heat-induced epitope retrieval was performed by immersion in boiling citrate buffer pH 6.0 for 2 min after reaching maximum pressure in a pressure cooker. Endogenous peroxidase was quenched by immersion in 3% H_2O_2 in methanol for 10 min. The monoclonal mouse anti-human ERα (clone 1D5; Dakocytomationman, Denmark) diluted 1:50, was used with a streptavidin - biotin - peroxidase complex technique (LSAB Peroxidase Universal kit, Dakocytomation). All further IHC staining procedures were according to the instructions of the test kits. Then section was counterstained with Mayer's hematoxylin. For positive control human breast carcinoma was used. Negative control was obtained by omitting the primary antibody.

Macroscopically, the mass has a fluctuant steadiness, several parts with firm and other sites with soft consistency and measuring 5×4×5 cm in diameter. The cross section was yellow and many cavernous structures were existed which grayish fluid were filled them.

Histopathological evaluation of the tumor revealed two distinct areas; First part which was located in the center of the mass was composed of large mature cartilage structures with lacunae and chondrocytes (*Fig. 1* and *2*). Chondrocytes were in various sizes and shapes that indicated pleomorphism but tumoric giant cells did not shown. Some chondrocytes had large and euchromatic nuclei, prominent nucleoli and basophilic cytoplasm and others had small and dark nuclei with scant cytoplasm. In second part, which was in periphery, there were multiple new forming cartilages with chondroblastes. Mitotic figures were 0 to 2 per high- power field and also invasion to stroma was detected. IHC evaluation revealed that, this tumor was not expressed ERα *(Fig. 3)*.

DISCUSSION

Extra skeletal osteosarcoma and chondrosarcoma are very rare in human beings and domestic animals. Extra skeletal chondrosarcoma is an anaplastic mesenchymal neoplasm that produces a cartilage matrix with no involvement of bone and periosteum. This tumor can arise from many different tissues [12]. In human, extra skeletal chondrosarcoma is more common in the extremities than in the visceral organs [13].

Chondrosarcoma in mammary gland is often multi-lobulated. The neoplastic cells at the periphery of the lobules are small with round hyperchromatic nuclei. The

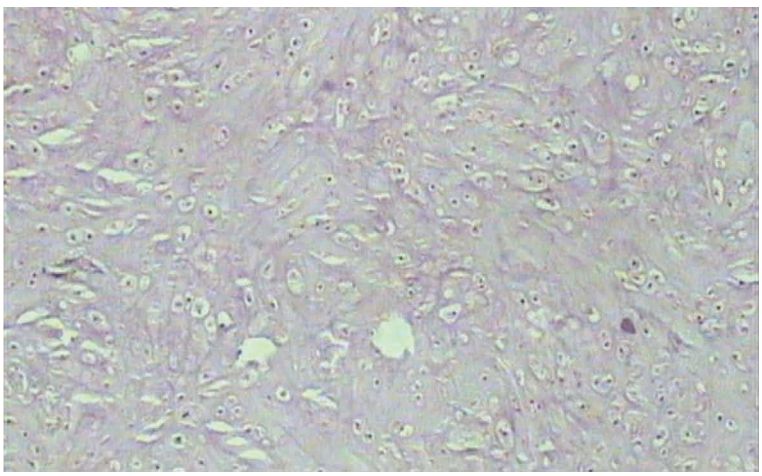

Fig 1. Chondrosarcoma. Note to cartilage structure with lacunae and chondrocytes (H&E, ×100)

Şekil 1. Kondrosarkom. Lakuna ve kondrosit ile karakterize kıkırdak yapısı (H &E, ×100)

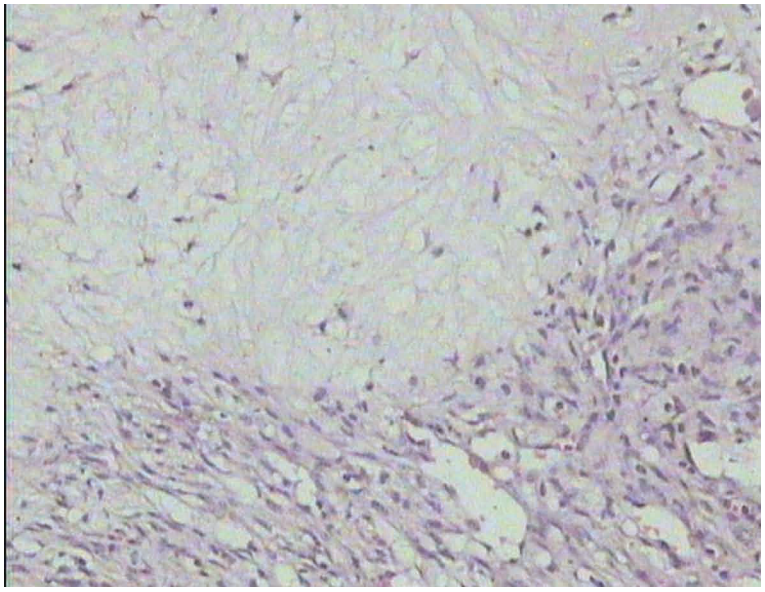

Fig 2. Chondrosarcoma. Note to cartilage structure with chondrocytes in center of slide and new formation of cartilage with chondroblasts in peripheral part (H&E, ×100)

Şekil 2. Kondrosarkom. merkezde kondrosit ve ve periferde kondroblastlardan oluşan kıkırdak yapısı (H & E, ×100)

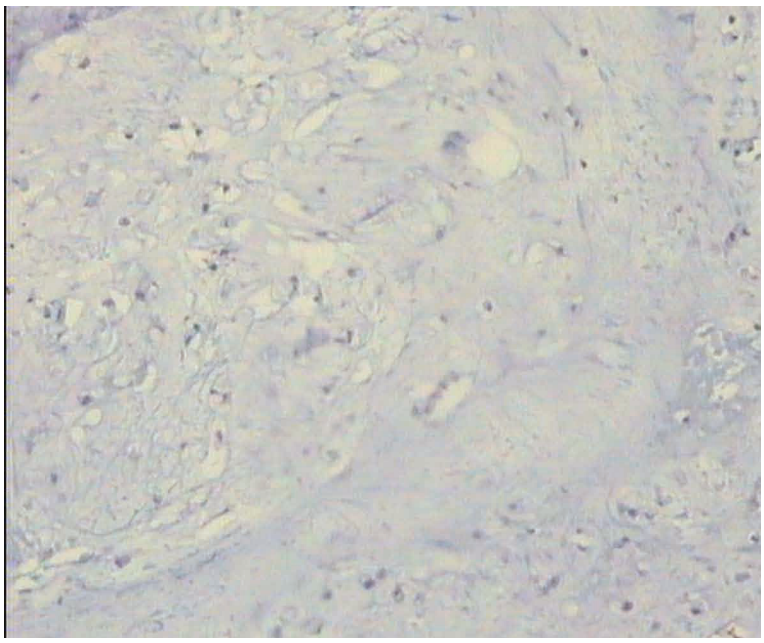

Fig 3. Chondrosarcoma. No expression of ERα in neoplastic cells was seen (IHC, ×100)

Şekil 3. Kondrosarkom. ER-α negatif neoplastik hücreler (IHC, ×100)

nuclear outline is often irregular, and nucleoli are prominent [14]. The mentioned histopathologic characteristics are in agreement with this case. According to the literature, number of mitoses and basophilic chondroid matrix are variable and are more common in less well-differentiated neoplasm. In this case, there were rare mitotic figures. Also the chondroid matrixes were in both forms, mature and immature, so this case was accounted for moderately differentiated.

Previous studies demonstrated, nearly all histologically normal canine mammary tissues contain ER as well as PR and ER expression remains high in canine dysplasia and benign tumors, with significant decreases in carcinomas [15]. In the present study immunohistochemical evaluation demonstrated that ER-α, was not expressed in chondrosarcoama.

ER-αpositive human breast tumors are associated with an improved prognosis, but studies with long-term follow-up have suggested that ER-αpositive tumors, despite having a slower growth rate, do not have a lower metastatic potential. Lack of ERα expression in the primary tumor was associated with the presence of histologically proven lymph node metastases [16]. But in present case no evidence of lymphatic metastasis was existed. Negative staining was not likely to be attributed to an experimental artifact because ERα immunoreactivity was seen in nonmalignant epithelial nuclei present in the same specimen.

Although a board age range is reported, the most common in middle aged to older dogs, the mean age of affected animals varying from 5.9 to 8.7 years [17]. The mean age of the dogs with extra skeletal chondrosarcomas is about 14 years [5].

Canine mammary sarcomas are considered to have a very poor prognosis. Chondrosarcoma in bitches tends to grow slowly and has limited metastatic rate [8]. In the few reports of metastatic chondrosarcomas, the lung is the most common site, with metastasis occurring 3 weeks to 17 months after surgical excision [8]. In another study of canine chondrosarcoma case series, local recurrence existed in 23% of cases, with a mean recurrence time of 143 days. The incidence of local recurrence was higher when complete surgical excision was not possible [8]. In a report of Serin and Aydogan [2], for treatment of mammary chondrosarcoma in dog, no recurrence or pulmonary metastasis of tumoral tissue was observed during the two months postsurgery. Post surgery assessment was not possible because there were not any information about the clinical status of this case.

According to immunohistopathologic and histopathologic characteristics, canine mammary chondrosarcoma was diagnosed. This case appeared to be the first reported case of chondrosarcoma in mammary gland of in a dog in Iran.

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CASE REPORT

Conjunctival Dermoid in A Belgian Malinois Dog

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Summary

In this report, the gross and histopathological findings and the surgical treatment of a rarely seen case conjunctival dermoid was described in Belgian Malinois dog. A 6 month-old female Belgian Malinois dog, weighing 8 kg was referred to the University animal hospital with the complaint an abnormal mass on the right eye. In ophthalmic examination of right eye, epiphora and conjunctival hyperaemia were observed. Mass in the right conjunctiva at the temporal canthus was surgically removed. In microscopic examination, resected tissue revealed that dermoid was originated from conjunctiva.

Keywords: *Malinois, Conjunctival dermoid, Choristoma, Dog, Sebaceous gland*

Belçika Malinois Irkı Bir Köpekte Konjuktival Dermoid

Özet

Bu makalede, Belçika Malinois ırkı bir köpekte nadir olarak görülen konjonktival dermoid olgusunun cerrahi tedavisi ile makroskopik ve histopatolojik bulguları tanımlandı. 6 aylık, 8 kg ağırlığında Belçika Malinois ırkı dişi bir köpek, sağ gözünde anormal bir kitle bulunduğu şikayeti ile üniversite hayvan hastanesine getirildi. Sağ gözün oftalmik muayenesinde aşırı göz yaşı akıntısı ve konjunktival hiperemi belirlendi. Sağ göz konjunktivasının temporal açısında izlenen kitle cerrahi olarak uzaklaştırıldı. Mikroskopik muayenede, rezeke edilen kitlenin konjunktivadan köken alan bir dermoid olduğu anlaşıldı.

Anahtar sözcükler: *Belçika Malinois, Konjuktival dermoid, Koristom, Köpek, Yağ bezi*

INTRODUCTION

Dermoids are examples of a choristoma or congenital circumscribed overgrowth of microscopically normal tissue in an abnormal place [1,2]. This congenital malformation may involve the ocular and periocular tissues [3] and may occur on lids, the conjunctiva or the cornea or as an inclusion cyst within the orbit [4]. Ocular dermoid contains many of the structures of normal skin such as epidermis, dermis, fat, sebaceous glands or hair follicles [5,6]. Ocular dermoid has been reported in several domestic animals such as dogs [6-10], cats [11], horses [12], sheep [13], cattle [14], rabbit [15,16], Guinea pigs [4], and birds [17].

The aim of the present report was to describe a rarely seen case of conjunctival dermoid and its surgical intervention in Belgian Malinois dog.

CASE HISTORY

A 6 month-old female Belgian Malinois dog, weighing 8 kg was referred to the University animal hospital with an abnormal mass on the right eye. Ophthalmic examination revealed that epiphora and hyperaemia at the right conjunctiva were evident. Moreover, a dermoid in the right conjunctiva at the temporal canthus was observed *(Fig. 1A)*. Fluorescein stain test was performed to identification of any corneal ulceration. No corneal ulceration was evident. Then the surgical intervention was considered. The patient was premedicated by subcutaneous administration of 0.04 mg/kg atropine sulphate (Atropin 2%, Vetas, Turkey) 30 min before the surgery. The sedation was performed by 2 mg/kg intramuscular administration of xylazine HCl (Alfazyne 2%, Egevet, Turkey). General anaesthesia was

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induced by intramuscular administration of 10 mg/kg ketamine HCl (Alfamine 10%, Egevet, Turkey). Cephalic vein was cannulated for the administration of lactated Ringer's solution (10 mL/kg/h) during the surgical procedure. Cefozolin sodium (20 mg/kg) was administered intravenously before surgery as a prophylactic treatment. Then aseptic surgery was performed. After fixation of globe, abnormal tissue at the conjunctiva was removed in a routine manner using a surgical blade and microsurgical instruments. The conjunctival defect was not sutured and the haemorrhage was controlled *(Fig. 1B)*. Postoperatively, tobramicine (Tobrex 0.3%, Alcon, Turkey) was applied topically twice daily for 7 days. Epithelialisation of conjunctival defect completed about 10 days after surgery.

Histopathologic Findings

Tissue (resected from the right conjunctiva) were fixed in 10% formalin solution and embedded in paraffin. Five micron sections were stained with haematoxylin and eosin (H&E) and examined under a light microscope (Olympus Bx51, Tokyo, Japan) and photographed by a digital camera (DP20, Tokyo, Japan). Histopathologically, the excised lesion showed characteristics of normal epidermis with a squamous epithelium and dense melanin pigmentation in basal cells. Moreover, it was observed that the conjunctival dermoid contained sebaceous and sweat glands and blood vessels *(Fig. 2)*. The examination of the resected tissue revealed that it was a conjunctival dermoid.

DISCUSSION

Ocular dermoid have been reported in various animals [4,12-17] however there are limited reports which describe the conjunctival dermoids in dogs [6,9]. It has been postulated that most dermoids seen in dogs are corneal dermoids [7-10]. Erdikmen *et al.*[9] reported that 16 of 22 dogs with dermoids presented corneal dermoids. Dermoids may be existed in the eyelids, conjunctiva (palpebral or bulbar) and nictitating membrane, unlike cornea is commonly affected [1]. Dermoids usually localize at the temporal limbal area and extend to the sclera and cornea [7]. In our case, the dermoid was originated from conjunctiva and localized at

Fig 1. Preoperative **(A)** and postoperative **(B)** appearance of the right eye of case.

Şekil 1. Olgunun sağ gözünün preoperatif **(A)** ve postoperatif **(B)** görünümü

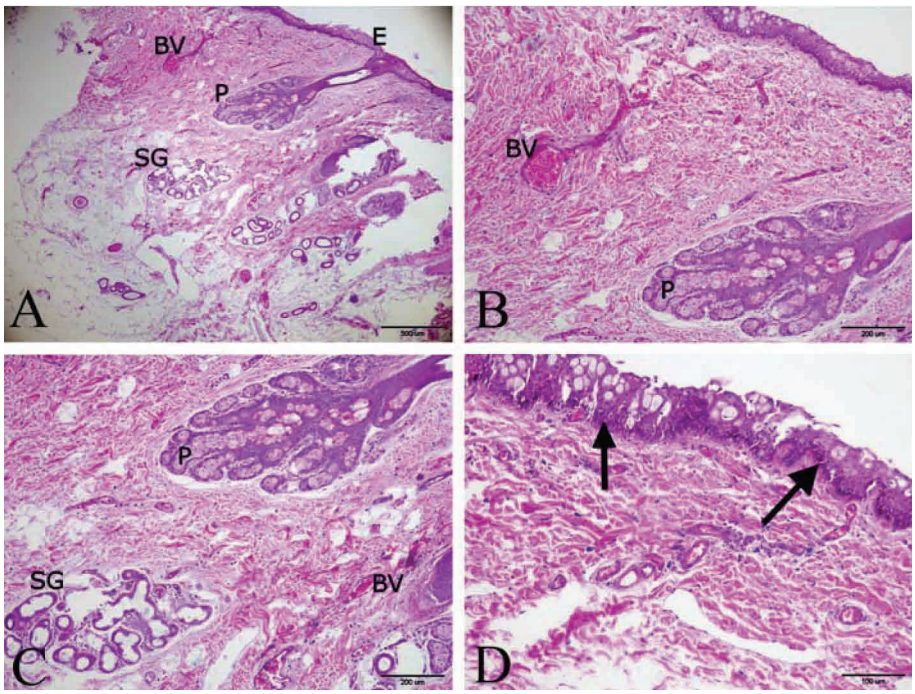

Fig 2. Histopathologic section of resected tissue from the conjunctiva

A- Epidermis (E), Sebaceous gland (P), sweat gland (SG), blood vessel (BV), H&E, x4, **B-** Sebaceous gland (P), blood vessel (BV), H&E, x10, **C-** Sebaceous gland (P), sweat gland (SG), blood vessel (BV), H&E, x10, **D-** Dense melanin pigmentation of basal cells (arrows), H&E, x20

Şekil 2. Konjuktivadan rezeke edilen dokunun histopatolojik kesiti

A- Epidermis (E), Yağ bezi (P), Ter bezi (SG), kan damarı (BV), H&E, x4, **B-** Yağ bezi (P), Kan damarı (BV), H&E, x10, **C-** Yağ bezi (P), Ter bezi (SG), Kan damarı (BV), H&E, x10, **D-** Bazal hücrelerde melanin pigmentinin birikimi (oklar), H&E, x20

the temporal limbal area as previously described above [7].

The ocular dermoids appear to be a breed predisposition in the German Shepherd dog [6,9], Saint Bernard, Golden Retriever and Dachshunds [9]. This malformation is generally congenital, but not hereditary [18]. Moreover, the ocular dermoids were determined in Anatolian Shepherd dog [8,9], Doberman Pincher, Dogo Argentina, Rottweiler, Gorden Setter [9]. To the authors' knowledge, this is the first case reported the conjunctival dermoid in Belgian Malinois dog in our clinics.

The epiphora and keratoconjunctivitis may occur due to the continuously irritation of hair to the cornea [6,8,18]. In our study, it has been observed that epiphora and mild conjunctivitis were seen on the right eye. Keratitis has not been determined due to the limited connection of hair and cornea. Our aforementioned findings suggest that ulcerative keratitis may not be observed in all cases of conjunctival dermoid in dogs. Histopathologically, the dermoids contain many of the elements of normal skin such as epidermis, dermis, fat, sebaceous glands, hair follicles and adipose tissue [2,7,9]. Similarly, in our case, sebaceous gland, sweat gland and blood vessels were observed in the microscopical examination of the resected tissue.

In conclusion, to the author's knowledge, this rarely seen conjunctival dermoid case is the first report in Belgian Malinois dog in our clinics and the data generated here may contribute to the current veterinary literature.

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CASE REPORT

Edwardsiella tarda Associated Subcutaneous Abscesses in A Captive Grass Snake (*Natrix natrix*, Squamata: Colubridae)

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Summary

Edwardsiella tarda is an opportunistic pathogen associated with aquatic environments. It has been identified as part of the normal flora of the digestive tract of healthy crocodiles, alligators, turtles, lizards and snakes. However, data on its association with different clinical diseases in reptiles, especially in snakes, is scarce. A female grass snake *(Natrix natrix)* was presented with low body condition and with multiple subcutaneous nodules. After surgical cleaning of a nodule, *E. tarda* was cultivated in pure culture from the abscesses. To the author's best knowledge, this is the first reported clinical case linked to *E. tarda* in snakes and one of the first case reports in grass snake.

Keywords: *Subcutaneous abscesses, Captivity, Edwardsiella tarda, Natrix natrix*

Bir Küpeli Yılanda (*Natrix natrix*, Squamata: Colubridae) *Edwardsiella tarda* İle Birlikte Seyreden Subkutan Apse Olgusu

Özet

Edwardsiella tarda sulak çevrelerle ilgili oportunist bir patojendir. Sağlıklı timsahlar, kaplumbağalar, kertenkeleler ve yılanların sindirim sisteminin normal florasının bir parçasıdır. Ancak, sürüngenlerde, özellikle yılanlarda, farklı klinik hastalıklarla olan ilişkilerine yönelik veriler sınırlıdır. Bir dişi çayır yılanı *(Natrix natrix)* düşük vücut kondüsyon ve çok sayıda subkutan nodüllerle birlikte sunuldu. Nodülün birinin cerrahi temizliğini takiben, apselerden *E. tarda*'nın saf kültürü izole edildi. Yazarın bildiğine göre, bu ilk defa rapor edilen yılanlarda *E. tarda* ile ilgili klinik olgudur ve çayır yılanında ilk raporlardan biridir.

Anahtar sözcükler: *Subkutan apseler, Gözetim, Edwardsiella tarda, Natrix natrix*

INTRODUCTION

Bacterial species of the genus *Edwardsiella* are associated with aquatic habitats, being frequently isolated from fresh-water fishes, amphibians, reptiles and their environment [1]. Two members of the genus, *E. tarda* and *E. ictaluri*, are important fish pathogens [2], while *E. hoshinae* was isolated from wild birds, lizards and turtles without reported pathogenicity [3,4].

E. tarda causes serious systemic disease, especially in eels *(Anguillidae)* and catfishes *(Ictaluridae)*, however recent studies on natural- and experimental infections suggested that probably all fish species are susceptible under certain conditions (e.g. stress, pollution) [2]. Besides fishes, the bacterium causes enteric disease in different aquatic bird species and invades opportunistically the sick and injured marine mammals [5,6]. It also has zoonotic potential, being associated with both gastrointestinal and extraintestinal infections in humans [7]. In reptiles, the

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bacteria was isolated both from oral cavity and cloaca of healthy individuals, however studies on its association with different clinical symptoms, especially in snakes, are scarce.

This report presents for the first time the clinical expression of *E. tarda* infection in snakes.

CASE HISTORY

A captive female grass snake *(Natrix natrix,* Squamata: Colubridae*)* was referred to the Department of Infectious Disease of the Faculty of Veterinary Medicine Cluj-Napoca. The animal belonged to the Vivarium of the University Babeş-Bolyai and was captured by fishermen in Mărtineşti, Cluj County, Romania. The animal was kept in a terrarium replicating the natural environment of the species and it was fed weekly with small crucian carps *(Carassius carassius)* and tadpoles.

At the clinical examination, the snake presented low body condition and several subcutaneous nodules of different sizes, dispersed almost all over the body surface *(Fig. 1)*.

After local anaesthesia with 2% lidocaine, one of the abscesses was opened and microbiological samples were collected in order to establish the microbial agents involved and to identify the optimal antibiotic treatment based on disk diffusion susceptibility test. The swabs were streaked for isolation onto Columbia agar supplemented with 5% sheep blood for fastidious Gram-negative and Gram-positive bacteria and onto Drigalski agar to select Gram-negative lactose-positive bacteria. After 24 h of incubation at 37°C, a pure culture of lactose-negative, Gram negative bacteria was obtained. The isolate was identified as *E. tarda* using Analytical Profile Index (API) 20E biochemical test strips in accordance with the manufacturer's directions (BioMérieux, France). The sensitivity of the isolate to various antimicrobials commonly used in exotic practices was evaluated as mentioned before.

Fig 1. Female grass snake *(Natrix natrix)* with multiple subcutaneous abscesses *(white arrows)*

Şekil 1. Dişi çayır yılanında *(Natrix natix)* çok sayıda subkutan apse odakları *(beyaz oklar)*

Based on susceptibility results a treatment was initiated with Enrofloxacin (10 mg/kg b.w. im; Bayer Corporation) for nine days, 5 ml Duphalyte (Pfizer) and 5 ml Glucose 5%, both administered by intra-coelomic route every second day for 2 weeks.

Despite the treatment, the condition and the health status of the animal deteriorated and ten days later, due to ethical considerations, we were forced to euthanize the snake using ketamine 20 mg/kg b.w. followed by freezing [8].

DISCUSSION

Previous studies suggested that *E. tarda* is part of the normal flora of the digestive tract of reptiles [9], the bacteria being isolated from American crocodiles *(Crocodylus acutus)* [10], American alligators *(Alligator mississippiensis)* [11], Chinese soft-shelled turtles *(Trionyx sinensis)* [12], Geoffroy's toadhead turtles *(Phrynops geoffroanus)* [4], tokay geckos *(Gekko gecko)* [13] and Brook's House geckos *(Hemidactylus brookii)* [14], garter snakes *(Thamnophis* spp.) [15], South American rattlesnakes *(Crotalus durissus terrificus)* [16] or tiger snakes *(Notechis scutatus)* [17]. Besides the healthy carrier status of different reptile species, septicaemia associated with *E. tarda* was reported in captive crocodiles and in Chinese soft-shelled turtles [18,19]. To our best knowledge, the present study is the first to associate clinical symptoms with *E. tarda* infection in snakes, causing multiple subcutaneous abscesses in a captive grass snake *(Natrix natrix)*.

Similarly to earlier studies reporting clinical edwardsiellosis in reptiles [18,19], the described animal was also a captive one. It has been shown that captivity increases the levels of corticosterone, which leads to decreased immunocompetence [20]. Such captivity related immunosuppression could have been underlying the infection with this opportunistic pathogen.

Although *E. tarda* was found as part of the bacterial flora in several snake species [15-17], previous attempts to isolate this microorganism from free-living grass snakes failed [21]. Since the captive grass snake was fed with tadpoles and fishes, both taxa being known as reservoirs for *E. tarda* [2,22], there is a strong argument for a food -borne infection in this case.

The grass snake is one of the most frequent snake species in Europe, and it is a common native reptile brought to veterinarians either as a wildlife casualty [8] or as pet [23]. Despite these facts, there are only two studies on bacterial diseases of this species [23,24]. While one of them reported the susceptibility of the grass snake to experimental infection with different *Mycobacterium* species [24], the second

presented a clinical case similar to the one described in the present study, associating *Salmonella typhymurium* with clinical symptoms [23]. Both reports stressed the importance of dermatologic problems in captive snakes.

The present study not only described a new clinical case in a common European native snake species, but also reports the first edwardsiellosis case in snakes.

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CASE REPORT

Congenital Giant Occipital Meningoencephalocele in A Holstein Calf Fetus

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Summary

A 6-month-old dead fetus, removed from the uterus of a 4-year-old Holstein cow, was referred to our department. A fluctuant sac, approximately 25x20x30 cm, covered with skin was observed over the occipital region of the cranium. Almost 4 liters lucid liquid was discharged from the sac. After the dissection of the soft tissue, a 1 cm diameter hole with irregular edges in connection with extracranial sac was determined on top of the occipital region of the left hemisphere in the cerebrum. The subject was diagnosed as meningoencephalocele due to the existence of prolapsed cerebellum in the sac. Also, a second channel connecting the fourth ventricle into the sac was also determined via MR images. An occipital meningoencephalocele in a 6-month-old holstein fetus was defined in this case report by pathologic-anatomic and radiological diagnosis.

Keywords: *Calf, Cranium anomaly, Meningoencephalocele*

Bir Holştayn Fetüste Görülen Konjenital Dev Oksipital Meningoensefalosel

Özet

Bu raporun materyalini; kesilen 4 yaşlı Holştayn ırkı bir sığırın uterusundan ölü olarak çıkarılan ve bölümümüze getirilen yaklaşık 6 aylık bir fetüs oluşturdu. Kraniumun oksipital bölgesinde fluktuan yapıda yaklaşık 25x20x30 cm boyutlarında deri ile kaplı bir kese gözlendi. Kese içinden yaklaşık 4 litre berrak akışkan sıvı boşaltıldı. Kafa yumuşak dokuların ayrılması sonrası beyinde sol hemisferin oksipital bölgesinin üst kısmında ekstrakranial kese ile bağlantılı kenarları düzensiz 1 cm çapında bir delik gözlendi. Kese içerisine beyinciğin prolobe olması olguya meningoensefalosel tanısını koydurdu. Ayrıca kese içerisine ventrikulus kuartusun bağlantısını sağlayan ikinci bir kanalın varlığı da MR görüntüleri ile tespit edildi. Bu raporda; 6 aylık holştayn fetüsta görülen oksipital meningoensefalosel patolojik-anatomik ve radyolojik bulgularıyla tanımlandı.

Anahtar sözcükler: *Buzağı, Kranium anomali, Meningoensefalosel*

INTRODUCTION

Cranial meningoencephalocele is a congenital anomaly and seen in domestic animals and especially in cattle [1,2]. Defects concerning crania bifida may be encountered in the form of encephalocele, meningocele or meningo-encephalocele [2,3]. Defective ossification of the skull and secondary herniation of the cerebrum is effective during morphogenesis. However, the actual defect has been considered to be related with the closure of the neural tube [2,3]. A hernia sac, made of skin without dura mater, is noteworthy in the defective region [2,4,5]. Defects have been reported to develop mostly in the frontal and occipital regions [2].

In the present report, occipital meningoencephalocele was seen in a 6-month-old holstein fetus was defined with anatomo-pathologic and radiological findings.

CASE HISTORY

A 6-month-old dead fetus, removed from the uterus of a 4-year-old Holstein cow, was referred to our department. A fluctuant sac, approximately 25x20x30 cm, covered with skin was observed over the occipital region of cranium. A systemic necropsy was performed to the fetus, samples

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were collected from each organ including extracranial sac. Collected samples passed through routine procedures and examined under light microscope.

In anatomopathological examination of the sac containing 4 liters of colorless liquid *(Fig. 1A)*. Under the oval extracranial sac on cranium, there was an irregularly edged 1.5-2 cm diameter passage at the fronto-occipital region of the cranium which was connecting the extracranial sac and upper part of the occipital region of left hemisphere *(Fig. 1B)*. Occurrence of a canal providing the connection between the sac and cavum cranii was determined when the incision was magnified and os frontale appeared *(Fig. 1C)*. Fissure longitudinal cerebri was not evident and this deep fissure which separates the hemispheres was transformed into a line form *(Fig. 1D)*. This transformation was thought to be formed by the increased intracranial pressure.

In the occipital lobe of left hemisphere, there was a 1 cm diameter canal with irregular edges connected to the extracranial sac. Its wall was covered by substantial alba *(Fig. 2A, 2B)*.

Velum medullare caudale was observed to have curled up and extended towards the canal connected to

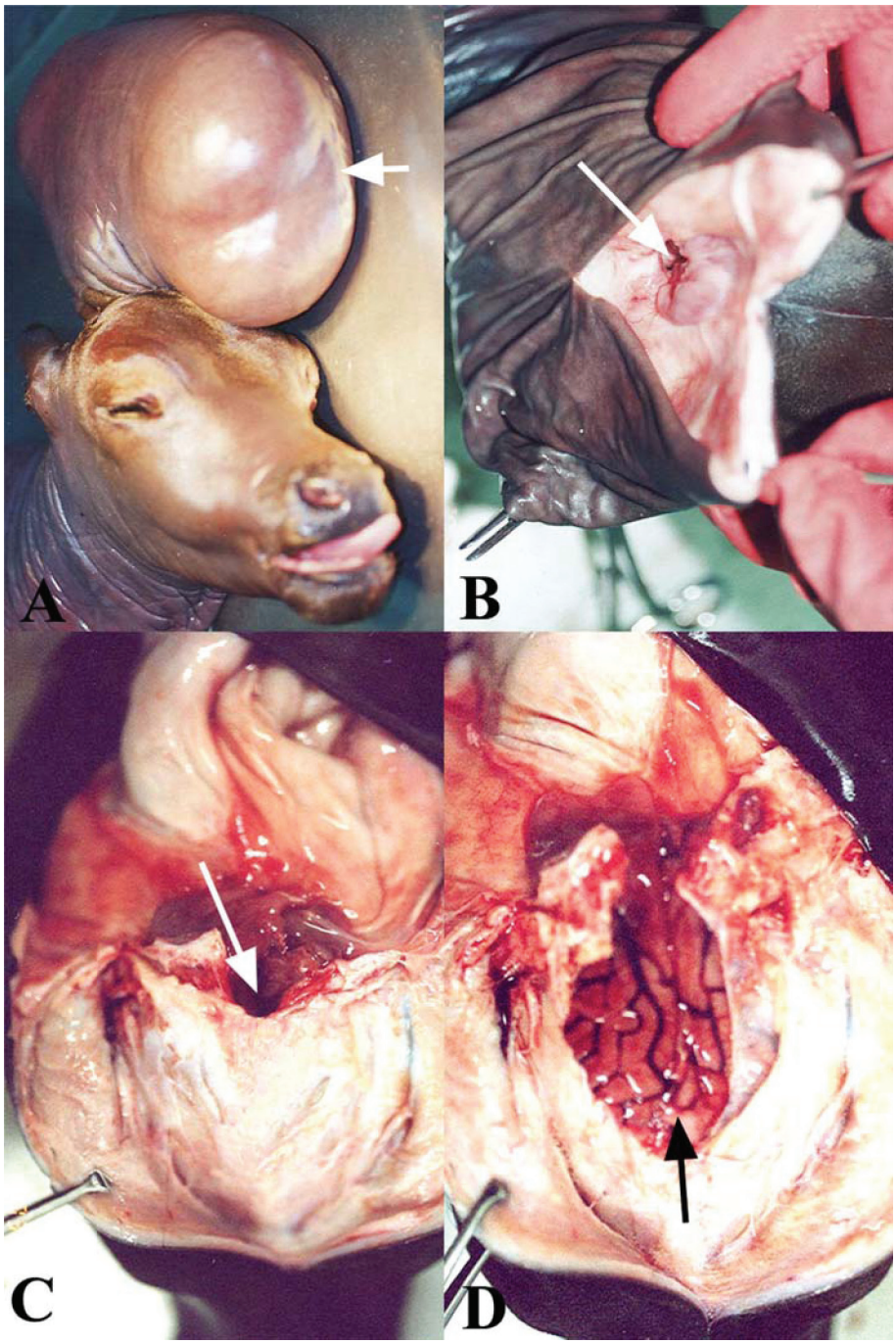

Fig 1. A- View of extracranial sac *(white arrow)*, **B-** View of the cranial attachment of the sac from the dorsal aspect *(white arrow)*, **C-** The canal between the sac and cavum cranii *(white arrow)*, **D-** Indistinct fissure longitudinalis cerebri *(black arrow)*

Şekil 1. A- Ekstrakranial kesenin görünümü *(beyaz ok)*, **B-** Kesenin kranial bağlantısının dorsalden görünümü *(beyaz ok)*, **C-** Kese ile kavum cranii arasındaki kanal *(beyaz ok)* **D-** Belirgin olmayan fissura longitudinalis cerebri *(siyah ok)*

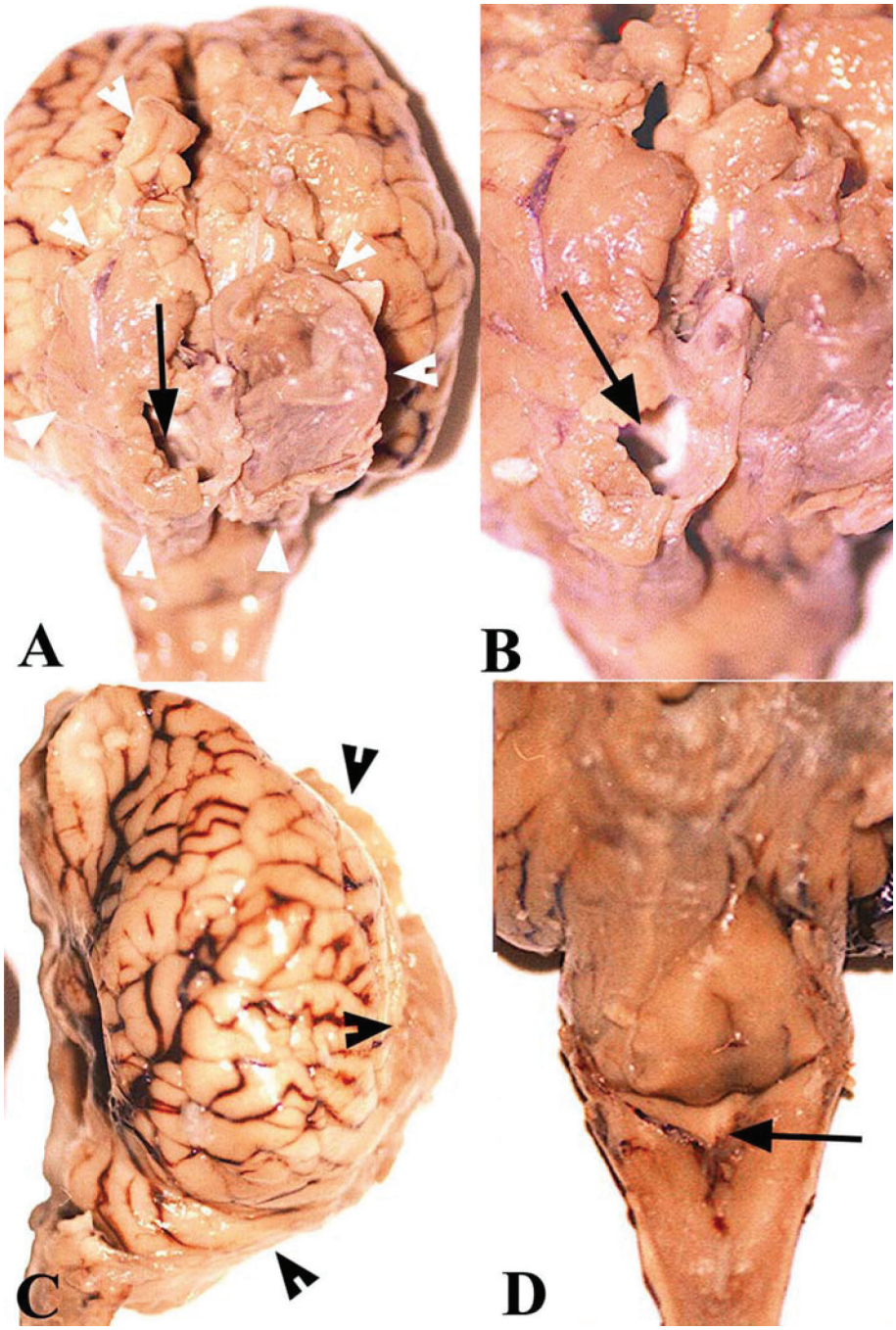

Fig 2. A- Canal formation in occipital lobe *(white arrow),* prolapsed cerebellar tissue *(white arrow heads),* **B-** Wall of the canal in the occipital lobe, composed of substantia alba *(black arrow),* **C-** View of the cerebellum different from the normal anatomic position *(black arrow heads),* **D-** View of fossa rhomboidea from the dorsal aspect *(black arrow)*

Şekil 2. A- Occipital lobdaki kanal oluşumu *(beyaz ok),* prolabe olmuş cerebellar doku *(beyaz ok başları),* **B-** Occipital lobdaki kanalın substantia alba'dan oluşan duvarı *(siyah ok),* **C-** Cerebellum'un normal anatomik pozisyondan ayrılmış görünümü *(siyah ok başları),* **D-** Fossa rhomboidea'nın dorsalden görünümü *(siyah ok)*

the sac. Fissura transversa and cerebellum could not be anatomically observed. Polus caudalis of the cerebrum cortex was observed to have been covered with a flat and large structure which had similar color and structure of cerebellum cortex *(Fig. 2A)*. In other words, the cerebellum separated from its usual anatomic position and, flattened and extended towards the frontal and upper parts *(Fig. 2C)*. Due to the difference in the structure and position of the cerebellum, fossa rhomboidal, forming the base of fourth ventricle**,** could be observed when looked at dorsally *(Fig. 2D)*.

Belt like nerve tissue at both sides of velum medullare caudale starting pars dorsalis pontis which formed the posterior and peripheral walls of the canal extending to the sac was observed. Occurrence of epiphysis could not be determined macroscopically among colliculus rostralis. An independent, flattened disc like tissue which paler than the tissue around was observed at the basement of sulcus medianus lamina tecti.

Left ventriculus lateralis expanded and formed cavitations in every direction in the rostral. This ventriculus was observed to have united with the canal opening to extracranial sac in the caudal. Cavitations in the right ventriculus lateralis were only at the anterior and posterior side but not as many as those of the left one.

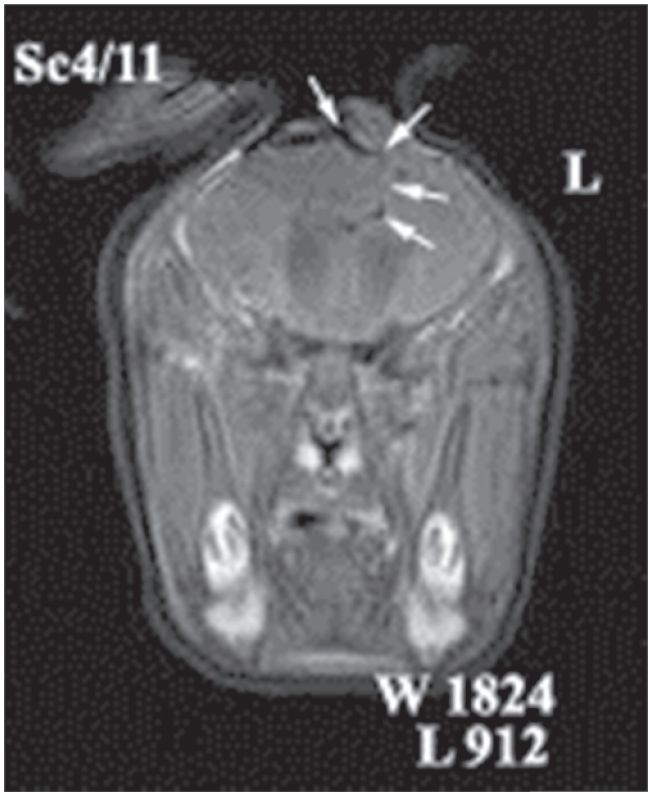

Fig 3. Sagittal section of the MR image. Canal connecting the left ventriculus lateralis to the extracranial sac *(white arrows)*. View of the second canal providing the connection through ventriculus quartus to extracranial sac *(white arrow heads)*

Şekil 3. MR görüntüsü sagital kesit. Sol ventriculus lateralis ile ekstrakranial keseyi bağlayan kanal *(beyaz oklar)*. Ventriculus quartus'un ekstrakranial kese ile bağlantısını sağlayan ikinci kanalın görünümü *(beyaz ok başları)*

Belt like nerve tissue determined on the walls of the canal extending to the sac was observed to be the cerebellum. The disc shaped flattened structure at the base of sulcus medianus laminae tecti were determined to be belonging to the epiphysial tissue.

Besides it was noteworthy that there was no pathological lesion other part of the brain and no pathological result was encountered in the histological examinations of tissues belonging to other systems.

Magnetic Resonance (MR) scanning of the cranium was performed using a 1.5 Tesla superconducting magnet (Philips Gyroscan Intera, Best, The Netherlands). T_2 weighted turbo spin echo (TSE) (TR/TE, 5800/110 ms) sagittal and transversal plan images were obtained. A canal in the occipital lobe of the left hemisphere was determined in the sagittal and transversal sections obtained from MR investigations. This canal with irregular edges, which has a diameter of 1 cm, was observed to have extended between the extracranial sac and ventricles laterals (*Fig. 3* and *Fig. 4*). In the sagittal MR images a second canal providing the connection between extracranial sac and fourth ventricle was determined *(Fig. 3)*.

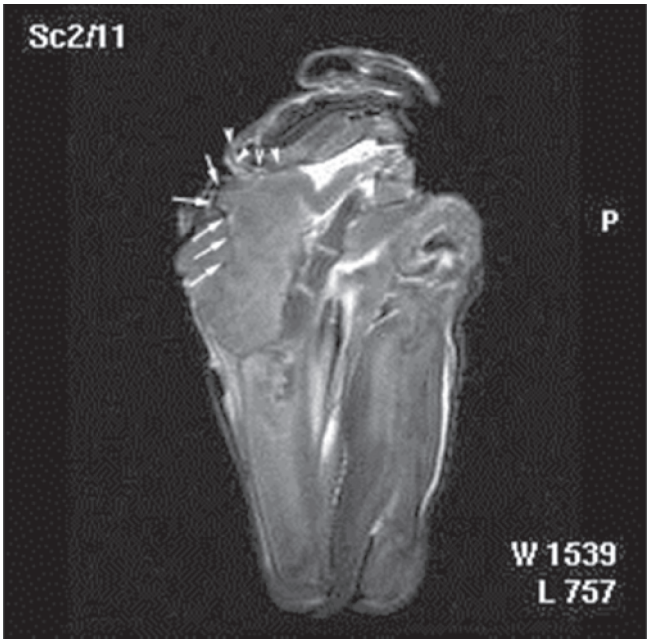

Fig 4. Transversal section of the MR image. View of the canal between the left lateral ventriculus and the sac *(white arrows)*

Şekil 4. MR görüntüsü transversal kesit. Sol lateral ventrikül ile kese arasında uzanan kanalın görüntüsü *(beyaz oklar)*

DISCUSSION

It has been reported that defects concerning crania bifida in cattle are encountered in the form of meningocele or meningoencephalocele [1-3]. In the present report occipital meningoencephalocele in a 6-month-old Holstein fetus was defined with anatomo-pathological and radiological findings.

Meningoencephalocele has also been described in other domestic animals such as horses, cattle, pigs, dogs, lambs and cats [4,6-11]. Cranial localization is generally formed as frontal and occipital [1-3]. Meningocele may sometimes located at cervical [12], thoracic [5], lumbar [13] vertebral regions in other animals. Settlement of occipital meningoencephalocele case in the present study was in agreement with the literature data.

Etiologically congenital defects of central nervous system formed during intrauterine development depending on either genetic or environmental factors same as general congenital defects [2,11]. Hereditary meningocele and meningoencephalocele cases have been reported in researches made in pigs and cats [11]. In the present report as the mother was slaughtered, the etiology of the case could not be determined.

In the previous studies concerning meningocele, liquid might go out of a normal cavity (such as anterior fontanelle) by the effect of interior pressure induced by the increase in the cerebrospinal fluid, which caused by a defect in closing the neuronal tube [3]. In this report it

was observed that particularly left and right ventriculus lateralis were widened and cavitations formed in every direction especially on the left of the rostral. It was noteworthy that the left ventricle opened to extracranial sac via a canal in the caudal like a porencephalic defect. Increased cerebrospinal fluid herniated in the form of a sac into the anterior fontanelle, with the help of canal situated in the left ventriculus. Contrary to other studies [4,10], the prolapsed cerebellum into the sac verified that the subject was not meningocele but meningoencephalocele. Besides, a second channel providing the connection of fourth ventricle into the sac was determined with MR images [2,4,10,11]. Cerebrospinal fluid, increasingly collected in the fourth ventricle and the sac, was considered to be the most important reason of the separation of the cerebellum from its anatomical position.

In conclusion, sagittal and transversal sections obtained from tomography and magnetic resonance imaging techniques are routinely applied to human for the diagnosis of cranium anomalia, if these techniques would have been available routinely in the veterinary medicine, the diagnosis of meningocele or meningoencephalocele and differential diagnosis of other mixed cranial anomalies would be easier as presented in this report.

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YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup, derleme ve çeviri türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.
2- Dergide yayımlanması istenen yazılar Times New Roman yazı tipi ve 12 punto ile A4 formatında, 1.5 satır aralıklı ve sayfa kenar boşlukları 2.5 cm olacak şekilde hazırlanmalı ve resim, tablo, grafik gibi şekillerin metin içindeki yerlerine Türkçe ve yabancı dilde adları ve gerekli açıklamaları mutlaka yazılmalıdır.
Dergiye gönderilecek makale ve ekleri (şekil vs) *http://vetdergi.kafkas.edu.tr* adresindeki online makale gönderme sistemi kullanılarak yapılmalıdır.
Başvuru sırasında yazarlar yazıda yer alacak şekilleri (13 X 18 cm boyutlarından büyük olmamalı) online makale gönderme sistemine yüklemelidirler. Yazının kabul edilmesi durumunda tüm yazarlarca imzalanmış *Telif Hakkı Devir Sözleşmesi* editörlüğe gönderilmelidir.
3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.
4- ***Makale Türleri***
Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır.
Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, giriş, materyal ve metot, bulgular, tartışma ve sonuç ile kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 10 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.
Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 4 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.
Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 2 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.
Gözlem, uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri giriş, olgunun tanımı, tartışma ve sonuç ile kaynaklardan oluşmalı ve 4 sayfayı geçmemelidir.
Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 1 sayfayı geçmemelidir.
Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri giriş, metin ve kaynaklardan oluşmalı ve 10 sayfayı geçmemelidir.
Çeviri, makalenin orijinal formatı dikkate alınarak hazırlanmalıdır.
Yazarla ilgili kişisel ve kuruma ait bilgiler ana metin dosyasına değil, on-line başvuru sırasında sistemdeki ilgili yerlere unvan belirtilmeksizin eklenmelidir.
5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.
6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır.
Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.
Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.
Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.
Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.
Örnek: **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.
Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi kaynak bilgilerinin sonuna eklenmelidir.
Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.
Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.
7- Bakteri, virus, parazit ve mantar tür isimleri ve anatomik terimler gibi latince ifadeler orijinal şekliyle ve italik karakterle yazılmalıdır.
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