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RESEARCH ARTICLES

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RESEARCH ARTICLE

Assessment of Time Periods of Exogenous Development of *Trichostrongylus tenuis* Nematodes, Parasitizing in Domestic Goose

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ABSTRACT

The aim of present study was to assess the time periods of exogenous development of *Trichostrongylus tenuis* nematodes that parasitize in domestic goose, and their survival *in vitro*. To do that, eggs of *Trichostrongylus* were obtained from excrement of infected birds and cultured in laboratory to the third stage larvae (L3). Culturing was performed at 15^{0}C, 20^{0}C and 25^{0}C. Time periods of the parasitic development was analyzed, as well as their survival. According to results, the optimum temperature of embryonic and postembryonic development of *T.tenuis* is 25^{0}C. The time period of exogenous development at 25^{0}C was the shortest (five days), and survival was 83.67%. Decreasing temperature resulted in slower development of infectious larvae (10 days) of lower survival (58.67%). Developmental processes in eggs and larvae of *Trichostrongylus* were accompanied by changes in their sizes regardless of the temperature conditions. Parasitic embryogenesis was characterized by increasing length and width of eggs. Postembryonic development of nematodes was associated with inflating length and width of second stage larvae, but inflating length and decreasing width of third stage larvae.

Keywords: domestic goose, trichostrongylosis, exogenous development

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Evcil Kazlarda Parazitlenen *Trichostrongylus tenuis* Nematodların Ekzojen Gelişim Sürelerinin Değerlendirilmesi

ÖZ

Bu çalışmanın amacı, evcil kazlarda parazitlenen *Trichostrongylus tenuis'in* eksojen gelişim ve in vitro ortamda canlı kalma sürelerini değerlendirmektir. Enfekte kuşların dışkısından izole edilen *Trichostrongylus* spp. yumurtaları laboratuvar ortamında 3. dönem larva elde edilmek üzere 15^{0}C, 20^{0}C ve 25^{0}C olmak üzere üç farklı sıcaklıkta kültür edilmiştir. Farklı sıcaklıklardaki larva gelişimi ve canlı kalma süreleri analiz edilmiştir. Elde edilen analiz sonuçlarına göre, *T. tenuis*' in embriyonik ve postembriyonik gelişimi için optimum sıcaklık 25^{0}C ' dir. Larvalar, in vitro ortamda en hızlı gelişimi 5 günde 25^{0}C'de göstermişler ve % 83.67 hayatta kalmışlardır. Azalan sıcaklık, enfektif larvaların daha yavaş gelişimine (10 gün) ve daha kısa süre canlı kalmalarına (% 58.67) sebep olmuştur. Trichostrongylus yumurta ve larvaları her üç sıcaklık ortamında, farklı değerlerde olsa da gelişim göstermiştir. Parazitik embriyogenez, yumurtaların uzunluğunun ve genişliğinin artması ile karakterize edilmiştir. Nematodların postembriyonik gelişimi sırasında, ikinci dönem larvalarda uzunluk ve genişlikte artış gözlenirken, üçüncü dönem larvalarda uzunlukta artış, genişlikte ise azalma gözlemlenmiştir.

Anahtar Kelimeler: Kaz, trichostrongylosis, ekzojen gelişim

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INTRODUCTION

The poultry farming may develop not only by creating new highly productive breeds of geese, improving diets for nutrients, but by also ensuring sustainable epizootic control (Romanov 1999, Elmberg et al. 2017). The common helminth infections of geese include gastrointestinal nematodoses. One of the most widespread gastrointestinal nematodoses is trichostrongyloidosis, associated with significant losses for poultry farming (Enigk and Dey-Hazra 1971, Shutler et al. 2012).

Epizootological features of trichostrongyloidosis have been mostly studied in wild birds (Anseriformes, Galliformes, Gruiformes, and Otidiformes), and this infection is widespread throughout Europe, Asia, North America, Africa, Australia, New Guinea, New Zealand. There, the infection rates can reach 100 %, and the intensity of infection can be up to 2471 nematode specimens per bird (Calvete et al. 2003, Webster et al. 2007, Bhat et al. 2014).

Most of the scientific works are studies of the parasitization of *Trichostrongylus tenuis* in partridges. The pathogen destabilizes the dynamics of partridge population, leading to its decline. Moreover, the prevalence of infection in partridges reaches 90%, the intensity of infection is up to 30.000 nematode specimens, and infection rates are higher (in 30 times) in adult birds than in young animals (Wilson 1983, Fox and Hudson 2001, Newborn and Foster 2002, Seivwright et al. 2004).

It has been proven that *T. tenuis* nematodes evolved from the free-living ancestors of modern rhabditates. This is indicated by their partial free-living lifestyle, namely the part of the life cycle and metamorphoses that occur outside the host's body, in the external environment. The parasite's development is direct involving one definitive host. The wild birds and poultry that are susceptible to the causative agent of trichostrongyloidosis include *Anser albifrons*, *Anser anser dom.*, *Gallus gallus dom.*, *Anas platyrhynchos dom.*, *Meleagris gallopavo*, *Anas platyrhynchos*, *Branta canadensis*, *Chen caerulescens*, *Lagopus scoticus*, *Lagopus muta*, *Numida meleagris*, *Otis tarda*, *Perdix perdix*, *Pavo cristatus*, *Phasianus colchicus*. Moreover, such a wide distribution of this pathogen in birds is explained by the peculiarities of its development, where exogenous preservation of the parasite at the stages of the egg and larva allows parasites to survive as a species (Watson et al. 1988, Cattadori et al. 2005, Skírnisson et al. 2012).

Thus, a comprehensive study of *Trichostrongylus tenuis* parasites of domestic goose is not only of general biological interest, but it has a great importance in the ensuring of trichostrongylosis epizootic control at poultry farms also.

MATERIAL and METHODS

Studies were conducted at the Laboratory of the Parasitology and Veterinary Expertise of the Department of Veterinary Medicine of the Poltava State Agrarian Academy (Poltava, Ukraine, 49 ° 35'42 " N 34 ° 33'19 " E) in 2019.

The time periods of the exogenous development of *T. tenuis* nematodes were studied in laboratory conditions. In order to do that, helminth eggs were isolated using Kotelnikov-Khrenov's method of coproscopic examination of domestic goose reared in private farms of Poltava region (Kotelnikov, 1984), from excrement of infected birds. The obtained washed eggs were transferred to Petri dishes, 100±5 eggs per dish, and cultured in a thermostat at various temperatures (15^0C, 20^0C and 25^0C) until the formation of third stage larvae (L3). Samples were examined daily under a microscope. The stage of development of eggs and larvae of nematodes was determined by morphological structure, considering the timing of their development and taking into account the number of dead eggs and larvae. Each experiment was carried out in triplicate.

The metric parameters of *T. tenuis* eggs and larvae during their development were studied using ImageJ for Windows® software (version 2.00). Microphotography was performed using a digital camera to a MikroMed 5Mpix microscope (China).
The research protocol of the current study was approved by the Ethic Committee of the Poltava State Agrarian Academy (Approval number: 2019/02).

Statistical analysis
Statistical processing of the experimental results was carried out using Statistica 10 (StatSoft Inc., USA) software. Standard deviation (SD) and average values (M) were calculated. Significance of difference between average values in studied eggs and larvae cultures of *T. tenuis* was established using one-way analysis of variance and F-test. The statistically significant rate was set as $p<0.05$.

RESULTS

The exogenous development of *T. tenuis* was shown to include five stages: blastomere cleavage (Fig. 1 a), larval formation in egg (embryonic) (Fig. 1 b), and postembryonic formation of first stage (L1) (Fig. 1 c), second stage (L2) (Fig. 1 d) and third stage (L3) larva (Fig. 1 e). The timing of development and survival rates of exogenous stages of parasites depended on the temperature regime (Table 1).

The most optimal temperature for the successful development of eggs to the infectious L3 was 25^{0}C. The time periods of development were the shortest, five days at this temperature, and the survival rate was 83.67±3.51%. A decrease in temperature contributed to longer development of *Trichostrongylus*, and nematode survival was reduced to 75.33±1.53% at 20^{0}C and to 58.67±2.08% at 25^{0}C.

The morphometric parameters of *T.tenuis* eggs were studied during their embryonic development. Regardless of the temperature of cultivation, their length and width changed (Table 2).

At 15^{0}C, the length and width of *Trichostrongylus* eggs increased by 3.77% ($p<0.01$) and 6.39% ($p<0.001$), respectively. At 20^{0}C, the parameters of eggs during cultivation significantly ($p<0.001$) increased: length by 4.62%, width by 8.08%. Cultivation at 25^{0}C also led to an increase in their size ($p<0.001$): length by 5.44%, width by 7.82%. During postembryonic development of *T.tenuis* larvae, their sizes changed at all temperature conditions (Table 3).

At 15^{0}C, the length and width of the second stage larvae (L2) increased relative to the sizes of the first stage larvae (L1) by 22.10 and 8.55% ($p<0.001$), respectively. Similar data were obtained for cultivation at 20^{0}C and 25^{0}C. The length of L2 increased by 22.21 and 20.26% ($p<0.001$), and the width increased by 8.26 and 8.72% ($p<0.001$). At the same time, infectious larvae of the third stage (L3) had a longer and narrower body in comparison with L1. At 15^{0}C, length of L3 increased by 26.74% ($p<0.001$), and the width decreased by 5.20% ($p<0.01$). At 20^{0}C, L3 were longer (by 26.34%, $p<0.001$) and narrower (by 7.88%, $p<0.001$). The same tendency in L3 body size changes during postembryonic development was also observed at 25^{0}C. Infectious larvae were longer by 24.27% ($p<0.001$) and narrower by 11.60% ($p<0.001$) relative to L1.

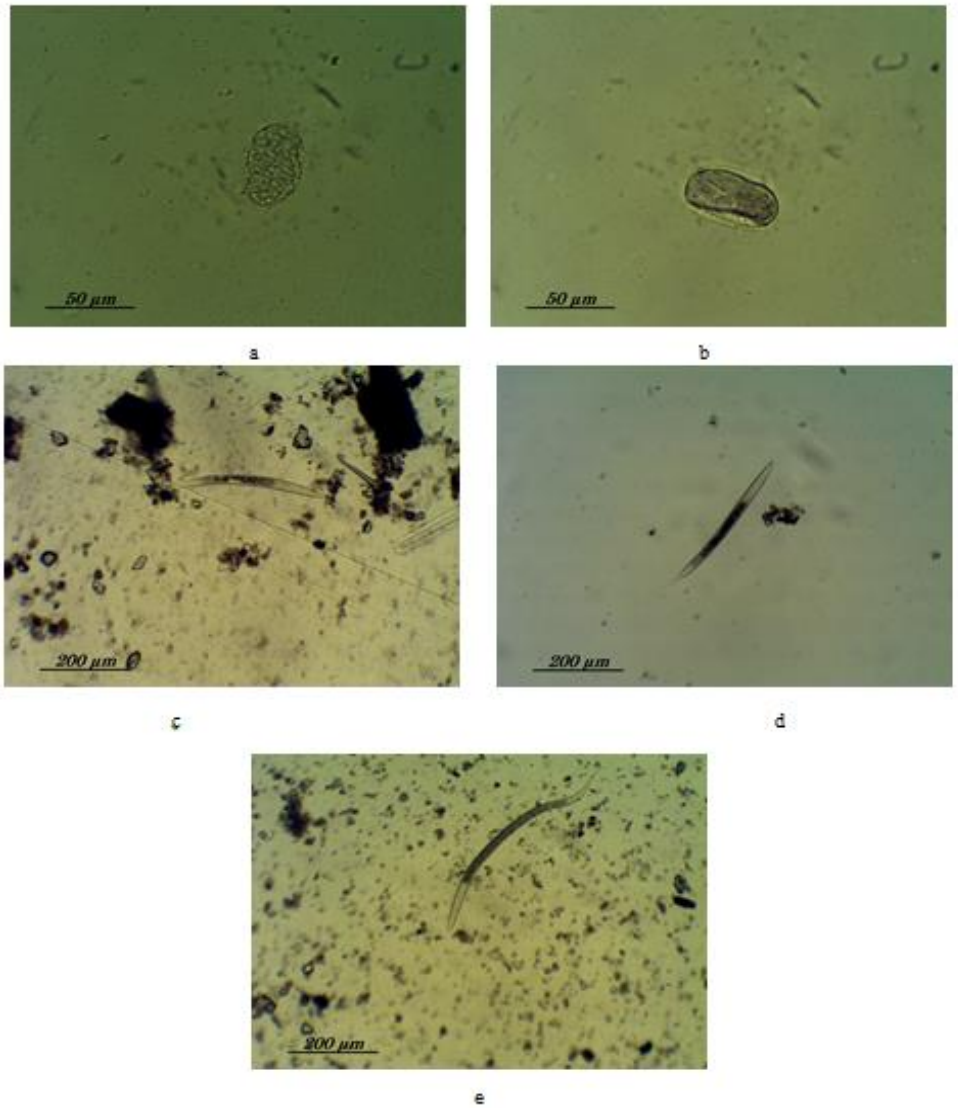

Fig. 1. Stages of embryonic and postembryonic development of nematodes *Trichostrongylus tenuis,* which parasitize domestic geese: a – blastomere cleavage; b – formation of larvae in the egg; c – L1; d – L2; e – L3

Table 1. The effect of temperature on the timing of exogenous development and survival of *Trichostrongylus tenuis* in laboratory culture, n=100, M±SD

Temperature (^{0}C)	Duration of development (days)	Rates of formation of L3 (%)
15	10	58.67±2.08
20	7	75.33±1.53
25	5	83.67±3.51

Table 2. Morphometric parameters of *Trichostrongylus tenuis* eggs during embryonic development, n=10, M±SD

Parameters (μm)	Temperature (^{0}C)	Stages of egg development	
		Blastomere cleavage	Formation of larva in egg
Len/gth	15	69.21±1.56	71.92±1.93**
	20	69.03±1.29	72.37±1.85***
	25	68.82±1.51	72.78±1.60***
Width	15	37.23±1.15	39.77±1.69***
	20	37.19±1.36	40.46±0.95***
	25	37.74±1.28	40.94±1.15***

Note: ** – p<0.01; *** – p<0.001 – compared to values for eggs at the stage of blastomere cleavage

Table 3. Morphometric parameters of *Trichostrongylus tenuis* larvae during postembryonic development, n=10, M±SD

Larval stages	Parameters, μm	Temperature (^{0}C)		
		15	20	25
L1	Length	367.55±17.44	372.46±13.44	385.19±15.66
	Width	23.65±0.57	23.99±0.48	24.39±0.68
L2	Length	471.84±6.87***	478.81±6.02***	483.04±5.74***
	Width	25.86±0.70***	26.15±0.71***	26.72±0.62***
L3	Length	501.70±2.89***	505.63±3.33***	508.65±4.90***
	Width	22.42±1.06**	22.10±1.32***	21.56±1.28***

Note: ** – p<0.01; *** – p<0.001 – compared to values for first stage larvae (L1)

DISCUSSION

According to scientific evidence, trichostrongyloidosis is a significantly common disease of birds, both wild and domestic. Its pathogens are nematodes of the species *Trichostrongylus tenuis*. The disease occurs in both waterfowl and terrestrial birds in many countries of the world (Wascher et al. 2012, Cervantes-Rivera et al. 2016, Denizhan and Karakuş 2019). The high prevalence of pathogen in various bird species is explained by the biological specifics of *T.tenuis* that enhance its adaptation to environmental factors, which affect the exogenous development of the parasite (Dobson and Hudson 1992, Connan and Wise 1993). Thus, further studies are needed to determine the timing of the development of these nematodes in the external environment and the influence of temperature on the rate of L3 formation and survival. This will allow to use the correct and more effective preventive and therapeutic anthelmintic measures in poultry farming.

We found that the temperature factor significantly affects the timing of exogenous development of *T.tenuis* nematodes, and is also one of the factors determining their survival. The most favorable temperature for the formation of the largest number of infectious larvae (up to 83.67±3.51%) under laboratory conditions is 25^{0}C. In these conditions, the exogenous development of *T. tenuis* occurs in five days. With decreasing temperature, the formation of

L3 slows down to seven days at 20^{0}C, or 10 days at 15^{0}C. At the same time, the survival of L3 decreases to 75.33±1.53% at 20^{0}C, or 58.67±2.08% at 15^{0}C. Such dependence of the development time and survival of *T.tenuis* on temperature both in the laboratory and in the environment has been noted before (Shaw et al. 1989). The authors indicate that eggs do not develop to L3 and die at low temperatures of winter, while mass formation of infectious larvae occurs in summer, at an average monthly soil surface temperature of 7.6 to 10.3^{0}C. At the same time, it has been observed that temperature indirectly affects the development of infectious larvae. A colder temperature (10^{0}C) provides better humidity conditions on the surface of the vegetation for migration of larvae (Saunders et al. 2000).

We also obtained new data on the morphometric parameters of *T.tenuis* during exogenous development. The embryonic development is characterized by an elongation (by 3.77–5.44%) and widening (by 6.39–8.08%) of eggs. In the process of postembryonic larval development, we noted an increase in L2 in length (by 20.26–22.21%) and width (by 8.26–8.72%), and further in L3, an increase in their length (by 24.27–26.74%) with a decrease in width (by 5.20–11.60%). Such changes, in our opinion, contribute to maximum survival in the external environment, and the possibilities of migration on plants for further entry into the host organism.

CONCLUSION

The timing of exogenous development and survival are determined in different temperature conditions for *Trichostrongylus tenuis* nematodes isolated from domestic geese. It is established that the optimal temperature for the formation of infectious larvae is 25^{0}C, at which their formation occurs in five days, and survival reaches 83.67%. The growth and development of exogenous parasitic stages is accompanied by a change in their morphometric characteristics. That is an adaptive mechanism to environmental conditions, which contributes to the widespread occurrence of trichostrongyloidosis in birds.

Conflict of Interest: The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Estimating Roughage Quality with Near Infrared Reflectance (NIR) Spectroscopy and Chemometric Techniques

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ABSTRACT

Near-Infrared Spectroscopy has been commonly adopted in feed quality evaluations. The majority of studies have been performed on the detection of fibrous components with the help of NIR. Model development studies are not yet common for different roughage quality characteristics such as Relative Forage Value (RFV), Relative Forage Quality (RFQ), and Net Energy Lactation (NEL), which are used in the evaluation of roughage quality. The purpose of this study is the detection of RFV, RFQ, and NEL values with NIR spectroscopy in different roughage samples, and to investigate the effect of wavelength selection on the model's success. In this study, spectral data belonging to silage, dry alfalfa, dry oat, and wheat straw samples and laboratory analysis results were used to develop estimation models according to the partial least square regression (PLSR) method. A variable importance projection (VIP) method was used as the wavelength selection method. Estimation models, which were developed according to study results, were obtained from the VIP-PLS model combination (RMSE=12.7, Bias=0.000, R2=0.804, RPD=2.28) for RFV. VIP method has increased the estimation of success for all variables. Based on the study results, it was recognized that it is possible to use NIR in the calculations used in roughages quality evaluation parameters.
Keywords: Near-İnfrared Reflectance (NIR), Roughage quality, Wavelength Selection, Chemometrics

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Kaba Yem Kalitesinin Near Infrared Reflectance (NIR) Spektroskopisi ve Kemometrik Teknikler ile Tahmin Edilmesi

ÖZ

Yakın Kızılötesi Spektroskopisi yem kalite değerlendirmelerinde yaygın olarak kullanılmaya başlanmıştır. Yapılan çalışmaların büyük kısmı lifli bileşenlerin NIR ile tespitine yönelik olarak yürütülmüştür. Kaba yem kalitesinin değerlendirilmesinde kullanılan Nisbi yem değeri (Relative Forage Value, RFV), Nisbi yem Kalitesi (Relative Forage Quality, RFQ) ve Net Enerji Laktasyon (Net Energy Lactation, NEL) gibi farklı kalite özelliklerine yönelik ise model geliştirme çalışmaları henüz yaygınlaşmamıştır. Bu çalışmada farklı kaba yem örneklerinde NFV, RFQ ve NEL değerlerinin NIR spektroskopisi ile tespiti ve dalgaboyu seçiminin model başarısı üzerine etkisinin araştırılması amaçlanmıştır. Araştırmada silaj, yonca kuru otu, yulaf kuru otu ve buğday samanı örneklerinde alınan spektral veriler ve laboratuvar analiz sonuçları kullanılarak kısmı en küçük kareler regresyon (PLSR) yöntemine göre tahmin modelleri geliştirilmiştir. Dalga boyu seçim yöntemi olarak variable importance projection (VIP) metodundan yararlanılmıştır. Araştırma bulgularına göre geliştirilen tahmin modellerinin RFV için VIP-PLS model kombinasyonundan (RMSE=12.7, Bias=0,000, R2=0,804, RPD=2,28) elde edilmiştir. VIP yöntemi bütün değişkenler için tahmin başarısını yükseltmiştir. Araştırma bulgularına dayanarak, kaba yem kalite değerlendirme parametrelerinde kullanılan hesaplamaların NIR ile tespitinin mümkün olduğu anlaşılmıştır.
Anahtar Kelimeler: Yakın Kızılötesi Yansıma, Kaba yem kalitesi, Dalga boyu seçimi, kemometri

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INTRODUCTION

Near-Infrared Reflectance (NIR) spectroscopy is one of the analytical devices that has widespread use in the content analysis of different agricultural products, and it has been commonly used in feed analyses (Sinnaeve et al. 1994). In feed quality analyses, characteristics such as moisture, protein content, and digestibility has been the main center of focus. As an alternative to reference analyses that are used to evaluate feed quality, NIR spectroscopy allows us to analyze multiple variables simultaneously without using chemicals and in a shorter time (Undersander 2006).

Measuring feed quality with NIR spectroscopy has originatedat at the end of the 1970s and the first years of the 1980s. In this period, various studies have been performed, which showed it was possible to use NIR spectroscopy devices for the analysis of protein and fibrous materials in feed samples (Norris et al. 1976, Shenk et al. 1981, Marten et al. 1983, Jones et al. 1976). These studies are still ongoing in our day, and it is investigated whether it is possible to use NIR to measure feed quality in different plant types with newly developed devices. In this scope, estimation models have been developed to ensure the measurement of parameters of feed quality such as ADF, NDF, CP and crude fat in soybean (Asekova et al. 2016), Italian ryegrass (Yang et al. 2017) , corn (Gümüştaş and Bayram 2018) and wheat straw (Nielsen et al. 2019) with the help of NIR. These parameters are determined with wet chemical methods recognized by international standard organizations, history of which depends on the detergent system of analysis recommended by Von Soest (1991) and Wendee's system of analysis (Ergün et al. 2004). Instead of wet chemical methods used in the determination of feed quality parameters, NIR determination methods have been adopted, and these methods have been accepted by ISO (ISO 12099). The number of NIR spectroscopy studies about novel statistical methods has been increasing on chemometric approaches covering efficient wavelength selection methods and pretreatment on spectral data.

Chemometric methods are used mainly in three stages in the development of calibration models with NIR devices. These stages consist of the pretreatment for the removal of noise and unwanted changes in spectral data, the selection of wavelengths related to the target variable, and the formation and validation of the estimation model (Kahrıman and Egesel 2018). While each of these stages has a separate importance on the model's success, the use of efficient wavelength selection methods to enable the determination of variables that should be used in the model can noteworthily increase the model's success. Different wavelength selection methods have been used on scientific literature in studies about spectral analyses. Some of those are Variable Importance Projections (VIP), Selectivity Ratio (SR), Competitive Adaptive Resampling Method (SPA), and Random Frog (RFOG) methods (Wold et al. 1993, Galvão et al. 2008, Li et al. 2014). There is a high number of studies reporting that the success of the established model increases by applying wavelength selection methods on data obtained from NIR devices (Cécillon et al. 2009, Kahrıman et al. 2017).

In the literature review, it was observed that mostly general parameters such as fiber analyses (ADF, NDF) or total protein content are used as target variables in the studies that use NIR on the determination of feed quality. From this point forth, it was considered that it would be beneficial to investigate whether it is possible to estimate RFV, RFQ, and NEL parameters directly with NIR and to study how wavelength selection will affect the success of the established model.

The purpose of this study is to determine whether it is possible to determine RFV, RFQ, and NEL values with NIR in different roughage samples, and to study the effect of wavelength selection on the estimation success of the models established for these parameters.

MATERIALS and METHODS

Samples

The samples of roughage used (n=52) in the study (Corn silage, Alfalfa, Oat grass hay, Wheat straw) were obtained fresh from farms in 0.5 kg weight. They were transported to the laboratory after being placed in airtight bags, and stored at -20 °C until analyses were performed. Dried feed samples were milled (Retsch ZM 200 ultra centrifugal mill, on 1 mm screen) before reference and spectral analyses.

Reference (REF, Chemical Analyses) Analyses

Reference (REF) (chemical) analyses of roughage samples from the study were performed in Balıkesir University Faculty of Veterinary Animal Nutrition and Nutritional Diseases Laboratory by using dry matter (method 934.01), ash (method 942.05), crude protein (method 990.03), crude oil (method 920.39) AOAC (1997) methods in compliance with the reported method, and reference analysis values (nutrient values) were determined. NDF and ADF analyses of feed samples were performed according to the methods reported by Van Soest et al. (1991) by using automated Gerhard FT12 Fiber Analyzer (Gerhardt Analytical Systems Documents, 2012). RFV, RFQ, and NEL levels were calculated according to equations reported in NRC (2001) by using nutrient values obtained from the chemical analyses of roughages samples.

Development of NIR Models and Evaluation

Spectral measurements were performed on a desktop NIR device (Spectrastar 2400D, Unity Scientific, USA) with the samples after completing laboratory

analyses. Rotational measurement cup mode was used in measurements, and spectral data were recorded at each 1 nm between 1200-2400 nm with approximately 50 g of ground sample. For model development, sample files were compiled in jdx format by using the InfoStar program.

Spectral data were imported to an Excel file with Unscrambler 10 x (Camo, Oslo, Norway). Afterwards, these files were transferred into the program named R (R Development Team, 2018), and modeling studies were performed by using the mdatools package (Kucheryavskiy, 2020). First of all, spectra were converted to line charts in order to observe the change in spectral data (Figure 1). Partial least square regression (PLSR) method was used as the modelling method, and two separate models were established with spectral data for each variable. In the first model, all spectral data was used to develop the estimation models. In the second model, efficient wavelengths were selected according to the VIP method (Wold et al. 1993), and estimation models were developed by using these wavelengths. Estimation success and reliability of established models were evaluated according to the following parameters.

$$RMSEP = \sqrt{\frac{\sum (Ypred - Yref)^2}{n}}$$

$$SEE = \sqrt{\frac{n}{n-1}(RMSEP^2 - Bias^2)}$$

$$RPD = \frac{STD_{ref}}{SE_{pred}}$$

Where; RMSEP: the square of the mean value of estimated squares; Ypred: the calculated value; Yref: the value obtained by standard analyses; n: the number of observations; Bias: the mean difference between estimated and standardized analyses; SEE: the standard error of the predicted values in the calibration set; STDref: the standard deviation of reference analyses, and SEpred, the standard error of prediction values. Moreover, the coefficient of determination was calculated for the models. These calculations were performed separately for the calibration and validation set, and the internal validation method was used as the validation method.

Determination of Relative Feed Value (RFV), Relative Forage Quality (RFQ) and Net Energy Lactation (NEL) Values

The estimated values of dependent variables in prediction models were calculated by the equations in Table 1. Proposed equations were obtained from previous studies (Samiei et al. 2015, Romero et al. 2014; Jaranyama and Garcia 2004, Van Dyke and Anderson 2000).

Table 1. Calculation formulas for RFV, RFQ, and NEL values and the related equation components

Equation for Target Varibale	Equaiton Components
RFV = DDM x DMI x 0.775	$DMI = \frac{120}{NDF\%}$ $DDM = 88.9 - (0.779xADF\%)$
$\boldsymbol{RFQ} = \frac{(DMIxTDN)}{1.23}$	$DMI = \left(\frac{120}{NDF\%}\right) + (NDFD - 45)x\left(\frac{0.374}{1350}\right)x100$ $TDN = (NFC \times 0.98) + (CP \times 0.93) + (FA \times 0.97 \times 2.25) + (NDFn \times (NDFD/100)) - 7$ $NFC = 100 - (NDFn + CP + EE + ash)$ NDF = else estimated as NDFn= NDF ^{x} 0.93 FA= fatty acid (% of DM)= ether extract – 1 NDFD = 45 is an average value for fiber digestibility of alfalfa and alfalfa/grass mixtures
$\mathbf{NE_L} = [0.866 - (0.0077xADF)] * 2.2$	

DDM = Digestible Dry Matter, DMI = Dry matter intake (% of Body Weight), DM=Dry Matter, TDN = Total Digestible Nutrients (% of DM), NDF= Neutral Detergent Fiber (% of DM), ADF=Acid Detergent Fiber (% of DM), NDFD = 48-hour in vitro NDF digestibility (% of NDF), CP = Crude Protein (% of DM), NDFn = Nitrogen Free, NFC = Non Fibrous Carbohydrate (% of DM), NE_L= Net Energy Lactation.

RESULTS

Descriptive statistics of the laboratory analysis results are presented in Table 2 for the samples used in the study. A noteworthy variation was also observed in the raw spectral data obtained from the samples (Figure 1). Depending on those, it can be stated that both the variation in spectral data and the variation in target variables were suitable for estimation model development in the established models.

The evaluation parameters are presented in Table 3 for the estimation models established in the study. While 99.9% of the variation in spectral data can be explained by models for all feed quality parameters, 34.20% to 80.43% of the variation independent variables could be explained in established models (Table 3). Root-mean-square error (RMSE) was found to be at an acceptable level for the measured feed quality evaluations. While slope values of the estimations varied between 0.60 and 0.80, estimations without any deviation could be provided in calibration models (Bias=0.00), and in contrast, various levels of deviations were observed in internal validation sets although it changed according to models. RPD values belonging to calibration and validation sets were determined to be between 1.25 and 2.22 (Table 3). The studies on spectral model development reported that models with RPD values above 2 could be used for scans. It was observed that the wavelength selection method (VIP) used in the study increased the estimation success in calibration sets for NEL and RFV parameters, and reduced it for RFQ parameter (Table 3). These data are being confirmed with the relation between reference and NIR estimations of the models developed by using all spectral data (Figure 2 a, b, c) and the results of the models that used wavelengths selected with VIP (Figure 2 d, e, f). There are various studies on literature that reported a positive correlation between RFV and RFQ (Undersander and Moore 2004). Accordingly, a positive connection may be expected in the NIR estimations of RFV and RFQ values. It was observed in the study that the use of the VIP method did not meet this expectation for the external validation set, and it was understood that wavelength selection might lead to deviations in NIR estimations for the RFQ parameter.

On the other hand, the fact that there were different main components in RFQ and RFV calculation formulas may have an important effect on NIR estimations. While DMI and TDN are main estimators in RFQ calculations, ADF and NDF are effective in RFV calculation. It is considered that the wavelength selection method poses a positive effect on the selection of areas related to ADF and NDF, however, it leads to a number of deviations in the detection of wavelengths related to DMI and TDN.

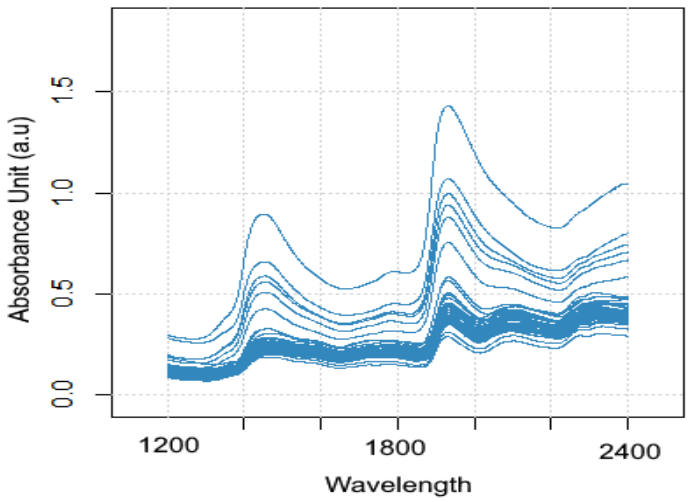

Figure 1. The plot of spectral data obtained from roughage samples.

Table 2. Descriptive statistics for RFV, RFQ, and NE_L calculations in the sample set used

	n	Mean	Minimum	Maximum	Standard Deviation
RFV	53	91.4	47.9	158.0	29.0
RFQ	53	86.1	48.3	160.9	27.0
NE_L	53	1.23	0.90	1.56	0.19

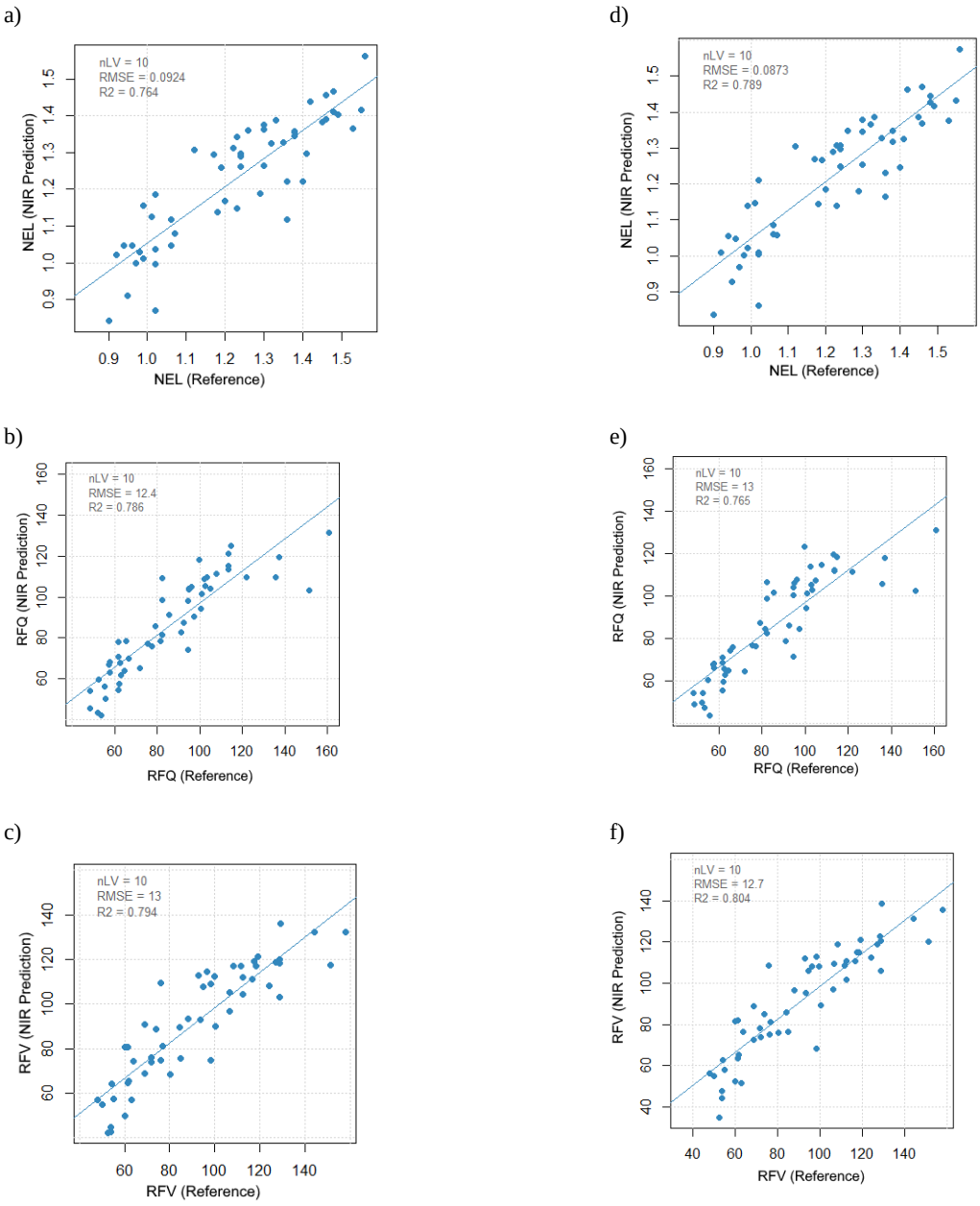

Figure 2. The relation between reference and NIR estimations in regression models established with the entire spectral region (a, b, c) and selected wavelengths (d, e, f).

Table 3. Evaluation parameters for PLS models established with all spectral data and wavelengths selected with the VIP method

		X cumexpvar	Y cumexpvar	RMSE	Slope	Bias	RPD
RFV	PLS-Cal	99.99	79.36	13.043	0.79	0.0000	2.22
	PLS-Val	99.97	45.40	21.080	0.66	1.0494	1.38
	VIP-Cal	99.99	80.43	12.700	0.80	0.0000	2.28
	VIP-Val	99.98	36.17	22.859	0.70	0.6319	1.27
RFQ	PLS-Cal	99.9	78.61	12.371	0.79	0.0000	2.18
	PLS-Val	99.97	35.10	20.883	0.61	0.7463	1.29
	VIP-Cal	99.99	76.48	12.973	0.76	0.0000	2.08
	VIP-Val	99.99	34.86	21.056	0.63	0.5881	1.28
NE_L	PLS-Cal	99.99	76.35	0.092	0.76	0.0000	2.08
	PLS-Val	99.98	34.20	0.154	0.62	0.0062	1.25
	VIP-Cal	99.99	78.90	0.087	0.79	0.0000	2.20
	VIP-Val	99.98	39.88	0.147	0.67	0.0014	1.31

DISCUSSION

It was determined that there was a sufficient variation for RFV, RFQ, and NEL values in the sample set that was used as a dependent variable in the modelling study (Table 2). The variation determined in target variables overlaps with the limit values reported in previous studies for the calculation of these characteristics with roughage samples (Undersander and Moore 2004, Güney et al. 2016).

The results of the study are similar to the results obtained from other studies on feed quality evaluations in roughage samples. Nevertheless, there is a prominent change in calibration success according to the sample type used in scientific literature. It was observed in the study of Doğusoylu and Bayram (2019) that estimation success was low in the calibration model they established for determining NDF content in corn kernel, and it was determined that R-value was 0.3353, R2 was 0.1124, and the standard deviation was 3.4691 in the calibration set. In another study, NDF, ADF, crude ash, crude fat, and crude protein values were compared in locust bean with chemical and spectroscopic (NIRS) methods, and it was stated that different results had been obtained ($P< 0.05$, $P<0.01$) (Pehlevan and Özdoğan 2015). Asekova et al. (2016) found R2 values above 80% and RPD values above 2 for the calibration models established for crude protein, crude fat, and NDF content in soybean, and they have calculated R2 value as 78.9% and RPD value as 1.79 for ADF content. While RFV, RFQ, and NEL parameters were not addressed in these studies, it is observed that there are prominent changes due to sample type in the analysis results belonging to the characteristics used in the calculation of these parameters. In our study, roughages of different plant types have been used as the material. Among these materials, silage samples have relatively different characteristics compared to other roughage samples and were separated from other samples in spectral data (Figure 1). This may have caused deviations in the NIR estimations of RFV, RFQ, and NEL parameters. In the study performed by Rushing et al. (2016), apart from important feed characteristics, the estimation success for RFV value ($n = 55$, $R2 = 0.916$, $RSCD = 3.45$, $RSCIQ = 3.04$) was found to be higher than the model established in our study for the determination of RFV value with NIR. This may have caused by the fact that samples of only one plant type (Elymus glabriflorus) were used in that study. These results suggest that calibration models should be established separately for samples belonging to different roughage types. The number of samples in this study did not allow this to be performed.

CONCLUSION

In this study, it was investigated whether it was possible to determine the indirectly calculated RFV, RFQ and NEL parameters with NIR spectroscopy in roughage feed quality measurements, and the effect of wavelength selection on the model's success was examined. According to study results, it was recognized that RFV, RFQ, and NEL parameters could be determined with NIR at an acceptable accuracy. It was observed that the wavelength selection that was used (VIP) affected estimation success positively, and it will be useful for estimation models to only use the wavelengths related to the target variable in the scanned spectral area as the indicator variable. Since the number of samples used in this study is limited, the samples that negatively affected the model success could not be eliminated. It is estimated that increasing the number of samples in the calibration set and using different chemometric methods in future studies may allow determining RFV, RFQ, and NEL values more accurately in roughage samples.

Conflict of Interest: The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Morphologic and Molecular Detection of *Dactylogyrus* Species in *Cyprinus Carpio* and *Ctenopharyngodon Idella* in Southwestern Iran

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ABSTRACT

Monogeneans gill parasites of the diversified *Dactylogyrus* genus are host-specific to freshwater fish of the Cyprinidae family. Accurate detection of various *Dactylogyrus* species is very time-consuming and requires highly experienced personnel; to overcome this problem, the genomic DNA technology has been recently used to detect *Dactylogyrus* spp. In this study, 23 *Cyprinus carpio* and 19 *Ctenopharyngodon idella* isolates were collected from different farms and local markets of Ahvaz, southwest of Iran. The gill arches of each fish were carefully examined under a light microscope. *Dactylogyrus* species was identified morphologically. Identical species were kept in ethanol for molecular study. Molecular analysis was performed using amplification of the ITS-1 region of the ribosomal RNA gene of the parasite. Then, the amplified PCR products were sequenced. The aligned nucleotide sequences were analyzed to construct the phylogenetic tree of the identified species. The results revealed two lineages including *D. extensus* isolated from common carp and *D. lamellatus* taken from grass carp fish. Phylogenetic analysis showed that the detected *D. extensus* and *D. lamellatus* isolates from Khuzestan region were clustered with some Iranian (Guilan), Chinese and Czech isolates. Importing fingerlings from Guilan Province could lead to introducing monogenean infections to native cyprinid fish.

Keywords: *Dactylogyrus*, Common Carp, Grass Carp, Fish, PCR.

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İran'ın Güneybatısında Yer Alan *Cyprinus Carpio* ve *Ctenopharyngodon İdella*'daki *Dactylogyrus* Türlerinin Morfolojik ve Moleküler Tespiti

ÖZ

Çeşitlendirilmiş Dactylogyrus cinsinin solungaç monojenleri, Cyprinidae ailesinin tatlı su balıklarına ev sahipliğine özgüdür. Çeşitli Dactylogyrus türlerinin doğru tespiti çok zaman alıcı olduğundan ve çok deneyimli personel gerektirdiğinden, genomik DNA teknolojisi son zamanlarda Dactylogyrus spp. Bu çalışmada, Güneybatı İran'ın Ahvaz'ın farklı çiftliklerinden ve yerel pazarlarından 23 Cyprinus carpio ve 19 Ctenopharyngodon idella toplandı. Her balığın solungaç kemerleri, ışık mikroskobu altında dikkatle incelenir. Dactylogyrus türlerinin morfolojik olarak tanımlanması morfolojik olarak belirlenmiştir. benzer türler moleküler çalışma için etanol içinde tutuldu. Moleküler analiz, parazitin ribozomal RNA geninin ITS-1 bölgesinin amplifikasyonu kullanılarak yapıldı. Daha sonra amplifiye PCR ürünleri dizildi. Hizalanmış nükleotit sekansları, tanımlanan türlerin filogenetik ağacını yürütmek için analiz edilir. Sonuçlar, ortak sazandan izole edilen D. extensus ve çim sazan balıklarından alınan D. lamellatus da dahil olmak üzere iki soyun ortaya çıktığını göstermiştir. Filogenetik analiz, Khuzestan bölgesinden tespit edilen D. extensus ve D. lamellatus izolatlarının bazı İran (Guilan), Çin ve Çek Cumhuriyeti izolatlarıyla kümelendiğini gösterdi. Guilan Eyaletinden balıkların ithal edilmesi, yerli Cyprinid balıklarına monojenik enfeksiyonların ortaya çıkmasına neden olabilir.

Anahtar Kelimeler: Sazan, Çim Sazan, Balık, PCR.

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INTRODUCTION

Various species of cyprinid fish have been imported from the Far East (China), Russia and Eastern European (Romania and Hungary) countries into the fish culture industry of Iran over the past 60 years. The growth rate of this sort of freshwater fish in aquaculture has been on the rise due to the increasing interest of Iranian consumers and the economic and nutritional values of this type of fish (Zolfinejad et al. 2017, Shamsi et al. 2009). When transferring fish to farm ponds and natural habitats, different pathogenic parasites are introduced to native species.

The cyprinid fish naturally harbor various species of monogenean gill parasites. *Dactylogyrus* Diesing 1850 (Monogenea*)*, is the most common gill parasitic worm of freshwater fish (Woo 2006). This ectoparasite is known to be highly cyprinid fish-specific (Simkova et al. 2007). The host specificity of this species is very high such that each *Dactylogyrus* species attaches to a distinct niche of the gill arch. Sometimes, this specificity of the host and parasite species is such that they approach phylogenetically close to each other (Simkova et al. 2007, Kadlec et al. 2003). According to the studies by Shamsi et al. (2009) and Daghigh Roohi et al. (2019), each grass carp can carry at least two species of *Dactylogyrus* on its body surface. Severe parasitism of the gill filaments leads to epithelial hyperplasia followed by serious respiratory failure and negative effects on fish growth. It can also cause a high rate of mortality in small fish (Lu et al. 2012, Tu et al. 2015, Thoney and Hargis 1991). The infected fish are more susceptible to secondary infestation, which increases the intensity of pathogenicity of the *Dactylogyrus* species, and eventually, the economic loss (Woo et al. 2002, Tu et al. 2015). *Dactylogyrus* direct life cycle accelerates the rate of parasitic infection distribution, and it can cause disease transmission to reach its epizootic level (Thoney and Hargis 1991). Therefore, early detection of the causative microorganism plays a significant role in controlling and preventing fish infections and economic losses. Also, the taxonomic study of parasites in a specific geographical region helps to find common lineal species, and it informs fisheries organizations of the provinces or countries of where this parasitic infection originates from.

Morphological identification of *Dactylogyrus* species directly depends on the accurate observation of copulatory components with different shapes and sizes in various species. Since *Dactylogyrus* is a very small (usually less than 1 mm) parasite, it is difficult to differentiate various species, especially when flatted marginal hooks occur in viscous gill media during the mounting process (Sharma et al. 2011). Also, the exact identification of closely resembling species of *Dactylogyrus* based on morphological characteristics is extremely time-consuming and requires highly experienced personnel. The genomic DNA technology has recently been used for the accurate detection of various microorganism species such as *Dactylogyrus*.

Previous molecular studies have shown that the ribosomal DNA (rDNA) of Platyhelminthes is a highly conserved region and can be used as a suitable marker for the identification and evolution study of different species (Olson et al. 2003). Most molecular studies have been carried out on the 28S rDNA region in cyprinid fish in Iran (Ahmadi et al. 2017, Mozhdeganlou et al. 2011, Daghigh Roohi et al. 2019), while there are limited studies on the internal transcribed spacer 1 (ITS-1) region of rDNA. In the present study, we evaluated the genomic sequence of the internal transcribed spacer 1 (ITS-1) region to detect closely-related *Dactylogyrus* species in Khuzestan Province, Iran.

MATERIAL and METHODS

Parasite collection

A total of 42 freshwater cultured common and grass carp, including 23 *C. carpio* and 19 *C. idella*, were acquired as dead but fresh from different aquacultures and local markets of Ahvaz, southwest of Iran. After transferring the fish to the parasitology laboratory of the Faculty of Veterinary Medicine of Shahid Chamran University of Ahvaz, the gill arches of each fish were placed on a plate and examined under stereomicroscope. Then, the identified *Dactylogyrus* species were separated and collected by the routine methods. Fish sampling continued until 10 *Dactylogyrus*-infected fish from each species were collected (of the 42 fish, 20 infected fish were found and investigated).

Identification of each parasite was performed based on a survey of the structures of sclerotic attachment and reproductive organs according to Gusev (Gusev 1985). The parasites were observed on clear slides in glycerin using a light microscope equipped with digital image analysis (Carl Zeiss Axiovision LE 4.5). Some similar species were placed in 70% ethanol before DNA extraction.

Molecular studies

DNA extraction and PCR amplification

Each ethanolic specimen was washed in distilled water to remove alcohol from its tissues. The genomic DNA was extracted using the CinnaPure ™DNA kit (CinnaGen Co., Iran) according to the manufacturer's instructions. The ITS-1 region of ribosomal RNA genes was amplified with forward (5`-CTGCGGAAGGATCATTATC-3`) and reverse (5`-GATCCACCACTTGCAGTTGT-3`) primers (Mozhdeganlou et al. 2011). Polymerase chain reaction (PCR) was carried out in a thermocycler (A&E, England) for 38 cycles as follows: 5 min at

94°C (initial denaturation) followed by 45 s at 94°C for further denaturation, annealing for 45 s at 56°C, 45 s at 72°C and a final extension of 72°C for 10 min. The PCR products were investigated on 1.5% agarose-Tris-acetate-EDTA (TAE) gel, visualized with safe stain under a UV illuminator. Distilled water was used instead of genomic DNA of PCR as negative control.

DNA sequencing

For morphologic confirmation, the amplified PCR products were purified and sequenced at Bioneer-Korea Co. using both sets of primers. After reading the sequences, the data were analyzed using BioEdit V. 7.0.5.3 software and the Basic Local Alignment Search Tool (BLAST) programs and databases of the NCBI (National Center for Biotechnology Information, BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignment sequences were analyzed by the Molecular Evolutionary Genetics Analysis (MEGA) software, version 10, using default parameters to construct the phylogenetic tree of the isolated species.

Phylogenetic analysis

The evolutionary history was inferred by using the maximum likelihood estimation method and Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood (-2522.07) has been evaluated. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then the topology with superior log likelihood value was selected. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 1.6401]). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.00% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 23 nucleotide sequences. There were 227 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

RESULTS

Twenty *Dactylogyrus* parasites were isolated from 42 collected fish (19 grass carp and 23 common carp). Microscopic identification of the species revealed that two species of *Dactylogyrus* including *D. extensus* (Figure 1) and *D. lamellatus* (Figure 2) were isolated from the gills of common and grass carp, respectively. The PCR products with the bond lengths of 515 and 618 base pairs were visible on the gel-electrophoresis in *C. carpio* and *C. idella* samples, respectively (Figure 3).

The nucleotide sequence of the PCR products was aligned and compared with other registered *Dactylogyrus* species in the GenBank. According to the phylogenetic analysis, the detected isolates belonged to two distinct clusters. The Khuzestan isolate of *D. extensus* was 95% homologous with the Chinese (KM277459.1) and Czech (AJ564129) isolates of this species. The Khuzestan isolate of *D. lamellatus* was also 99% homologous with the Chinese (KX369221 and KX369218), Czech (AJ564141.1) and Guilan, Iran (MG907490.1) isolates of this species in the GenBank. The results of phylogenetic analysis on the ITS-1 region of rDNA showed that *D. extensus* (Khuzestan isolate) was grouped together with the same species isolates collected from China and Czech Republic, along with the closely related sister species, *D. minutus* isolate from Guilan, north of Iran. Also, *D. lamellatus* isolated from Khuzestan Province was clustered with the Iranian isolate (Guilan Province, north of Iran) and the same species isolates collected from China and Czech Republic with high bootstrap (99%) (Figure 4).

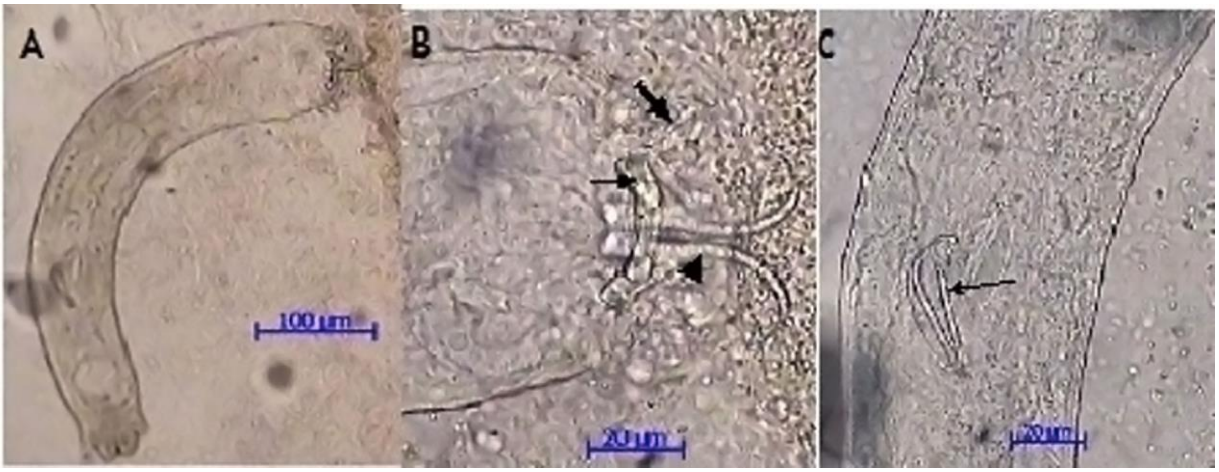

Figure 1. Photomicrograph of *Dactylogyrus extensus*: A: Adult whole mount worm, B: Haptor of *D. extensus*; Anchors (arrow head), Dorsal bar (narrow arrow), marginal hook (thick arrow), C: male copulatory organ (arrow).

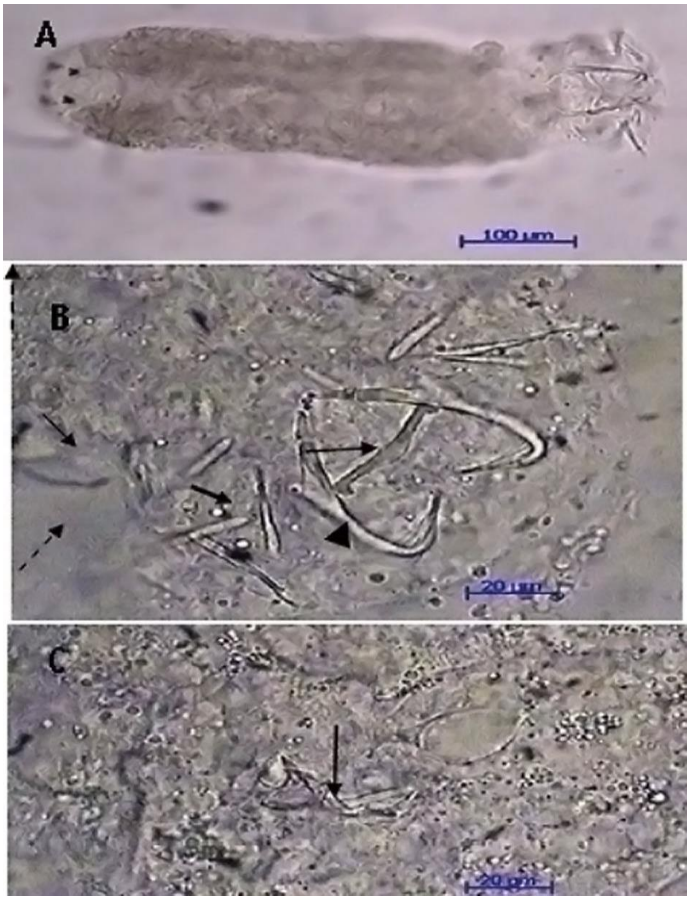

Figure 2. Photomicrograph of *Dactylogyrus lamellatus*: A: Adult whole mount worm, B: Haptor of *D. lamellatus*; Anchors (arrow head), Dorsal bar (narrow arrow), marginal hook (thick arrow), C: male copulatory organ (arrow).

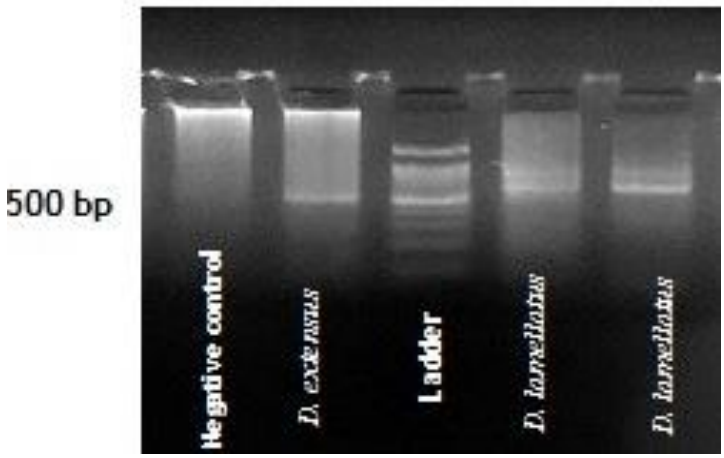

Figure 3. Agarose gel electrophoresis of PCR amplification products from *D. extensus* and *D. lamellatus* under UV light.

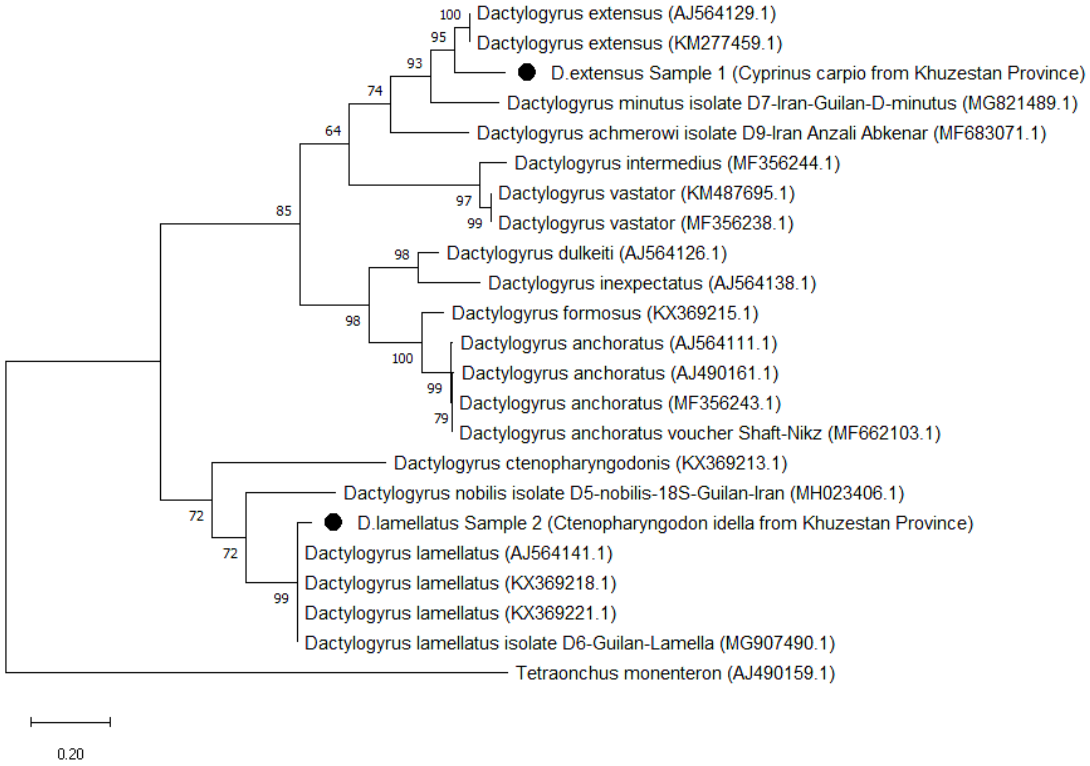

Figure 4. Phylogenetic tree based on the partial sequence of 18S, ITS-1 region and 5.8S rDNA, constructed according to the maximum likelihood method. Black dots represent the Iranian *Dactylogyrus* isolates (Khuzestan province). Numerals above the branches indicate bootstrap values (%) from 1000 replicates. The scale bar indicates the proportion of sites changing along each branch.

DISCUSSION

The routine identification of *Dactylogyrus* species was performed based on morphological characteristics during the process of appropriate preservation, fixation and staining of genital organs of the parasite (Strona et al. 2009). For this purpose, highly experienced personnel and powerful microscopic instruments are often required due to the minute size of parasites. Thus, the identification of *Dactylogyrus* species is difficult and time-consuming. Today, molecular techniques using genomic DNA of parasites are used as a more accurate and reliable method, which has significantly aided with the detection of these parasites. In the freshwater fish of Iran, more than 70 species of *Dactylogyrus* have been identified (Mozhdeganlou et al. 2011), which are mainly specific to cyprinid fish. These parasites have been introduced to the Iranian aquaculture and farming systems during the import of the cyprinid fish from the Far East (China), Russia and Eastern Europe (Romania and Hungary) over the past 40 years (Shamsi et al. 2009).

Shamsi et al. (2009) studied the geographical distribution of *Dactylogyrus* species in cyprinid fish of different fauna of Iran. They could isolate the species of *D. anchoratus, D. extensus* and *D. vastator* from *Barbus sharpeyi*, *Carassius auratus gibelio* and *C. carpio*, respectively, in Khuzestan region (Mesopotamian fauna). Therefore, the present study is the first report of the presence of *D. extensus* in common carp and *D. lamellatus* in grass carp fish of Khuzestan Province, Southwestern Iran. Borji et al. (2012) detected the species of *D. extensus* and *D. anchoratus* in *C. carpio* (Borji et al. 2012) under a light microscope from the ponds around Mashhad, Northeastern Iran. Also, in another study in Northeastern Iran, the species of *D. anchoratus* from *C. carpio* and *C. idella, D. extensus* from *C. carpio* and *D. lamellatus* from *C. idella* were detected using molecular analysis (Ahmadi et al. 2017). The identified species in Northeastern Iran were similar to Khuzestan species, but since the molecular study was performed on the 28S rDNA region, it has not been considered for comparison with Khuzestan isolates in the GenBank. In the north of Iran (Guilan Province), the *D. lamellatus* species was reported in 95% of the farmed grass carp using molecular methods (Daghigh Roohi et al. 2019). The molecular study of *Dactylogyrus* species in Guilan Province was the only study from Iran on the ITS-1 region of rDNA. As the freshwater fish of farming systems and local markets in Khuzestan Province are provided by internal and external imports from Guilan Province and other

countries, monogenean infections are also introduced to native cyprinid fish of the southwest of Iran.

Molnar found that the evolution and maturation time of the *D. lamellatus* parasites increase with rising water temperature (Molnar 1971). Khuzestan Province is located in southwest of Iran at 29°57'-33°0'N and 47°38'-50°32'E and this area has a hot desert climate. In the present study, the maximum and minimum temperatures of the Province during the fish sampling period (December 2017 to March 2018) varied from 13 to 31°C on different days of months. Therefore, to provide an appropriate habitat for *D. lamellatus* in the intensive farming system, the proliferation of the parasite and the rate of fish involvement should increase. Among *Dactylogyrus* species, *D. extensus* has the lowest host specificity and broadest host range. Thus, the parasite can be easily transmitted from other species of fish to common carp (Gibson et al. 1996). Also, *D. extensus* has a high tolerance to a wide range of temperatures (from 1 to 30°C) and salinity (up to 1.3%) of water throughout the year (Jalali 1998, Mahdipour et al. 2004, Jalali and Barzegar 2005). Therefore, the incidence of *D. extensus* infection in these favorable environmental conditions for the parasite habitat is acceptable.

The phylogenetic analysis showed that the *D. extensus* and *D. lamellatus* isolates detected from Khuzestan region had a close affinity with some Iranian (Guilan), Chinese and Czech isolates. Since Khuzestan fish farmers usually provide the need of their cyprinid fish from East (China), Russia and Eastern Europe (i.e., Romania and Hungary) as fry or fingerlings, the *Dactylogyrus* species can be easily introduced to Iranian water resources and native fish of southwest of Iran. The biodiversity of the detected *Dactylogyrus* species might be caused by the host specific features and high diversity of these monogenean parasites in various geographical and environmental conditions.

CONCLUSIONS

This was the first study to demonstrate the occurrence of *D. extensus* and *D. lamellatus* in common and grass carp from Khuzestan Province, Southwestern Iran. Also, we found that PCR is a reliable and feasible diagnostic tool for the detection of *Dactylogyrus* species, and it can be applied for large-scale assessments in screening programs of customs and fisheries organization to ensure the health of imported aquatic species. Further molecular investigations are needed for the diagnosis and prevention of *Dactylogyrus* infections in other freshwater fish.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Evaluation of ECG and EMG Findings in Dogs Undergoing Abdominal Ultrasonography

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ABSTRACT

In this study, it was investigated that how the abdominal ultrasonography (USG) affect the ECG (for heart rate) and EMG data and whether it would be possible to apply both ECG and EMG simultaneously while applying USG. ECG and EMG data were recorded at least 15 seconds before and after 10 seconds during the USG procedure in 12 dogs. The biosignal recording system was set for 1 channel EMG and 1 channel ECG. The common mode rejection ratio of the amplifiers was over 85 dB. The transmission band of the EMG amplifier was set to 5-450 Hz according to the amplitude analysis, and the transmission band of the ECG amplifier was set to 0.5-40 Hz for heart rate detection. The system's 12-bit Analog digital converters were averaged 128 consecutive sampling data and transferred to the Android 7.0-based recording system. Then datas were processed with Matlab 2018 analysis program. As a result, when the EMG and ECG datas of the dogs included in the study were evaluated, a significant contraction was detected in the affected muscle groups, also heart rates increased statistically. However, it was observed that such applications did not cause any complications that would affect the patient's health.

Keywords: Dog, Ultrasonography, EMG, ECG

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Abdominal Ultrasonografi Uygulanan Köpeklerde EKG ve EMG Bulgularının Değerlendirilmesi

ÖZ

Bu çalışmada, köpeklerde uygulanan abdominal ultrasonografinin, eş zamanlı uygulanan EKG (kalp hızı için) ve EMG (kas kasılması için) verilerini ne şekilde etkilediği araştırılmış, ayrıca USG uygulanırken aynı anda hem EKG hem de EMG uygulanmasının her hangi bir komplikasyon oluşturup oluşturmayacağının ortaya konulması da amaçlanmıştır. Esaote My Lab Five VET marka renkli Doppler Ultrasonografi cihazı ve bu cihaza ait 5.0/8.0 MHz multi frekanslarında tarama yapabilen mikrokonveksprob kullanılarak yapılan 12 köpekteki USG işlemi esnasında en az 15 saniye öncesi ve 10 saniyesi sonrası EKG ve EMG verileri kayıt altına alınarak değerlendirildi. Biyosinyal kayıt sistemi, 1 kanal EMG ve 1 kanal EKG verisi için ayarlandı. Amplifikatörlerin ortak gürültüden kurtulma oranı 85 dB'in üzeri idi. EMG amplifikatörünün geçirme bandı amplitüd analizine uygun şekilde 5-450 Hz, EKG amplifikatörünün geçirme bandı ise 0.5-40 Hz olarak ayarlandı. Sistemin 12-bit Analog dijital çeviricileri 128 kez ardışık örneklemenin ortalamasını alarak veriyi, Android 7.0 temelli kayıt sistemine aktardı. Veriler daha sonra Windows temelli bir bilgisayarda Matlab 2018 bilimsel analiz programı ile işlendi. Sonuç olarak çalışmaya alınan köpeklerde EKG verileri değerlendirildiğinde kalp hızının istatistiksel olarak arttığı ayrıca EMG sonuçlarına göre de etkilenen kas gruplarında anlamlı derecede kasılma saptandı. Ancak bu tür uygulamaların hastanın sağlığını etkileyecek herhangi bir komplikasyona yol açmadığı görüldü.

Anahtar Kelimeler: Köpek, Ultrasonografi, EMG, EKG

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INTRODUCTION

Ultrasonography (USG) is the technique of examining soft tissues with high frequency sound waves (Nyland et al. 2002). With this method; the identification of the abdominal pathologies, imaging, recording of the images and taking biopsies can be applied by the surgeons (Alkan 1999, Şındak and Biricik 2006).

Electrocardiography (ECG) is the recording of the electrical signal that develops during the beating of the heart muscle (Tilley 1979, Başoğlu 1992), which created by the action potentials that occur during the contraction (depolarization) and relaxation (repolarization) of the heart. Electromyography (EMG) is a technique that results from physiological changes in muscle fibril membranes, develops, records and analyzes myoelectric signals. Superficial kinesiological EMG records signals from the targeted muscle with electrodes adhered to the skin. Superficial EMG focuses on exercise selection, training, functional movements and neuromuscular activation of muscles in postural positions. Kinesiological EMG applications are used in laboratory conditions to understand the contraction patterns of the muscles, to measure the amount of activation of the muscles, to evaluate the muscle synergistic activation profile and to define the formation of fatigue (Basmajian and De Luca 1985, De Luca 1997, Cerrah et al. 2010).

In this study, the aim was emphasized to determination of heart rate frequency via ECG technique and analysis of muscle contraction by quantitative processes by EMG during abdominal USG examination in healthy dogs which were handled to Afyon Kocatepe University (AKU) Veterinary Faculty Animal Hospital. Also, to reveal any complications that arises whether ECG and EMG application at the same time concurrently while applying USG.

MATERIAL and METHODS

The study was performed under the approval dated numbered 49533702/16 of the local ethics committee for animal experiments of Afyon Kocatepe University, in Turkey.

The material of the study consisted of 12 dogs between the ages of 1-9 who were brought to AKU Veterinary Faculty Animal Hospital for ovariohysterectomy, castration operation and general examination. General examination was performed to all dogs than after a 30-minute rest period; they were taken to the USG room following the shaving of the hair in the abdominal region.

USG, EMG and ECG Examinations

All dogs were placed in the lateral position and then the mid part of the *M. biceps femoris* muscles was clipped. After the skin was wiped, Ag / AgCl sticky electrodes were parallelly placed to the muscle fibers. The distance between two electrodes was 2 cm away from. For the ECG, two electrodes were sticked to both sides of the heart. After the EMG and ECG recording system was connected, waited for 5 minutes until the dog calmed down in this position. Later, Esaote My Lab Five VET color Doppler Ultrasonography device and a micro-convex probe capable of scanning at 5.0 / 8.0 MHz multi frequencies of this device were used at least 15 seconds before and 10 seconds after the abdominal USG examination. These signals were used in data analysis. The modular prototype device produced for this study was used for ECG and EMG examinations (Figure 1). The biosignal recording system was set for 1 channel EMG and 1 channel for ECG recording. The common mode rejection ratio of the amplifiers was higher than 85 dB. The transmission band of the EMG and ECG amplifier were 5-450 Hz and 0.5-40 Hz respectively. 1000 Hz sampled signals transferred to a Windows-based computer for analysis with the Matlab 2018 (Mathworks, USA) software. Artifact rejection and rms (root-mean-square) filters were applied to the EMG signals. Then, the maximum value of the first 5-second of the processed EMG; the maximum value of the first 5-second of the processed EMG before the USG probe touched the dog and the maximum value of the 5-second of processed EMG after the USG probe touched the dog (excluding the 1-second portion after touch) were used in the statistics (Figure-1). Heart rates were calculated from the same 5-second ECG segments as range (= max-min) using QRS wave peaks. In this way, the data of 5-second intervals; rest, the probe was labeled before and after touch.

Statistical Analysis

Descriptive statistics of the data obtained were determined by determining the average and standard deviation values. In dogs with abdominal USG, statistical findings of heart rate were evaluated with the ECG device by Student-t test. The EMG findings detected in the same dogs were processed with the Mann Whitney-U test and the data before and after the examination were statistically revealed.

RESULTS

ECG Measurements

Variance analysis for repeated measurements was used for evaluating the heart rate period statistics (Geisser-Greenhoused correction was also applied).

The periods of the heart rates after touching the probe was reduced ($p < 0.01$) when detecting by duration (milliseconds). It means that number of heart rates was significantly increased in 12 cases after the probe touched. When the number of heart beats taken 15 seconds before and 10 seconds after the probe touched (Table 1, Figure 2).

EMG Measurements

According to the data obtained from the electrodes placed on the *M. biceps femoris* of the study group dogs, when the muscle activation values occurred 15 seconds before the probe was touched and 10 seconds after being touched, it was observed that the muscle activation values increased statistically in ten cases after the probe was touched ($p < 0,02$) (Table 2, Figure 3, Figure 4).

Figure 1. Prototype moduler ECG and EMG device.

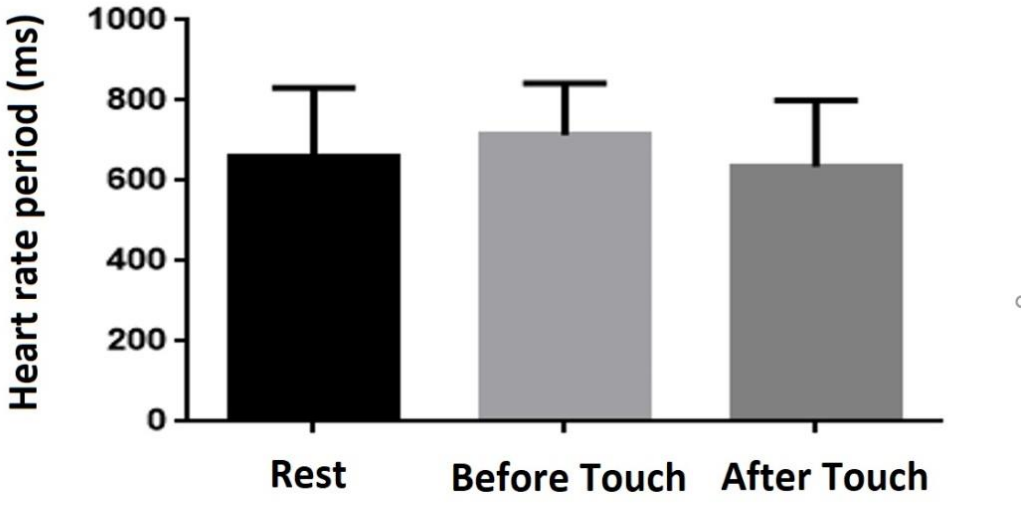

Figure 2. Heart rate periods (Mean,SD).

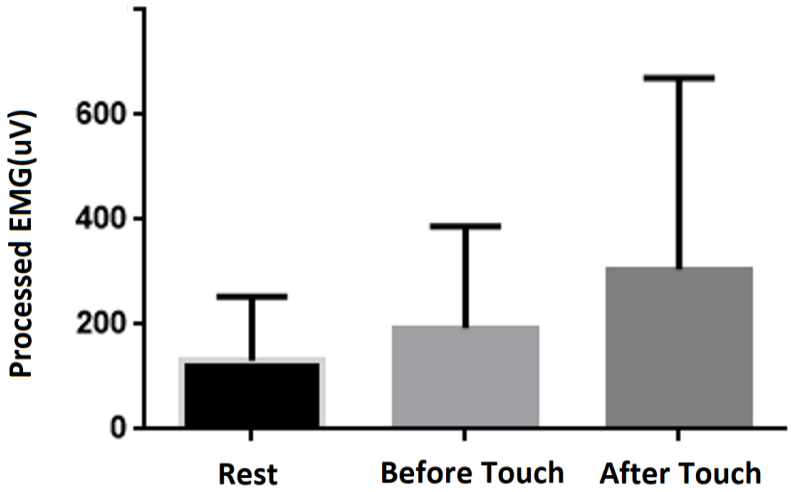

Figure 3. Processed EMG (Mean, SD).

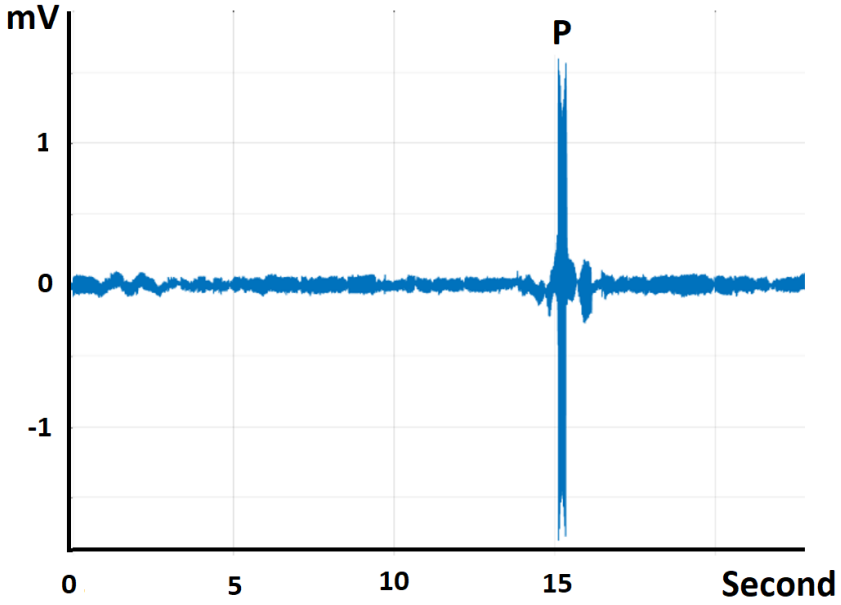

Figure 4. Raw EMG signal from a dog. P: USG probe touch artifact

Table 1. Heart rate period in dogs

Heart Beat Period (ms)		
Rest	**Before Touch**	**After Touch**
498	567	542
697	697	652
526	666	607
608	622	545
640	629	554
525	661	517
549	691	524
679	673	676
679	753	538
1029	906	983
960	1025	958
501	681	534

Table 2. EMG Datas in Dogs

EMG (MikroVolt)		
Rest	**Before Touch**	**After Touch**
20	21	97
62	61	102
85	93	124
126	381	478
118	147	165
153	173	146
267	362	770
51	53	58
30	29	39
30	47	68
434	651	1232
207	308	390

DISCUSSION and CONCLUSION

When faced with a live source of stress, the body shows war or run away response due to the activation of the sympathetic nervous system. As a result of the physical and chemical changes in the body during the war or run away response, the condition develops as the acceleration of heartbeat, high blood pressure, acceleration of breathing and sudden secretion of adrenaline (Güçlü 2001). Even if stress is experienced for a short period of time, it could cause permanent results in a short time such as tension and increased heart rate (Kahn and Cooper 1993). In this study, the results of the increase in heart rate following the probe contact were formed in all dogs with ECG accompanied by USG, and results consistent with the findings of sudden cardiac increase in environmental compliance were obtained.

Fear could be divided into two sections which are called conditionally and unconditionally. Stimuli that cause an unconditional fear response generate fear without the creature's learning experience related to these stimuli. In conditions of conditional fear, it is now possible for the organism to emerge conditional fear responses in the living as a result of the temporal-spatial matching of a previously neutral stimulus with an unconditional stimulus (such as electric shock). The main reactions used in measuring conditional fear used in the studies conducted are freezing behavior, tachycardia and ultrasonic vocalization (Borszcz 1995, Fendt and Fanselow 1999). In this study, USG application was accepted as an external stimulus and although the sound and light control was provided in the environment, following the touching of both the supine position and a solid material such as a probe to the abdominal muscles, the heart responds to this situation, which will increase the number of beats per minute.

EMG measurement results can be affected by many factors. Unlike invasive needle or thin wire EMG, where probes can be placed precisely in specific areas of the muscle, sEMG shows the total signal of target and nearby muscle activities. The formation of cross talk from other active muscles caused by skin movement is another problem. In addition, extensive research has proven the importance and validity of surface EMG measurements in humans (Bockstahler et al. 2012). Expressed as the maximum average and minimum average ratios to compare muscle activity data, a lower Min-average ratio is a rather low maximum activity value (indicative of less muscle activity during the rest phase) or a significantly higher average activity value (Zaneb et al. 2009). In this study, surface EMG was used to measure muscle activity during USG in dogs. Apart from these, the extent to which stress and excitement factors will affect muscle activation during the USG examination of the dogs, and considering the external factors, when we look at the EMG results obtained in 12 cases, it was determined that the amplitude showed significant increases after the probe touch. As a result, it was observed that the muscle contractions of the dogs increased excessively in accordance with the previous literature data ($p < 0.02$).

As a result; portable ECG and EMG device provides convenience to veterinary clinicians and academicians due to its compact structure. It was concluded that the device used in the study can be used safely in dogs for neuromuscular diseases and in detecting physiotherapy effectiveness above muscle activity. When the ECG data of the dogs included in the study were evaluated, the number of beats per minute was statistically increased and quantitative data were easily detected. In addition, according to the results of EMG, significant contraction was determined in the muscle groups, and in dogs, muscle contractions were displayed graphically by EMG during USG. It has been demonstrated that ECG and EMG examinations performed simultaneously with USG application can be easily applied in dogs. Thus, it is thought that veterinarians will save time by applying multiple advanced technical diagnostic methods at the same time. In veterinary clinics, as the time spent in both diagnosis and treatment lengthens, this creates a stress factor on patients. For this reason, in our study, multiple diagnosis methods were used simultaneously, and the time spent in patients in veterinary clinics and hospitals was shortened and thus, the stress factor on patients was tried to be minimized. However, it was also observed that the application of all three diagnostic methods at the same time did not cause any complications in affecting the patient's health. Nevertheless, the determination of heart rate especially with ECG and muscle contractions with EMG will shed light on further researches.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Morphometric and Stereological Assessment of The Bovine Vesicular Gland

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ABSTRACT

The vesicular gland is an important genital gland related to the fertility of bovine. In this study, the morphometric and stereological methods were sought to adapt to the bovine vesicular gland. For this aim, 8 healthy Holstein bull's paired vesicular glands were collected from the slaughterhouse. The weight and the dimensions of the glands were measured before the fixation. After the formalin fixation, all the glands were divided into 3 subgroups for the total volume estimation of the gland, cell counting and the volume estimation of the cell nucleus. The cell counting and the volume estimation of the cell nucleus were performed on the principal cells. The average weight, width, length, height, the volume of the glands, the mean number and nucleus volume of the principal cells were 30.9 g., 2.62 cm, 8.86 cm and 1.86 cm, 29.7 cm^3, 3.58.10^9 and 108 μm^3, respectively. The researchers believe that the findings will contribute to the literature and in particular, facilitate experimental and toxicological researches performed in the future.
Keywords: Cell number, Nucleus volume, Smooth fractionator, Stereology, Vesicular gland

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Sığır Veziküler Bezinin Morfometrik ve Stereolojik Olarak Değerlendirilmesi

ÖZ

Veziküler bez sığır fertilitesinde önemli bir üreme bezidir. Sunulan bu çalışmada sığır veziküler bezinin morfometrik ve stereolojik olarak incelenmesi amaçlanmıştır. Bu amaçla 8 sağlıklı Holstein boğasının çift olan veziküler bezleri mezbahadan toplanmış ve ağırlığı ile boyutları ölçülmüştür. Formaldehit tespitinden sonra tüm bezler bezin toplam hacminin, hücre sayısının ve çekirdek hacminin ölçülmesi için 3 alt gruba ayrılmıştır. Hücre sayımı ve hücre çekirdek hacmi ölçümü bezin en çok bulunan ana hücresi olan principal hücreler üzerinde gerçekleştirilmiştir. Bezin ortalama ağırlığı, eni, uzunluğu, yüksekliği, ortalama hacmi, principal hücrelerin ortalama sayısı ve ortalama çekirdek hacmi sırasıyla 30,9 gr., 2,62 cm, 8,86 cm, 1,86 cm, 29,7 cm^3, 3,58,10^9 ve 108 μm^3 olarak bulunmuştur. Araştırmacılar bu bulguların literature katkı sağlayacağına ve gelecekte yapılacak olan deneysel ve toksikolojik çalışmalara ışık tutacağına inanmaktadır.
Anahtar Kelimeler: Çekirdek hacmi, Hücre sayısı, Smooth parçalama, Stereoloji, Veziküler bez

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INTRODUCTION

The accessory genital glands are fully developed in the bull (Budras et al. 2003). The paired vesicular gland is one of the accessory genital glands (Frandson et al. 2003) and this gland contributes to the bulk of the seminal fluid (Pakurar and Bigbee 2004). The fluid of this gland is the source of energy for the spermatozoon motility (Gartner and Hiatt 2006). The vesicular gland of the bovine is an irregular, lobulated gland surrounded by a fibromuscular capsule. It is more or less solid with narrow branching lumina (Aughey and Frye 2001, Frandson et al. 2003, Marettova and Legath 2010, Rahman et al. 2010). The ducts are separated in the lobules and terminated as elongated and coiled tubules (Cons 1957). The mucosa of these tubules is made of an epithelium and lamina propria surrounded with a muscular layer (Badia et al. 2006, Rahman et al. 2010). The epithelium of these tubules has double layer and contains three different types of cells; principal (formerly called type A), basal (formerly called type B) and dense (formerly called type C) cells. The principal cells are the most abundant main cells, which form an inner lining. These cells are tall, columnar, and have a large oval nucleus (Amselgruber and Feder 1986, Cons 1957) (Figure1).

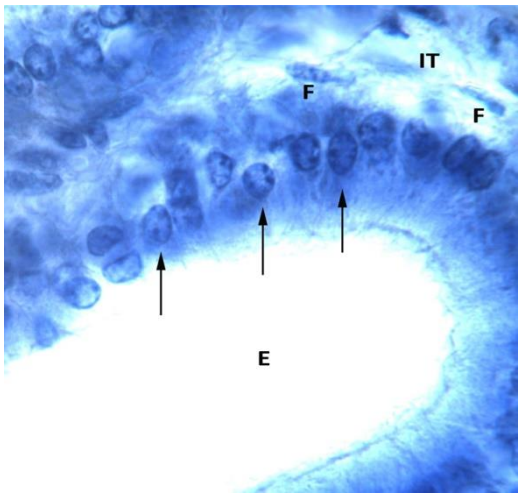

Figure 1: The vesicular gland histology (40 µm thick section, x100 objective). Arrows indicate the principal cells, (E) Endpiece, (F) Fibroblast and, (IT) Interstitial tissue.
Şekil 1: Veziküler bez histolojisi (40 µm'luk kalın kesitler, x100). Oklar principal hücreleri, (E) Endpiece, (F) Fibroblastları ve (IT) Intersitisyel dokuyu işaret etmektedir.

The structure investigation of the organ is important in the morphological and experimental researches (Altunkaynak et al. 2007, Aslan et al. 2006). The changes in the organ structure are directly related to its functional reserve as an indicator of the health status. The stereological methods produced unbiased estimates about the tissue and highly statistically efficient, precise results without any assumptions (Michel and Cruz-Orive 1998, Santos et al. 2009, Sørensen 1989, 1991). The stereological methods could use on the morphological, experimental, toxicological and tumoural studies. The stereological methods are unbiased, precise and independent from the tissue shrinkage (Santos et al. 2009). One of the stereological methods used in this study is the Cavalieri method. This method could easily estimate the total volume on the selected sections obtained from the organ (Gundersen et al., 1988). The second method was the optical fractionator method. It is a precise, unbiased and modern stereological method for the nucleus counting and combines the optical disector with the fractionator method (West et al. 1991). Optical disector is a 3D probe for particle counting in the thick sections (West et al. 1991, Gundersen et al. 1999). The fractionator is a sampling design that samples the organ systematically and randomly (West et al. 1991, Gundersen et al. 1988). However, the smooth fractionator technique is a modification of the fractionator method. This modification has gained increased efficiency to the fractionator method. In order to increase efficiency, the items are arranged (slab, sections, etc.) in symmetrical design with a peak and minimal jumps (Gundersen 2002). The third method is the nucleus volume estimation of the cell. The nucleus volume estimation of an organ is clearly and significantly important for the tumoural grading. The stereological methods could estimate the nucleus volume even in the irregular shaped particles and produced efficient and precise results (Sørensen 1989, 1991). In this study, the volume estimation method was combined with the vertical sectioning procedure (Baddeley et al. 1986, Sørensen 1991).

In the present study, the morphological and stereological evaluations were performed on the

bovine vesicular gland. The stereological methods were sought to adapt to the bovine vesicular gland for the first time according to our knowledge. The obtained findings of this study and the stereological applications may facilitate the future experimental, toxicological and tumoural researches.

MATERIAL and METHODS

In this study, the morphometric and stereological data were obtained from the vesicular gland of the healthy Holstein bulls (2.5-3 years, 650-700kg). The morphometric data were collected from the paired glands of the 8 Holstein bulls. For this aim 16 vesicular glands of 8 animals were obtained from the slaughterhouse. All the glands were weighed (Kern, Balingen-Germany) and the dimensions were measured using vernier calliper before the fixation (Labomar, 304B-01- Turkey).

After the morphometric measurements, all the 16 glands were fixed with the neutral buffered 10% formaldehyde during two weeks. After the fixation, all the glands were divided into three subgroups for explaining the methods clearly. The first group was the glandular volume estimation (n=6) group, the second group was the cell counting (n=5) group and the third group was the cell nucleus volume estimation (n=5) group.

Firstly, the Cavalieri principle (Gundersen et al., 1988), was performed to estimate the mean gland volume. Therefore six fixed glands were systematically sliced into 3 mm intervals with a random start to obtain ~8 slabs per gland. The area of these slabs was measured with 0.3 cm spaced point grid. The calculations per gland were performed by the formula below;

$V = [t \times a/p \times \sum P]$ cm^3,

t; is the slab thickness,

a/p; is the area represented by one point,

$\sum P$; is the total counted points.

In the cell counting (Gundersen, 2002) group, the glands were sliced into 3 mm intervals (from a random start in the first 3mm distance) to obtain ~8 slabs per gland and every third slab was chosen to obtain a fraction of 1/3 (Figure 2/A). The smaller slabs were placed peripherally and the larger ones were placed centrally (Figure 2/B). After the paraffin embedding, the length of the tissue in the paraffin block was measured and separated with a knife into nine equal pieces from a random start in the first interval (Figure 2/C). Every piece was turned 90^0 to the same side, collected in the metal holder and re-embedded into paraffin block (Figure 2/D). This block was sliced into 40 μm sections and the slices were chosen which passed through the centre of the tissues and then staining with the Giemsa's azure eosin methylene blue solution (Figure 2/E). The counting procedure was performed on the nine tissues per gland.

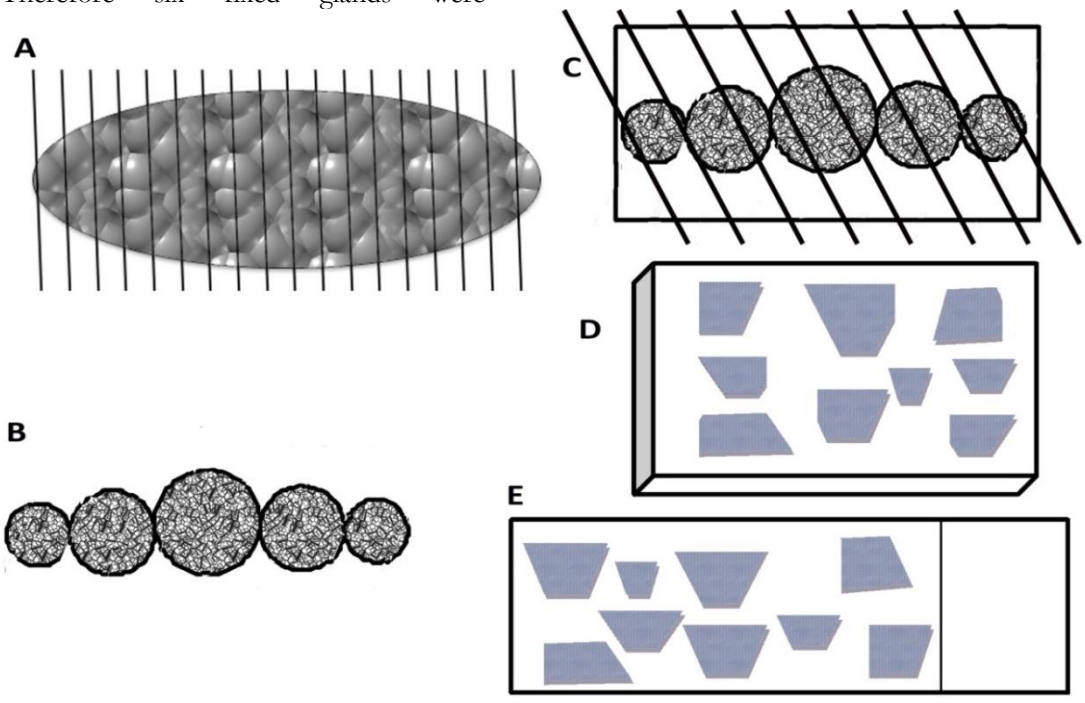

Figure 2: The diagram of the baseline sampling scheme for the cell counting: A; The gland was sectioned from a random start, B; The slabs were arranged according to the smooth fractionator principle and totally embedded in paraffin, C; The length of the tissue was measured and divided D; The pieces were totally embedded in paraffin at the metal holder, E; The paraffin block was sliced for the cell counting.

Şekil 2: Hücre sayımı için örnekleme şeması: A; Bez rastgele bir başlangıçla dilimlendi, B; Dilimler smooth fractionator metoduna göre dizildi, C; Doku uzunluğu ölçülüp, bölündü, D; Parçalar parafine gömüldü, E; Hücre sayımı için blok dilimlendi.

Finally, in the (n=5) nucleus volume of the cell estimation group (Baddeley et al. 1986, Møller et al. 1990, Sørensen 1989, 1991), each gland was sliced into 3 mm intervals and every third slab was chosen. These slabs (with a random start) were re-sliced into 3 mm bars and every 7th bar was chosen. They were rotated around their vertical axis and embedded into the paraffin. The paraffin block was sectioned into 40 µm thickness and the sections were selected which pass through the centre of the tissue.

Stereological Analysis

The number of the principal cell was estimated using a computer loaded Shtereom I software (Oguz et al. 2007), attached to a light microscope with a motorized stage. The thickness of the tissues on the slides was measured by microcator. The researchers sought to count between 100-200 nuclei per gland according to the disector principle (Gundersen et al. 1999). The counting frame area, step lengths along the X and Y axis and the disector height were set at about 76.9 µm², 1250 µm and 10 µm respectively (Figure 3).

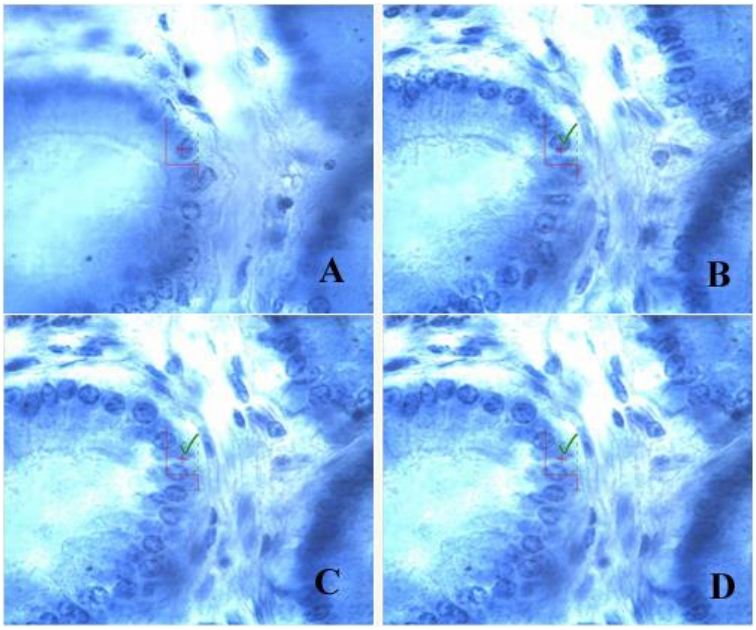

Figure 3: The principal cell counting process with optical disector in the vesicular gland (x100) A; (0 µm), B; (8 µm), C; (13 µm), D; (21 µm).
Şekil 3: Optik disektör sondasıyla veziküler bezde principal hücre sayımı (x100) A; (0 µm), B; (8 µm), C; (13 µm), D; (21 µm).

The total principal cell number was estimated according to the following formula (West et al. 1991).

$N = 1/F_{ssf} \times 1/F_{hsf} \times 1/F_{asf} \times 1/F_{slab} \times \sum Q^-$

F_{ssf}; the sampling fraction of the section,

F_{hsf}; the sampling fraction of the height (the mean section thickness divided by the height of the dissector,

F_{asf}; the sampling fraction of the area (multiplying the step lengths and divided by the area of the frame,

F_{slab}; the converted value to the micron of the tissue length in the paraffin, and this value is divided by the height of the slicing section,

$\sum Q^-$; the counted principal cells.

The coefficient error of the counted nucleus and the mean section thickness (the mean section thickness measured with the microcator per area during counting principal cells on the glass slide) were estimated according to Gundersen's formula (Gundersen et al. 1999):

$$CE(N) = \sqrt{CE^2(Di\,sector) + CE^2(t)}$$

The volume of the principal cell nucleus was estimated on the live image reflected the computers screen. The orientation frame and the test probe were both placed on the monitor. The nucleus that fell inside the orientation frame and intersected with the transparent test probe was evaluated. This transparent test probe also guided the ruler for measuring the nucleus. The ruler was in 15 classes and the total length of the ruler was 35 mm. The final magnification of the cell on the monitor was calculated as x1960. Almost 250 principal cell nuclei were estimated per gland (Figure 4). The formula for the nucleus volume estimation was:

$V_v = \pi/3 \times (L_{15} \times 1000/3.00 \times Magnification)^3 \times l_0^3$ (Sorensen,1991).

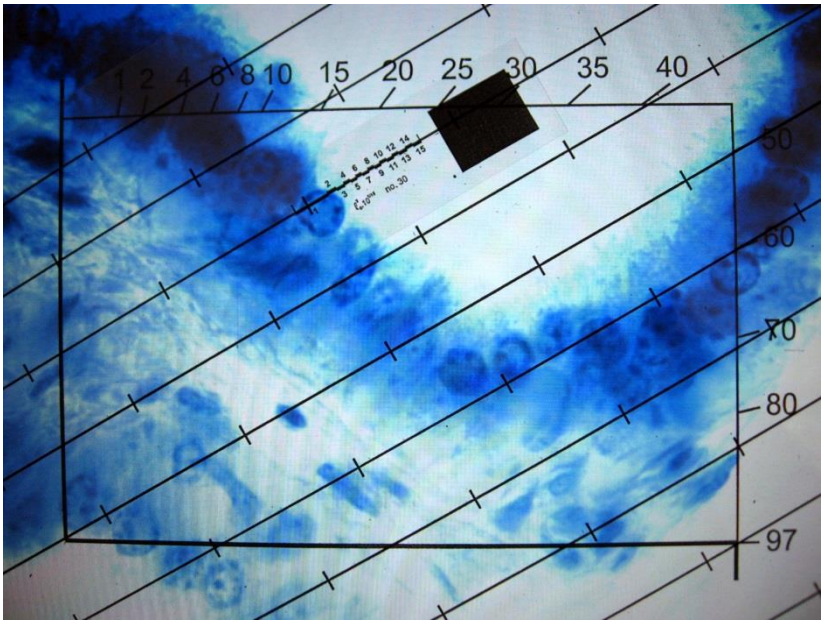

Figure 4: The nucleus volume estimation of the principal cells with ruler in the vesicular gland.
Şekil 4: Cetvel vasıtasıyla veziküler bez principal hücrelerinde çekirdek hacmi hesaplanması.

RESULTS

The mean weight of the gland was 30.9 gr. The mean width, length and height of the glands were 2.62 cm *(SD=0.30)*, 8.86 cm *(SD=1.18)* and 1.86 cm *(SD=0.32),* respectively (Table 1).

Tablo 1. Sol ve sağ veziküler bezler hakkında morfometrik veriler.
Table 1. The morphometric data about the left and right vesicular glands.

No.	Gland	Width	Length	Height	Gland	Width	Length	Height
1	left	2.56 cm	7.87 cm	1.47 cm	right	2.13 cm	7.68 cm	1.50 cm
2	left	2.67 cm	7.60 cm	1.80 cm	right	2.55 cm	7.24 cm	1.83 cm
3	left	2.45 cm	8.00 cm	1.50 cm	right	2.24 cm	8.30 cm	1.50 cm
4	left	2.83 cm	10.1 cm	2.07 cm	right	3.00 cm	10.6 cm	2.22 cm
5	left	2.60 cm	8.00 cm	2.20 cm	right	2.39 cm	8.86 cm	1.80 cm
6	left	3.37 cm	9.08 cm	2.76 cm	right	2.88 cm	9.05 cm	1.60 cm
7	left	2.51 cm	10.6 cm	1.96 cm	right	2.56 cm	10.6 cm	1.90 cm
8	left	2.78 cm	9.51 cm	1.88 cm	right	2.43 cm	8.63 cm	1.79 cm
Mean± SD		2.72 cm±0.29	8.85 cm±1.14	1.95 cm±0.41		2.52 cm±0.30	8.87 cm±1.22	1.77 cm±0.24

All the vesicular glands examined displayed normal histology, and the epithelial cells were visualized at every depth in all the sections. The mean volume of the gland was found as 29.7 cm^3 *(CE=0.09)* (Table 2). The estimated mean amount of the principal cell in the vesicular gland was $3.58.10^9$ and the mean coefficient of error (CE) was 0.09 (Table 2). The mean volume of the principal cell nucleus was 108 μm^3 (Table 2). The shrinkage of the sliced tissue on the glass slide where the cells were counted and the mean final thickness of the slice was 46.5 % and 21.4 µm (*SD=1.5*), respectively.

Table 2. The mean volume of the vesicular gland, principal cell number and the nucleus volume.
Tablo 2. Veziküler bezin ortalama değerleri, principal hücre sayısı ve çekirdek hacmi

No.	The Mean Volume of the Gland	CE Value	The Number of the Principal Cell in the Gland	CE Value	The Nucleus Volume of the Principal Cell in the Gland
1	37.9 cm^3	0.065	$4.28.10^9$	0.09	97 μm^3
2	24.8 cm^3	0.083	$3.39.10^9$	0.10	110 μm^3
3	26.7 cm^3	0.130	$3.89.10^9$	0.09	103 μm^3
4	36.5 cm^3	0.110	$2.83.10^9$	0.10	111 μm^3
5	27.0 cm^3	0.062	$3.50.10^9$	0.09	120 μm^3
6	25.5 cm^3	0.089	-	-	-
Mean± SD	29.7 cm^3±5.9	0.090	$3.58.10^9$±$5.44.10^8$	0.09±0.005	108 μm^3±8.7

DISCUSSION

In the present study the weight, volume, external dimensions, cell number and the nucleus volume of the principal cell in the vesicular gland were estimated. This genital gland of the bulls was investigated by several researchers, previously. In one of this, the vesicular gland of Bangladesh's indigenous bull was investigated. According to the morphological measurements, the weight of the gland was different from the Holstein bulls in this study. The Holstein bull's vesicular gland (~30 gr) was heavier than the indigenous bull (~20 gr). However, the other parameters such as length and width were the same except thickness. The Holstein bull's vesicular gland (~1.9 cm) was thicker than the indigenous bull (~1.5 cm). These differences may the reflection of the beef aimed breeding of Holstein bulls. However, Budras et al. (2003) and Dyce et al. (1999) reported different findings from the Holstein and indigenous bull. They were estimated the gland mean length was between 10 or 20 cm and width was 3 or 5 cm. However, the length (~9 cm) and the width (~2.6 cm) of the Holstein bull's vesicular gland were still in the range of the Rahman et al. (2010) and Dursun's (1998) reports.

Studies on the male accessory glands are generally concentrated on its morphometric and histological features (Badia et al. 2006, Budras et al. 2003, Chandolia et al. 1997, Frandson et al. 2003, Macmillan and Hafs 1969). In the literature, the stereological studies are not adequate. A study on the mice vesicular gland performed using stereological methods was revealed the harmful effects of alcohol usage (Gomes et al. 2002). The chronic alcohol intake has negative effects on the secretory process of the vesicular gland and causes morphological alterations on the epithelial cells. Besides the toxicological studies, in experimental research the triggering effect of the melatonin hormone on the reproductive activity of the inactive rams in the non-breeding season was investigated evaluating the principal cell height and the nucleus diameter (Mokhtar et al. 2016). The stereological methods were performed to the Holstein bull's bulbourethral gland, previously (Akosman et al. 2013). According to this research, there was $3.22.10^9$ principal cell in the bulbourethral gland and the nucleus volume of this cell was 59.1 μm^3. The epithelial cell number in the bulbourethral gland of the rabbit and guinea pig was also evaluated with the stereological methods (Vasquez and del Sol 2014). The development of the mice prostate gland (Singh et al. 1999) and the tumoural grading also investigated with the stereological methods (Santamaria et al. 2016). In another research, seasonal structural changes of the donkey vesicular gland were observed with estimating the nuclear/cell ratio and the interstitial/ glandular tissue ratio (Abou-Elhamd et al. 2020).

In this study, the tissues were embedded in paraffin. The paraffin embedding creates an almost 50% shrinkage in volume (Dorph-Petersen et al. 2001). In the present study, this value was estimated as 46.5%. When the square of the coefficient error divided by the square of the coefficient of variance, the result should between the values of the $0.25<X<0.5$. In this study, the obtained ratio was 0.36, which is in the acceptable range (Lodrup et al. 2008, West et al. 1991).

In conclusion, experimental investigations on the vesicular gland generally rely on calculating the height of the tubular epithelium and the tubule diameter (Archana et al. 2009). Besides the measurements, estimating the number and the nucleus volume of the

glandular cells using stereological methods will make a major contribution to the accuracy of the recent research.

Conflict of Interest: The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

The Effects Of Melatonin Treatment On Some Serum Immunoregulatory Cytokine Levels In Rats Exposed To Chronic Cadmium Toxicity

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ABSTRACT

The present study aimed to investigate the effect of melatonin treatment on some serum immunoregulatory cytokine levels in rats exposed to chronic cadmium toxicity. For this purpose, animals (n = 32) were divided randomly into four equal groups as untreated control (C), cadmium (Cd), melatonin (Mlt) and Cd + Mlt (CdMlt). The rats in Cd and CdMlt groups received cadmium chloride ($CdCl_2$) (2 mg/kg/day) orally by gastric gavage three times a week for 4 weeks. On the other hand, Mlt (100 mg/kg/day) was orally administrated to Mlt and CdMlt groups five times a week for 4 weeks. C group was not received any treatment. After the treatments, the animals were sacrificed and blood samples were taken to without anticoagulant tubes. Then, levels of IL-1β, IL-2, IL- 6, TNF-α, and INFγ in the serum were determined. It was not found any change among the groups according to IL-1β, IL-2, and IL-6 levels ($p>0,05$). Besides, the administration of Mlt ameliorated the TNF-α levels in CdMlt group compared to Cd ($p<0,05$). IFNγ levels were found the highest in C and Mlt groups compared to Cd ($p<0,05$). In conclusion, Mlt treatment caused a significant change only in TNF-α levels in rats exposed to Cd.
Keywords: Cadmium, Cytokine, Melatonin, Serum, Rat

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Melatonin Uygulamasının Kronik Kadmiyum Toksikasyonuna Maruz Kalan Sıçanlarda Bazı Serum İmmun-Regülatör Sitokinler Üzerine Etkisi

ÖZ

Bu çalışmada kronik kadmiyum toksikasyonuna maruz kalan sıçanlarda melatonin'in bazı serum immun-regülatör sitokin seviyeleri üzerine etkilerinin araştırılması amaçlanmıştır. Bu amaç doğrultusunda, hayvanlar (n =32); kontrol grubu (K) kadmiyum grubu (Cd), melatonin grubu (Mlt) ve kadmiyum + melatonin grubu (CdMlt) olmak üzere rastgele dört eşit gruba ayrıldı. Cd ve CdMlt gruplarındaki hayvanlara, 4 hafta boyunca haftada üç kez gastrik gavaj yoluyla oral kadmiyum klorür ($CdCl_2$) (2 mg/kg/gün) verildi. Öte yandan Mlt ve CdMlt gruplarına 4 hafta boyunca haftada beş kez oral Mlt (100 mg/ kg/gün) uygulaması yapıldı. Kontrol grubunda yer alan sıçanlara herhangi bir uygulama yapılmadı. Deney periyodundan sonra, sıçanlar sakrifiye edildi ve kan örnekleri antikoagulant içermeyen tüplere alındı. Deneme sonunda serum IL-1β, IL-2, IL-6, TNF-α ve INFγ konsantrasyonları belirlendi. IL-1β, IL-2 ve IL-6 seviyelerinde deney grupları arasında bir değişiklik bulunmadı ($p>0,05$). Ayrıca, Mlt uygulaması CdMlt grubundaki TNF-α düzeylerini Cd grubuna kıyasla iyileştirdi ($p<0,05$). IFNγ seviyeleri Cd grubu ile karşılaştırıldığında en yüksek oranda C ve Mlt gruplarında tespit edildi ($p<0,05$). Sonuç olarak, Mlt uygulaması Cd toksisitesine maruz kalan sıçanlarda sadece TNF-α düzeylerinde önemli bir değişikliğe neden olmuştur.
Anahtar Kelimeler: Kadmiyum, Melatonin, Serum, Sitokin, Sıçan

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INTRODUCTION

Cadmium (Cd) is, a toxic heavy metal, a worldwide significant environmental pollutant that exerts a variety of adverse effects on humans and also animal health (Dukic-Cosic et al. 2020). It is used in many industrial areas include the nickel-Cd batteries, sensors, televisions, metal-electro plating, pigments, plastics, and alloy (Klassen et al. 2009, Predes et al. 2010). Although living beings are mainly exposed to Cd through the intake of contaminated water, food, plants (vegetables) or air, dermal exposure (through the skin) is uncommon (IARC 2012, Kisadere and Donmez 2019). It has been reported that long-term Cd accumulation causes serious tissue damages in many organs and biological systems (Cuypers et al. 2010). Although Cd causes oxidative stress by increasing lipid peroxidation (LPO) and/or by changing intracellular glutathione (GSH) levels, recent studies have shown that it can also affect the immune system functions in humans and animals (Patra et al. 2011, Kisadere et al. 2019, Turley et al. 2019). Marth et al. (2001) have been reported that at low concentrations, Cd is able to stimulate the immune system, while at higher concentrations it has inhibitory and immune-suppressive properties.

Inflammation is a very complex process, a preventive response of the organism to injury (physical, chemical, etc.) or infections. In a part of this process, different types of immune cells (macrophages, B/T lymphocytes, mast cells, and endothelial cells, etc.) produce biochemical compounds of inflammatory and immune reactions called cytokines. They have a specific effect on the interactions (synergistically or antagonistically) and communications between cells (Zhang and An 2007). They are divided into two groups as pro-inflammatory and anti-inflammatory cytokines. Excessive secretion of these mediators (~5–20 kDa), however, cause functional problems in different type of immune cells (Moniuszko-Jakoniuk et al. 2009). One of them is interleukin-1β (IL-1β), a pro-inflammatory cytokine, is crucial for host-defense system responses to infection and injury (Dinarello 1997). Also, IL-2 plays a major role in the activation, proliferation, and differentiation of T, B lymphocytes and natural killer (NK) cells. Another important multifunctional pro-inflammatory cytokine is IL-6 that plays critical roles in host defense, acute phase response, B cell proliferation, and thrombopoiesis (Hirano 1998, Zhang and An 2007). An increased serum IL-6 values have been related to various pathological conditions, including infections, physical trauma, inflammations, auto-immune problems and different types of malignancies (Taga and Kishimoto 1997). Tumor necrosis factor-alpha (TNF-α) also plays major roles in microbial infections, cell death, inflammation, pain, and the growth of different malignant tumors. Also, elevated TNF-α levels have been implicated with some serious problems such as cachexia, septic shock, and autoimmune diseases (Ware et al. 1996). Interferon gamma (IFNγ) is, type two (II) interferon, a critical for innate and adaptive immunity of host against various viral, bacterial, and protozoal infections. It also has immunostimulatory and immunomodulatory effects include: induces of class I MHC and class II MHC in different cells, activates macrophages, neutrophils, natural killer cells (NK), promotes cell-mediated immunity (Zhang and An 2007). Szuster-Ciesielska et al. (2000) have been announced that some cytokines include TNF-α, IFNγ and IL-6 may be influenced by the exposure to Cd.

In recent years, it has been reported that the detrimental effects of Cd can be alleviated by using some substances that have antioxidant and metal-binding properties. Many chelating agents and antioxidants have been used to diminish tissue damages in chronic Cd exposed animals (Pourmorad et al. 2006, Karabulut-Bulan et al. 2008). One of them is Mlt (n-acetyl-5-methoxytryptamine), a neuro-hormone, was secreted mainly from the pineal gland. The control of biologic circadian rhythms, sleep pattern-induction, regulation of seasonal reproduction, food intake, and immune enhancement can be described as a biological function of Mlt (Maestroni 1998). It has been suggested that it is a powerful antioxidant and free radical consumer substance due to its small size and lipophilic properties. Besides, it has a metal binding function (Karbownik et al. 2001, Dan et al. 2018). In addition, recent studies have shown that Mlt (an immune modulator) has regulatory effects on immunity and anti-inflammation (Shin et al. 2014). On the other hand, Kim et al. (2000) have been reported that Mlt restores significantly the immunotoxic status induced by Cd in mice. Also, positive immuno-regulatory effects of Mlt on different heavy metal intoxications were reported by different researchers (Bali et al. 2016, Li et al. 2016, Dutta et al. 2018, Durappanavar et al. 2019).

The purpose of this study was to determine the effects of oral Mlt treatment on some serum immunoregulatory cytokine levels in rats exposed to low dose chronic Cd toxicity.

MATERIALS and METHODS

Animals and Experimental Design

Male albino Wistar rats (n = 32; 3 weeks old; body weight ~ 200 ± 30g) were obtained from Balıkesir University Experimental Medicine Research and Application Center (BUEMRAC). After one week of acclimatization period, animals were divided randomly into four groups as detailed below: untreated control (C), cadmium (Cd), melatonin (Mlt) and Cd + Mlt (CdMlt) groups; each group contained 8 animals. Animals were, housed in standard plastic rat cages (polypropylene), maintained in an air-conditioned room (BUEMRAC; temperature: 23 ± 2 C^0; humidity: 55 ± 10%) on a 12-h light/dark cycle

with fresh-water and food available ad libitum. The animals in Cd and CdMlt groups received cadmium chloride ($CdCl_2$) (2mg /kg/ day) orally by gastric gavage three times a week for 4 weeks (Almenara et al. 2013). On the other hand, Mlt (100 mg/kg/day) was orally by gastric gavage administrated to Mlt and CdMlt groups five times a week for 4 weeks (Haddadi et al. 2015). At the end of the 4-weeks experiment period, rats were sacrificed under anesthesia using an intramuscular injection of ketamine/xylazine (0.1 ml/100gm/body weight). Blood samples were drawn by cardiac puncture using without anticoagulant tubes. Then, they were centrifuged (at 3000 g for 20 min) after coagulation and serum were separated. The serum samples not used immediately were frozen at -80ºC until further analysis. The levels of IL-1β, IL-2, IL-6, TNF-α, and INFγ in the serum were determined.

Determination of Some Serum Cytokine Levels

The levels of IL-1β, IL-2, IL-6, TNF-α, and IFNγ in the serum were measured by enzyme immunoassay using ELISA kits from Sunred Biological Technology (Shanghai, China) according to the kit instruction. This assay based on a double-antibody sandwich ELISA assay to measure the levels of rat IL-1β, IL-2, IL-6, TNF-α, and IFNγ in the serum. In brief, serum samples and standards provided in the kit were extracted on an extraction plate, derivatized using an equalizing reagent, and subjected to ELISA in IL-1β, IL-2, IL-6, TNF-α, and IFNγ pre-coated microtiter strips. The absorbance of the solution in the wells was read at 450 nm within 15 min using a microplate reader (Thermo Scientific Multiskan FC, USA). The optical density was used to calculate the cytokine levels using a standard curve.

Statistical Analysis

The statistical analysis of the data was done using by analysis of variance (one way-ANOVA) followed by Duncan's test using the SPSS 25.0 package program (SPSS, Inc., Chicago, IL). Value for $P \leq 0.05$ were considered as statistically significant.

RESULTS and DISCUSSION

The results of the study were shown in Table 1. It was not found any significant change among the experimental groups according to IL-1β, IL-2, and IL-6 levels ($p>0,05$). On the other hand, serum TNF-α levels were detected the highest in the Cd group when compared to other experimental groups ($p<0,05$). Besides, the administration of Mlt ameliorated the TNF-α levels in CdMlt group compared to Cd ($p<0,05$). In addition, IFNγ levels were found the highest in C and Mlt groups when compared to Cd and CdMlt ($p<0,05$).

In the present study, Cd (2 mg/kg/p.o for 4 weeks) administration did not lead to any significant change in serum IL-1β, IL-2, and IL-6 levels in all experimental groups. Afolabi et al. (2012) reported that different doses of Cd (50-100 ppm/p.o for 7 weeks) treatment led to an increase in plasma IL-2 and IL-6 levels in rats. Also, Moniuszko-Jakoniuk et al. (2009) suggested that the levels of IL-6 elevated in rat serum only after oral treatment of Cd (50 mg/kg) for 16 weeks. In a previous study, Yücesoy et al. (1997) announced that long term (one year) exposure did not alter serum IL-2 levels in the factory workers who were directly exposed to Cd. It might be changed due to different dose only. On the other hand, Mlt (100 mg/kg) treatment did not cause any significant change in the levels of IL-1β, IL-2, and IL-6 in our study. It was not found any information about the effects of Mlt on serum IL-1β, IL-2, and IL-6 levels in rats exposed to chronic Cd in the literature. Bali et al. (2016) announced that Mlt (10 mg/kg/bw p.o) administration significantly decreased the serum levels of IL-6 in arsenic (As)-induced liver damaged rats. Also, Durappanavar et al. (2019) reported that per-oral Mlt administration (10 mg/kg/bw) suppressed the release of IL-1β, IL-6, and TNF-α in the brain tissue of Wistar rats that were exposed to As.

In our study, serum TNF-α levels were determined the highest in Cd group compared to C, CdMlt, and Mlt. Alghasham et al. (2013) suggested that Cd (40 mg $CdCl_2$/L) treatment significantly increased the plasma levels of TNF-α and IL-6 in rats exposed to Cd-polluted water for six (6) weeks. Moniuszko-Jakoniuk et al. (2009) also informed that levels of some main proinflammatory cytokines were remarkably increased in the serum of experimental animals after long term exposure to Cd. Also, another researchers have noticed that accumulation of Cd induces the production of TNF-α and IL-6 in some living beings (Kataranovski et al. 1998, Krocova et al. 2000). On the contrary, Yücesoy et al. (1997) noticed that long-term (one year) Cd administration (average 1.8-25.3 μg/l) did not alter serum TNF-α levels in the workers of battery production company. The differences could be occurred due to the chemical form of the heavy metal, application route, dosage regime, exposure time, and genetic properties of host. In the present study, Mlt treatment ameliorated the serum TNF-α levels in CdMlt group compared to Cd. It was not found any significant information about the effects of Mlt on serum TNF-α levels in rats exposed to chronic Cd in the literature. Besides, Dutta et al. (2018) reported that Mlt treatment reduced the levels of both TNF-α and matrix metalloproteinase-2 (MMP_2) in As intoxicated kidney injury. Our results were also corresponding with the previous studies (Li et al. 2016, Durappanavar et al. 2019).

In our study, IFNγ levels were found the lowest in Cd and CdMlt groups compared to C. Theocharis et al. (1991) also detected a decrease in IL-2 and IFNγ levels in the presence of 10^{-4} M Cd^{2+}. Besides, Szuster-Ciesielska et al. (2000) suggested that Cd

effect depended on the concentration used, and 1 and 10 μM CdCl2 partially, but 100 μM Cd completely inhibited the production of TNF-α and IFNγ in bovine aorta endothelial cells. On the other hand, Moniuszko-Jakoniuk et al. (2009) reported that exposure to Cd in both 5 and 50 mg Cd/l doses, alone or in combination with ethanol (EtOH), led to an increase in the serum levels of IL-1α, TNF-α, and INFγ in rats. Similarly, Yücesoy et al. (1997) detected an increase in the serum IFNγ levels in the long-term low-dose Cd-exposed workers. In the present study, low dose (100mg/kg for 4 weeks) oral Mlt treatment did not attenuate the IFNγ levels in CdMlt group compared to C. Although Mlt has anti-inflammatory effects, previous studies about the effects of Mlt on cytokine levels are sometimes contradictory. Srinivasan et al. (2005) have been reported that Mlt increases IL-2, IL-6, IL-12, and IFNγ levels by stimulating cytokine production in aged individuals and patients in an immunocompromised state. In the contrary, Broncel et al. (2007) have been suggested that Mlt decreases IL-6, IL-12, TNF-α, and IFNγ levels in patients who at risk of atherosclerosis. These different results may be occurred due to Mlt could not exhibit its metal-binding properties in that dose.

Table 1. Some serum immunoregulatory cytokine levels of experimental groups.

Parameters	C	Mlt	CdMlt	Cd
IL-1β (pg/L)	782,00±153,36	824,50±23,31	1280,58±330,59	1385,75±365,48
IL-2 (ng/L)	2,82±1,18	2,96±0,26	3,42±0,43	3,68±0,56
IL-6 (ng/mL)	32,63±0,45	32,69±0,61	32,77±0,44	33,41±0,27
TNF-α (ng/L)	576,60±2,33b	576,96±0,91b	578,75±2,06b	586,42±2,33a
IFNγ (ng/L)	61,81±5,10a	56,95±4,05a	40,28±4,92b	39,31±3,43b

Groups: C, control; Mlt, melatonin; Cd, cadmium; CdMlt, Mlt + Cd. [a–b]Means in the same line with different superscripts differ significantly ($p < 0.05$).

CONCLUSION

In conclusion, Mlt (100 mg/kg/day) treatment exhibited a very partial change in some serum immunoregulatory cytokine levels in rats exposed to chronic low dose Cd toxicity. Therefore, further investigations are required for the clarification of these important interactions.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

In Vitro Antimicrobial Susceptibility of *Corynebacterium pseudotuberculosis* Isolated from Sheep with Caseous Lymphadenitis

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ABSTRACT

Caseous lymphadenitis (CLA), caused by *Corynebacterium pseudotuberculosis,* is a highly prevalent disease of sheep and goats resulting in significant economic losses in the world. In this study, it was aimed to determine the *in vitro* antimicrobial susceptibility of *C. pseudotuberculosis* strains isolated from sheep with CLA to various antibiotics. A total of 16 *C. pseudotuberculosis* strains isolated from lung samples of 67 sheep with CLA lesions were tested. Tetracycline, oxytetracycline, streptomycin, enrofloxacin, cloxacillin, ampicillin/sulbactam, amoxicillin/clavulanic acid, penicillin/novobiocin, trimethoprim/sulfamethoxazole and neomycin/bacitracin/tetracycline discs were tested by disc diffusion method. A total of 14 (87.5%) *C. pseudotuberculosis* strains were susceptible to enrofloxacin, 13 (81.2%) strains to neomycin/bacitracin/tetracycline, 12 (75.0%) strains to penicillin/novobiocin, 11 (68.7%) strains to oxytetracycline, 11 (68.7%) strains to amoxicillin/clavulanic acid, 10 (62.5%) strains to cloxacillin, 9 (56.2%) strains to tetracycline, 5 (31.2%) strains to ampicillin/sulbactam and 3 (18.7%) strains to trimethoprim/sulfamethoxazole. None of the isolates were susceptible to streptomycin.

Keywords: Antimicrobial susceptibility testing, Caseous lymphadenitis, *Corynebacterium pseudotuberculosis*

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Kazeöz Lenfadenitisli Koyunlardan İzole Edilen *Corynebacterium pseudotuberculosis* Suşlarının *in-Vitro* Antibiyotik Duyarlılıkları

ÖZ

Koyun ve keçilerde *Corynebacterium pseudotuberculosis* tarafından oluşturulan kazeöz lenfadenitis (KLA), tüm dünyada yaygın olarak görülmekte ve önemli ekonomik kayıplara neden olmaktadır. Bu çalışmada, KLA'li koyunlardan izole edilen *C. pseudotuberculosis* suşlarının çeşitli antibiyotiklere *in vitro* duyarlılıklarının belirlenmesi amaçlandı. Çalışmada KLA lezyonları gösteren toplam 67 adet koyunun akciğerlerinden izole edilen 16 adet *C. pseudotuberculosis* suşu test edildi. Disk difüzyon yöntemiyle yapılan değerlendirmede; tetrasiklin, oksitetrasiklin, streptomisin, enrofloksasin, kloksasilin, ampisilin/sulbaktam, amoksisilin/klavulanik asit, penisilin/novobiosin, trimetoprim/sulfametaksazol ve neomisin/basitrasin/tetrasiklin diskleri kullanıldı. Suşların 14 (%87.5) adeti enrofloksasine, 13 (%81.2) adeti neomisin/basitrasin/tetrasikline, 12 (%75.0) adeti penisilin/novobiosine, 11 (%68.7) adeti oksitetrasikline, 11 (%68.7) adeti amoksisilin/klavulanik asite, 10 (%62.5) adeti kloksasiline, 9 (%56.2) adeti tetrasikline, 5 (%31.2) adeti ampisilin/sulbaktama ve 3 (%18.7) adeti ise trimetoprim/sulfametaksazole duyarlı bulundu. İzolatlardan hiçbiri streptomisine duyarlı bulunmadı.

Anahtar Kelimeler: Antibiyotik duyarlılık testi, *Corynebacterium pseudotuberculosis*, Kazeöz lenfadenitis

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GİRİŞ

Corynebacterium pseudotuberculosis Gram pozitif, hareketsiz, sporsuz, kapsülsüz, kokoid veya çomak morfolojisinde, fakültatif intraselüler bir bakteridir. Bakteri 37ºC'de, aerobik veya fakültatif anaerobik ortamlarda, 2-4 günde küçük, yuvarlak, sarımsı veya gri-beyaz renkte, agar yüzeyinde kayan koloniler oluşturmaktadır. Proteolitik etkisi olmayan etkenin, biyokimyasal aktivitesi zayıf ve değişkendir. *C. pseudotuberculosis*'in nitrata olan etkisi bakımından, nitrat negatif ve pozitif olmak üzere farklı suşları bulunmaktadır. Genel olarak koyun ve keçi orijinli suşlar nitrat negatif, at ve sığır orijinliler ise nitrat pozitiftir (Dorella ve ark. 2006, Quinn ve ark. 2011).

C. pseudotuberculosis insan dahil çeşitli canlı türlerinde değişik enfeksiyonlara neden olmaktadır (Kuria ve Holstad 1989, Dorella ve ark. 2006, Bastos ve ark. 2012). Bakteri daha çok koyun ve keçilerde kazeöz lenfadenitis (KLA)'in spesifik etkeni olarak bilinmektedir. KLA, özellikle eksternal lenf yumruları (eksternal form) ve akciğerler başta olmak üzere çeşitli iç organlarda kapsüllü apselerin oluşumuyla karakterize (internal form), kronik bir enfeksiyondur. Dünyanın birçok bölgesinde yaygın olarak görülen enfeksiyonda apseleşmeyle birlikte erken doğum, yavru atma, deri ve yapağı kalitesinde bozulma, ileri derecede zayıflamaya bağlı olarak et verimi ve kalitesinde düşme, genç hayvanlarda daha fazla olmak üzere nadiren de olsa ölümler görülebilmektedir (Middleton ve ark. 1991, İlhan 2001, Dorella ve ark. 2006, Parin ve ark. 2018).

KLA'in tedavisinde çeşitli antimikrobiyal maddeler kullanılmaktadır. Etkenin çeşitli antibiyotiklere *in vitro* duyarlılığına yönelik bazı çalışmalar yapılmıştır. Kanada'da yapılan bir çalışmada, KLA'li keçilerden izole edilen toplam 26 adet *C. pseudotuberculosis* suşu test edilmiş ve izolatlardan 3'ünün neomisine, tamamının ise ampisilin, kloramfenikol, linkomisin, gentamisin, tetrasiklin, penisilin G ve trimetoprim/sulfametaksazole duyarlı olduğu rapor edilmiştir. Aynı çalışmada tüm izolatların streptomisine ise dirençli olduğu bildirilmiştir (Muckle ve Gyles 1982). Fransa'da 6 farklı coğrafi bölgede yetiştirilen KLA'li koyun ve keçilerden elde edilen 22 adet *C. pseudotuberculosis* suşuyla yapılan çalışmada, izolatlardan birinin yüksek düzeyde streptomisine dirençli olduğu ifade edilmiştir (Pepin ve ark. 1989). İspanya'da subklinik mastitisli koyun orijinli 10 adet *C. pseudotuberculosis* suşunun incelendiği araştırmada; amoksisilin, penisilin, tetrasiklin, kanamisin, gentamisin, eritromisin, rifampisin, linkomisin, siprofloksasin, doksisilin ve kloramfenikol test edilmiştir. Araştırmada sonuç olarak, izolatların tamamının MİK değerlerinin oldukça yüksek olduğu rapor edilmiştir (Fernández ve ark. 2001). Sa ve ark. (2013), Brezilya'da KLA'li koyun ve keçilerden izole ettikleri toplam 398 adet *C. pesudotuberculosis* suşuyla yaptıkları antibiyogram testinde; izolatların %100'ünün florfenikol ve tetrasikline, %99.2'sinin linkomisin, enrofloksasin ve siprofloksasine, %98.9'unun sefalotine, %98.7'sinin norfloksasine, %97.7'sinin gentamisine, %94.2'sinin ampisiline, %91.2'sinin penisilin G'ye ve %89.2'sinin neomisine duyarlı; %100'ünün ise novobiyosine dirençli olduğunu bildirmişlerdir.

Yapılan çalışmalar dikkate alındığında, KLA'li hayvanlardan izole edilen *C. pseudotuberculosis* suşlarının *in vitro* antibiyotik duyarlılıklarının belirlenmesinde, çeşitli antimikrobiyal maddelerin kullanıldığı ve bu çalışmalardan oldukça farklı sonuçların alındığı görülmektedir. Bu çalışmada, Balıkesir ilinde KLA'li koyunlardan izole edilen *C. pseudotuberculosis* suşlarının çeşitli antimikrobiyal maddelere *in vitro* duyarlılıklarının belirlenmesi amaçlandı.

MATERYAL ve METOT

Materyal

Çalışma kapsamında toplam 67 adet koyun akciğer örneği materyal olarak kullanıldı. Materyaller, Bigadiç Belediye Mezbahasında Eylül 2018-Nisan 2019 tarihleri arasında kesimi yapılan koyunlardan toplandı. Kesimi yapılan hayvanların akciğerleri, veteriner hekimler tarafından kontrol edildi. Örnekler, makroskopik olarak pnöymoni belirtileri gözlenen ve apseleşmiş lenf yumruları (bronşiyal ve mediastinal) saptanan hayvanlardan alındı. Kesim sonrasında yaklaşık 10-15 gr. ağırlığında temiz plastik kaplara konulan örnekler, soğuk zincirde ve kısa sürede Balıkesir Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı Laboratuvarına ulaştırıldı.

İzolasyon

Akciğer örneklerinden steril svaplarla %5-7 defibrine koyun kanlı agara (1.10886, Merck, Darmstadt, Germany) ekimler yapıldı. Petri kutuları 37°C'de ve aerobik ortamda 1-3 gün inkube edildi. Besiyerinde şekillenen koloniler makroskopik ve mikroskopik morfolojileriyle birlikte, çeşitli biyokimyasal özelliklerine göre *C. pseudotuberculosis* olarak identifiye edildi (Dorella ve ark. 2006, Quinn ve ark. 2011).

Antibiyotik duyarlılık testi

Bu amaçla, akciğer örneklerinden identifiye edilen *C. pseudotuberculosis* suşları brain heart infusion brotta (CM1135, Oxoid, Basingstoke, England) aerobik atmosferde, 37°C'de üretildi (McFarland Standart Tüp No: 0.5). Test, plate count agarda (105463, Merck), Kirby-Bauer Disk Difüzyon Yöntemime göre yapıldı (Bauer ve ark. 1966). Teste; tetrasiklin (30 µg, Oxoid), oksitetrasiklin (30 µg, Bioanalyse), streptomisin (10 µg, Bioanalyse), enrofloksasin (5 µg, Bioanalyse), kloksasilin (5 µg, Oxoid), ampisilin/sulbaktam (10/10 µg) (Bioanalyse), amoksisilin/klavulanik asit (2/1) (30 µg, Oxoid), penisilin/novobiosin (40 µg, Oxoid), trimetoprim/sulfametaksazol (1.25 µg/23.75 µg,

BBC) ve neomisin/basitrasin/tetrasiklin (30 µg/10 IU/30 µg, Mast Diagnostic) diskleri kullanıldı. Sonuçlar, besiyerleri 37°C'de 24 saat inkübe edildikten sonra değerlendirildi (CLSI 2013).

BULGULAR

İzolasyon

İncelen 67 adet akciğer örneğinin 16 (%23.8)'sından *C. pseudotuberculosis* izole edildi. Etken 9 (%56.2) örnekte saf kültür olarak, 7 (%43.8) örnekte ise çeşitli Gram pozitif ve negatif bakteriyel etkenlerle birlikte üredi.

Antibiyotik duyarlılık testi

Toplam 16 adet *C. pseudotuberculosis* suşunun kullanıldığı bu çalışmada, test edilen antimikrobiyal maddeler arasında en yüksek duyarlılık (%87.5) enrofloksasine karşı saptanırken, izolatların tamamının streptomisine dirençli olduğu görüldü (Tablo 1).

Tablo 1: *C. pseudotuberculosis* suşlarının *in vitro* antimikrobiyal duyarlılığı.
Table 1. *In vitro* antimicrobial susceptibility of *C. pseudotuberculosis* strains.

Antimikrobiyal Madde	İzolat Sayısı (%)		
	Duyarlı	Orta Duyarlı	Dirençli
Enrofloksasin	14(87.5)	1(6.2)	1(6.2)
Neomisin/basitrasin/tetrasiklin	13(81.2)	2(12.5)	1(6.2)
Penisilin/novobiosin	12(75.0)	0(0.0)	4(25.0)
Oksitetrasiklin	11(68.7)	1(6.2)	4(25.0)
Amoksisilin/klavulanik asit	11(68.7)	2(12.5)	3(18.7)
Kloksasilin	10(62.5)	3(18.7)	3(18.7)
Tetrasiklin	9(56.2)	4(25.0)	3(18.7)
Ampisilin/sulbaktam	5(31.2)	0(0.0)	11(68.7)
Trimetoprim/sulfametaksazol	3(18.7)	1(6.2)	12(75.0)
Streptomisin	0(0.0)	1(6.2)	15(93.7)

TARTIŞMA

KLA tüm dünyada yaygın olarak görülen ve küçük ruminant endüstrisinde önemli ekonomik kayıplara neden olan bir enfeksiyondur. OIE'nın raporuna göre 1996-2004 yılları arasında 201 farklı ülkeden alınan veriler değerlendirilmiş ve 64 ülkede enfeksiyonun varlığı bildirilmiştir. Bu ülkelerin 19'u Amerika Kıtasında, 18'i Afrika Kıtasında, 14'ü Avrupa Kıtasında, 11 Asya Kıtasında ve 2'si de Okyanusya'da bulunmaktadır (OIE 2009). Ülkelere göre enfeksiyonun prevalansıyla ilgili oldukça farklı değerler bulunmaktadır. Bu değerler yaklaşık %8-90 arasında değişmektedir (Kuria ve Holstad 1989, Middleton ve ark. 1991, Stanford ve ark. 1998, Ali ve ark 2016). KLA'in prevalansıyle ilgili değerlendirmeler, genellikle enfeksiyonun eksternal formunda görülen lezyonlara göre yapılmaktadır. Ancak hastalığın gerçek prevalansının saptanmasında internal forma ait lezyonların da mutlaka dikkate alınması gerektiği bildirilmektedir (Abebe ve Tessema 2015). Gerçekleştirilen bu çalışmada, Balıkesir İlindeki koyunlarda KLA'in prevalansı %23.8 olarak saptanmıştır. Bu bulgu; incelenen hayvan sayısının az olması, istatistiksel bir örnekleme yönteminin/lerin uygulanmaması ve bazı nedenlerden dolayı enfeksiyonun eksternal formuna ait semptom ve lezyonların yeteri düzeyde incelenememesi nedenleriyle, gerçek prevalans değeri olarak görülmeyebilir. Yine de bulgunun, konuyla ilgili bir ön çalışma olarak değerlendirilmesi olasıdır.

KLA'li koyun ve keçilerden izole edilen *C. pseudotuberculosis* suşlarının *in vitro* antimikrobiyal maddelere duyarlılıklarının belirlenmesinde, Türkiye'de daha az olmak üzere, bazı ülkelerde değişik çalışmalar yapılmıştır. Konya'da KLA'li koyunlardan izole edilen *C. pseudotuberculosis* suşlarının *in vitro* antimikrobiyal duyarlılığının araştırıldığı bir çalışmada; izolatların florfenikol (%98.6), telitromisin (%91.6), enrofloksasin (%83.3), penisilin G (%83.3), oksitetrasiklin (%81.9), rifampisin (%81.9), gentamisin (%81.9), amoksisilin (%77.7), ampisilin/sulbaktam (%76.3), eritromisin (%69.4), spiramisin (%58.3), kloksasilin (%55.5) ve ampisiline (%37.5) duyarlı olduğu ifade edilmiştir (Sakmanoğlu ve ark. 2015). Mısır'da yapılan bir çalışmada, klinik olarak KLA semptomları gösteren 80 adet koyun ve 46 adet keçinin ekstermal lenf yumrularından alınan örneklerin analizinden elde edilen 52 adet *C. pseudotucerculosis* suşu test edilmiştir. En yüksek duyarlılığın siprofloksasin (%96.2), amikasin (%90.4), neomisin (%88.5) ve streptomisine (%80.8) karşı olduğu çalışmada; penisilin (%96.2) ve eritromisine (%92.3) karşı ise en yüksek dirençliliğin saptandığı rapor edilmiştir (Algammal 2016). Etiyopya'da 82 adet koyun ve keçiden alınan örneklerin bakteriyolojik

analizinde, 59 (%71.9) hayvandan *C. pseudotuberculosis* izole edilmiştir. Yapılan antibiyogram testinde izolatların 46'sı (%77.9) norfloksasine, 43'ü (%72.8) doksisilin ve tetrasikline, 41'i (%69.4) kanamisin ve vankomisine, 35'i (%59.3) ise ampisilin ve klindamisine duyarlı bulunmuştur (Abebe ve Tessema 2015). Sudan'da koyun ve keçilerden izole edilen toplam 66 adet *C. pseudotuberculosis* suşunun disk difüzyon yöntemiyle yapılan değerlendirilmesinde; %86.7'sinin nitrofurantaine, %78.8'inin kloramfenikol ve rifampisine, %72.1'inin eritromisine, %66.2'sinin ampisiline, %65.2'sinin gentamisine, % 59.1'inin ise tetrasikline duyarlı olduğu bildirilirken; izolatların %100'ünün nalidiksik asit ve kolistine, %87.9'unun penisiline ve %72.7'sinin ise streptomisine dirençli olduğu ifade edilmiştir (Abdel Wahab ve Shigidi 2013). Robaj ve ark. (2017), Kosova'da 284 adet hayvandan oluşan bir koyun sürüsünde klinik olarak KLA semptomları gösteren 38 hayvandan aldıkları örnekleri kültüre ederek, 32 (%84.2) hayvandan *C. pseudotuberculosis* izole ettiklerini bildirmişlerdir. Araştırıcılar yaptıkları antibiyogram testinde; izolatların amoksisilin/klavulanik asit ve oksitetrasikline %100, kloksasiline %95.8, gentamisine %91.7, trimetoprim/sulfametaksazole %83.3 ve streptomisine %16.7 oranında duyarlı olduğunu rapor etmişlerdir. Gerçekleştirilen bu çalışmada, izolatların %87.5'i enrofloksasine, %81.2'si neomisin/basitrasin/tetrasikline, %75.0'i penisilin/novobiosine, %68.7'si oksitetrasiklin ile amoksisilin/klavulanik asite, %62.5'i kloksasine, %56.2'si tetrasikline, %31.2'si ampisilin/sulbaktama ve %18.7'si ise trimetoprim/sulfametaksazole duyarlı bulunurken; tamamının ise streptomisine dirençli oldukları saptanmıştır. Konuyla ilgili çalışmalar birlikte değerlendirildiğinde, *C. pesudotuberculosis* suşlarının çeşitli antibiyotiklere olan duyarlık/dirençliliğinin oldukça farklılıklar gösterdiği görülmektedir. Bu durum, çalışmanın yapıldığı ülke ve bölgelerin farklı olmasıyla açıklanabileceği gibi izolasyon yapılan hayvanlara yönelik antibiyotik uygulamaları ve çalışmalarda uygulanan yöntemlerin farklılığıyla da (değişik besiyerlerinin kullanılması gibi) ilgili olabilir.

Diğer bakteriyel enfeksiyonlarda olduğu gibi KLA'in tedavisinde de en önemli rolü antibiyotikler üstlenmektedir. İlk antibiyotik olan penisilinin keşfinden itibaren hem beşeri hem de veteriner hekimlikte enfeksiyöz hastalıklarla mücadelede antibiyotiklerle önemli başarılar elde edilmiştir. Ancak ilk antibiyotiğin kullanımıyla birlikte, muhtemelen ilk antimikrobiyal direnç de görülmeye başlamıştır. Günümüzde Dünya Sağlık Örgütü (WHO) başta olmak üzere, konuyla ilgili birçok kuruluş ve bilim insanı tarafından gelişen antibiyotik dirençliliğine dikkat çekilerek, gelecekte yeni antibakteriyellerin bulunmaması halinde, bazı bakteriyel enfeksiyonların tedavisinde çok önemli sorunların yaşanabileceği bildirilmektedir (Lee ve ark. 2012, WHO 2020). Bakterilerin antibiyotiklere karşı direnç geliştirmesinde çeşitli faktörler rol oynamaktadır. Bunlardan belki de en önemlisi bilinçsizce ve rastgele antibiyotik kullanımıdır. Gerçekleştirilen bu çalışmada, KLA'in internal formundan izole edilen *C. pseudotuberculosis* suşlarının oldukça yüksek oranlarda streptomisin (%93.7), trimetoprim/sulfametaksazol (%75.0) ve ampisilin/sulbaktama (%68.7) dirençli bulunması, bölgede koyunlara test yapılmadan antibiyotik uygulanmasıyla ilgili olabilir. Diğer yandan bu durum, koyun orijinli *C. pseudotuberculosis* suşlarında söz konusu antibiyotiklere karşı çoğul direnç gelişimiyle de açıklanabilir.

Streptomisin 1943 yılında *Streptomyces griseus*'tan elde edilen bir antibiyotik olup, daha çok *Brucella* spp. ve *Mycobacterium* spp. gibi fakültatif intraselüler bakteri enfeksiyonlarının tedavisinde kullanılmaktadır (Quinn ve ark. 2001; Alavi ve Alavi 2013). Bu çalışmada, fakültatif intraselüler bir bakteri olan *C. pesudotuberculosis*'in streptomisine yüksek oranda (%93.7) dirençli olması, dikkat çekici bulunmuştur. Benzer durum, Kanada'da keçi orijinli (Muckle ve Gyles 1982), Fransa'da ise koyun ve keçi orijinli (Pepin ve ark. 1989) *C. pesudotuberculosis* suşlarıyla yapılan çalışmalarda da görülmektedir. Muckle ve Gyles (1982) ile Pepin ve ark. (1989) tarafından yapılan çalışmaların yapıldıkları dönemler dikkate alındığında, antibiyotiklere ulaşım ve kullanım oranlarının günümüz ve yakın geçmişe göre oldukça düşük düzeyde olduğu düşünülebilir. Bu hipotezden hareketle, koyun ve keçi orijinli *C. pesudotuberculosis* suşlarının streptomisine yüksek düzeyde dirençli olması, söz konusu bakterinin streptomisine doğal dirençli olabileceği şeklinde değerlendirilebilir.

C. pseudotuberculosis bir hayvan sürüsüne bulaştığında, enfeksiyonun kontrol altına alınmasında çeşitli sorunlar yaşanmaktadır. Bunlar arasında, hastalığın bazı vakalarda sub-klinik formda seyretmesi nedeniyle fazla sayıda hayvanın kısa sürede enfekte olması ve bakterinin hücre duvarındaki kalın lipit tabakası nedeniyle bazı antibiyotiklere dirençli olması sayılabilir (Abebe ve Tessema 2015). Bu durum dikkate alındığında, koyunlardaki KLA'in tedavisinde mutlaka antibiyotik duyarlılık test sonuçlarına göre tedavi stratejilerinin belirlemesi gerekmektedir. Sonuç olarak, rastgele ve uygunsuz antibiyotik kullanımı dirençli suşların yayılmasına neden olabildiği gibi tedavi giderlerinin de artmasına yol açmaktadır.

Çıkar Çatışması: Yazarlar, çıkar çatışması olmadığını beyan eder

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RESEARCH ARTICLE

A Retrospective Study on Wild Orphan Animals in Afyon Kocatepe University Wildlife Rescue Rehabilitation, Training, Practice and Research Center (AKUREM)

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ABSTRACT

This research is the first study on wild orphan animals in Turkey. The aim of this study is to evaluate the reasons of the orphans' arrival, their health status and fate in the Afyon Kocatepe University Wildlife Rescue Rehabilitation, Training, Practice and Research Center. Data on 118 orphans accepted the center in 2017, 2018 and 2019 were evaluated. A total of 16 different species, including 12 birds and 4 mammals, were recorded. Most of the orphans (89%) were birds and few mammals (11%). Most of the orphans (45.8%) were accepted to the center during the breeding seasons, especially in July. Most of the orphan birds were from the urban areas (94.3%) while most of the orphan mammals were from the rural areas (84.6%). The majority of orphans (81.4%) admitted to the rehabilitation center were uninjured and healthy. The mortality rate in the rehabilitation center (47.5%) was higher than those who were successfully released into wildlife (40.7%), those in captivity (5.9%) and euthanasia (5.9%). Despite the high rate of healthy offspring, the high mortality rate indicates that the rehabilitation process is difficult and requires expertise. As a result, not all wild offspring found by humans are orphans. Leaving healthy offspring in their habitat in a controlled manner can increase their chances of survival. It has been concluded that increasing education and awareness-raising activities both in rehabilitation centers and in the community will reduce the problem of orphans in wildlife.

Keywords: Afyonkarahisar, biodiversity, conservation, rescue, Turkey, wildlife

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Afyon Kocatepe Üniversitesi Yaban Hayatı Kurtarma Rehabilitasyon Eğitim Uygulama ve Araştırma Merkezindeki (AKUREM) Yabani Öksüz Yavrular Üzerine Bir Retrospektif Araştırma

ÖZ

Bu araştırma Türkiye'deki öksüz yaban hayvanları üzerine yapılan ilk çalışmadır. Bu çalışmanın amacı, Afyon Kocatepe Üniversitesi Yaban Hayatı Kurtarma Rehabilitasyon Eğitim Uygulama ve Araştırma Merkezi'ne getirilen yaban hayatı öksüz yavrularının geliş nedenlerini, sağlık durumlarını ve akibetlerini değerlendirmektir. Merkeze, 2017, 2018 ve 2019 yıllarında getirilen 118 öksüz yavruya ait veriler değerlendirilmiştir. Onikisi kuş ve dördü memeli olmak üzere toplamda onaltı farklı tür kayıt altına alınmıştır. Öksüz yavruların çoğunluğu kuşlardan (%89), azınlığı ise memelilerden (%11) oluşmuştur. Öksüz yavrular üreme sezonunda, yoğun olarak da Temmuz ayında (%45.8) merkeze kabul edilmiştir. Öksüz kuşların büyük kısmı (%94.3) kentsel alanlardan, öksüz memeliler ise daha çok (%84.6) kırsal alanlardan getirilmiştir. Rehabilitasyon merkezine kabul edilen öksüz yavruların çoğunluğu (%81.4) zarar görmemiş ve sağlıklıydı. Rehabilitasyon merkezindeki ölüm oranı (%47.5), yaban hayatına başarıyla salınanlardan (%40.7), esaret altında tutulanlardan (%5.9) ve ötenazi yapılanlardan (%5.9) daha yüksek bulunmuştur. Sağlıklı yavru oranı yüksek olmasına rağmen ölüm oranının yüksek olması rehabilitasyon sürecinin güçlüğünü ve uzmanlık gerektirdiğini göstermektedir. Sonuç olarak, insanlar tarafından bulunan her yabani yavru öksüz değildir. Sağlıklı yavruların yaşam alanlarında kontrollü bir şekilde bırakılması hayatta kalma şanslarını artırabilir. Bu konuda, hem rehabilitasyon merkezlerinde hem de toplumda eğitim ve bilinçlendirme faaliyetlerinin artırılmasının yaban hayatındaki öksüz yavru sorununu azaltacağı kanaatine varılmıştır.

Anahtar Kelimeler: Afyonkarahisar, biyoçeşitlilik, koruma, kurtarma, Türkiye, yaban hayatı

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INTRODUCTION

Wildlife are under threat from many different kinds of human activities not only in Turkey but also all over the World. With the industrialization and technological developments, the increase in the human population has reduced the distance between wild life and human beings and in some areas, species extinctions have started to occur due to human activities. As the urbanization of areas with wildlife habitats continues to increase, oppression are enforced on the wild animals (Schenk and Souza, 2014; Mckinney, 2002). Wild animals are harmed in nature by anthropogenic and natural causes (Molina-Lo´pez et al., 2017). Nowadays, it is inevitable to take wildlife protection measures, as many species are already extinct and many are in danger of extinction. This 6th wave of extinction of life, which has previously disappeared five times, is accelerating with human influence. It is necessary to reduce the destructive pressure of humans on wildlife (Ceballos et all, 2010).

Wildlife Rehabilitation is described as the treatment and impermanent care of injured, diseased and displaced or orphaned wild animals, and ultimately they can be returned to their natural habitat (Miller, 2012). Treating and rehabilitating animals that are damaged and releasing them to nature is one of the methods of wildlife protection. Rehabilitated wild animals can get a chance to live in nature again (Grogan and Kelly, 2013). "Orphaned" means that chicks, fledgling or young animals, supposedly abandoned by their parents or fallen from their nest or brought by humans on the grounds that they are alone in nature. Especially juvenil animals are seriously admission to wildlife rescue centers (Mullineaux, 2014). Since every wild animal babies need unique care and diets, wild orphan animal raising and releasing is very difficult in the rehabilitation center. Wildlife rehabilitation specialists and veterinarians who care for wild babies must know how to care for wild animals. On the other hand, while people teach orphaned wild animals how to survive in wild, they are not as competent as wild parents (Moore and Joosten, 1997, Ruth, 2012, Miller, 2012). Wild babies raised by humans may have problems in detecting dangerous situations to be avoided, getting to know all the nutrients, caring for babies, and communicating with each other in the same kinds. Many wild animals offspring are unnecessarily rehabilitated since biological parents are more succesfull than even well trained rehabilitators (Robertson and Harris 1995). Many baby animals are kidnapped by well-intentioned people. People want to save the offspring when they see alone and think they are abandoned. Unfortunately the fact is that, most of the baby animals encountered in the wild are not orphaned or abandoned and they don't need help (Ruth, 2012, Miller, 2012). First of all we accept this situation and then decide if wild animal need help or not. If the mother is known to be dead, if the baby has obvious injuries or look sick, if the baby is crying and appears to be poorly fed and dehydrated, then wild baby animals need help and should be rescued. In some cases, the baby needs only little help. Maybe it can be dropped into the nest where it fell from or abondoned. Although they are not injured, there are three obstacles to the offspring being considered orphans by being taken away from nature. It is important that whether these orphans will survive in rehabilitation centers, will orphans gain sufficient skills to survive, will orphans find a living space in nature without harming others in the environment (Wimberger and Downs, 2010). Wild animals take care of their offspring in a variety of ways, that may look like unusual behavior to us but it is actually just a normal part of the animal's strategy for survival in nature. Unfortunately, human raising strategies remain insufficient in this regard. (Tseng, 2002).

Afyonkarahisar is a rich province about biodiversity, located on bird migration routes due to its large number of wetlands, with habitats suitable for different animal species as it is at the transition area between the Aegean and Central Anatolia regions. For this reason, Afyon Kocatepe University Wildlife Rescue Rehabilitation Education Practice and Research Center (AKUREM) was started their activity in 2017. AKUREM is one of the a few rehabilitation center, assigned the task of rescuing and rehabilitating wild animals in Turkey.

Apart from Afyonkarahisar, AKUREM also provides services to wild animals brought from Eskişehir, Kütahya, Uşak and Denizli provinces within the scope of the protocol signed with the V. Regional Directorate of Nature Conservation and National Parks (DKMP). Wild animals, which are damaged in nature, are picked up by the Nature Conservation and National Parks Authorities and sent to the center upon the notification of citizens and mostly sensitive citizens. Among the many activities of the AKUREM, the care and feeding of orphaned offspring occupies an important place. When the breeding season starts in spring, many wild offspring are brought to AKUREM that they are found sometimes injured, sometimes sick but mostly healty on the ground. In this study probable reasons and therefore the fate patterns of wild orphan animals are investigated in a wildlife rehabilitation center in Turkey.

MATERIALS and METHODS

Between the period from 2017 to 2019 a total of 640 injured wild animals were brought to AKUREM in which 118 of them (18,4%) was orphan animals. A retrospective study was conducted based on the data of 118 applications of wild orphan animals to evaluate their fate at the AKUREM. The rehabilitation center is operated under the direction of the Afyon Kocatepe University, who stipulates the management

protocols and Ethical Principles according to the DKMP legislation.

Species were grouped into the following broader taxonomic categories for statistical analyses purpose. These groups were clasified as 13 mammals (including Artiodactyla-Red Deer, Carnivora-Red Fox, Equidea-Feral Horse, Rodentia-Red Squirrel) and 105 aves (including Accipitriformes- Common Kestrel, Long- legged buzzard, Eurasian hobby, Imperial Eagle, Apodiformes- Common Swift; Ciconiformes - Stork; Columbiformes- Pigeon; Cuculiformes- Common Cuckoo; Passeriformes- Eurasian Magpie and Sparrow; Piciformes- Syrian Woodpecker; Strigiformes- Long Eared Owl).

Information that was collected on admission of orphaned animals included rescue center number, date of admission, location found, reason for presentation and fate status. Relevant data was organised into a computerised database (Microsoft Office Professional Plus 2010, Microsoft Corporation, Redmond, Washington, USA). Microsoft Excel was used to calculate the summary statistics (totals, means and proportions), and to create graphical outputs.

RESULTS

The evaluation process was made on 118 admission (18.4% of all the admissions) reports (*Figure 1*).

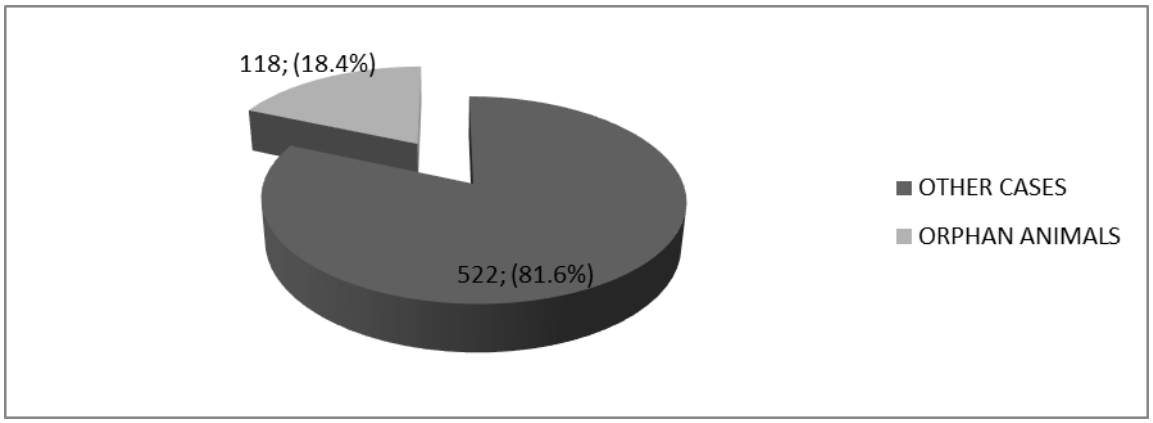

Figure 1. Percentages of total wild animals accepted to AKUREM from 2017 to 2019

Birds admission (89%) were more than the mamalian admission (11%) in the rehabilitation center (*Figure 2*).

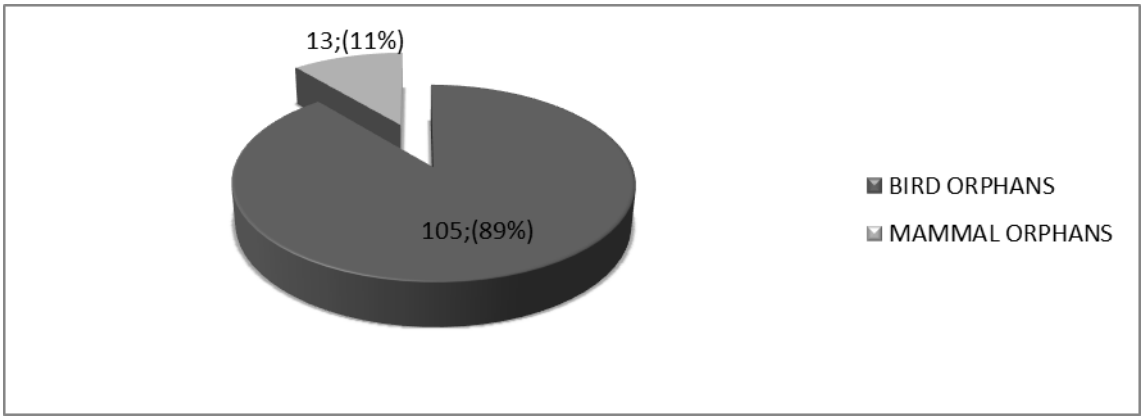

Figure 2. Percentages of orphan wild animals accepted to AKUREM from 2017 to 2019

The group under investigation was covered 16 different species. Four of these species were mammals and contained 13 animals under four orders namely Artiodactyla, Carnivora, Equidea and Rodentia. The remaining other 12 species were birds and contained 105 animals under 8 orders namely Accipitriformes, Apodiformes, Ciconiformes, Columbiformes, Cuculiformes, Passeriformes, Piciformes, Strigiformes. The distribution of the animals according to species and oders are given in Table 1. Only one animals, Imperial eagle (Aquila heliaca) was Vulnerable (VU) statues and the others were considered as Least Concerned (LC) categories

and by the IUCN (International Union for Conservation of Nature and Natural Resources) red list. Among the orphaned mammals accepted to the rehabilitation center, the highest rate were belongs to red deer (53.8%), followed by red fox (23.1%), red squirrel (15.4%) and feral foal (7.7%) respectively. Among the orphaned birds, the most frequently brought to the center, were common kestrel (Falco tinnunculus) (24.8%), common swift (Apus apus) (21.9%), pigeon (Columba livia) (19%) and eurasian magpie (Pica pica) (13.3%) respectively.

Table 1: Species in this research

MAMMALS

Artiodactyla	N	%
Red Deer (*Cervus elaphus*)	7	53.8
Carnivora		
Red Fox (*Vulpes vulpes*)	3	23.1
Equidea		
Feral Horse (*Equus caballus*)	1	7.7
Rodentia		
Red Squirrel (*Sciurus vulgaris*)	2	15.4
Total	**13**	**100**

BIRDS

Accipitriformes	N	**%**
Common Kestrel (*Falco tinnunculus*)	26	24.8
Long-legged buzzard (*Buteo rufinus*)	2	1.9
Eurasian hobby (*Falco subbuteo*)	1	0.95
Imperial Eagle (*Aquila heliaca*)	1	0.95
Apodiformes		
Common Swift (*Apus apus*)	23	21.9
Ciconiformes		
Stork (*Ciconia ciconia*)	5	4.8
Columbiformes		
Pigeon (*Columba livia*)	20	19.0
Cuculiformes		
Common Cuckoo (*Cuculus canorus*)	1	0.95
Passeriformes		
Eurasian Magpie (*Pica pica*)	14	13.3
Sparrow (*Passer domesticus*)	3	2.9
Piciformes		
Syrian Woodpecker (*Dendrocopos syriacus*)	1	0.95
Strigiformes		
Long Eared Owl (*Asio otus*)	8	7.6
Total	**105**	**100**

The distribution of orphaned mammals and birds admitted by the center by years and months are given in Table 2. In this study, the orphan rates were increased by the years (2017, 2018, 2019); 25, 36, 57 respectively. The breeding season (spring and summer months), especially in July, more orphan were admitted to center (45.8%). The proportion of orphaned accepted to the center regardless to the species in March, April, May, June, August, September and October were 0.9, 2.5, 23.7, 18.6, 5.9, 0.9 and 1.7% respectively. In autumn season, only juvenile pigeon were admitted to the center.

Table 2: Distribution of admitted orphans by season and months

Years	Orphan	MONTHS*								
		3.	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**TOTAL**
2017	Mammals	0	0	2	1	1	0	0	0	4
	Birds	0	2	2	2	11	4	0	0	21
									TOTAL	**25**
2018	Mammals	0	0	3	1	0	0	0	0	4
	Birds	0	0	4	11	14	2	1	0	32
									TOTAL	**36**
2019	Mammals	1	0	0	3	1	0	0	0	4
	Birds	0	1	17	4	27	1	0	2	53
									TOTAL	**57**
TOTAL		1	3	28	22	54	7	1	2	118
%		**0.9**	**2.5**	**23.7**	**18.6**	**45.8**	**5.9**	**0.9**	**1.7**	100
Seasons		**Spring**			**Summer**			**Autumn**		

*3. March, 4. April, 5. May, 6. June, 7. July, 8. August, 9. September, 10. October

The health status of orphaned animals brought to the center is shown in Figure 3 and 81.4% of orphans brought to the center were healthy while 18.6% of the orphans were found to be sick, frail or injured.

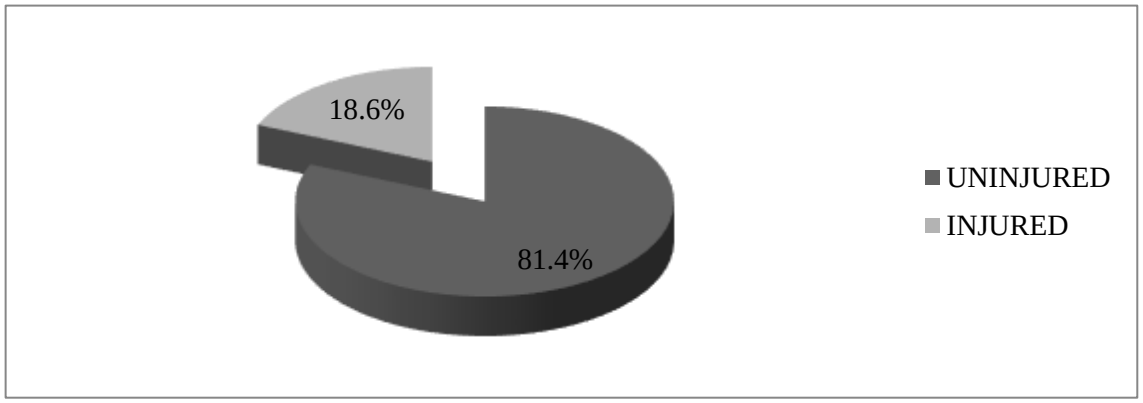

Figure 3. Health status of orphan animals

Information about the areas where the orphans found and brought to AKUREM is shown in Table 3. As it is easily seen from the Table 3, most of the orphans (85.6 %) came from the urban areas, while 14.4% came from rural areas. 94.3% orphans from urban areas were belongs to avian species while 15,4% were belongs to mammals. Opposite to this, 84.6% of the orphans from rural areas were belongs to mammals while only 5.7% were belongs to avian species.

Table 3: The areas where the orphans found

	URBAN AREA		RURAL AREA		TOTAL	
SPECIES	n	%	n	%	n	%
Mammals	2	15.4	11	84.6	13	**14.4**
Avian	99	94.3	6	5.7	105	**85.6**

The reasons for orphans' acceptance to the center are reported in Table 4. The causes can be classified as uninjured orphan 81.4%, animal attacked 2.5%, entangled or adhesive 4.2% and injured 11.9% were determined. When an overall assessment of the causes of orphans admitted to the center was made, the rate of uninjured orphans was found at the highest (81.4%). The rate of uninjured orphans were found to be high in both birds (81.9%) and mammals (53.8%).

Table 4. Descriptions of the reasons of the orphans to be accepted to the rehabilitation centre

Causes	Description	Case number				Total	
		Birds		Mammals			
		n	%	n	%	n	%
Uninjured	Orphan with no injuries	89	81.9	7	53.8	96	81.4
Animal attack	Attacked by dog, cat or non-domestic animals	1	0.9	2	15.4	3	2.5
Entangled or adhesive	Entangled in string, fishing line or smeared with adhesive	5	4.8	0	0	5	4.2
Injured	Broken bones, wounds, paralysed, blind or concussed	10	9.5	4	30.8	14	11.9
TOTAL		105	100	13	100	118	100

Descriptions of condition and fate patterns of orphaned animals admitted to the rehabilitation centre is shown in Table 5. After rescue, release to the wild life, captivity, euthanasia and mortality the rate were 42.9, 0.9, 6.7 and 49.5% for birds while 23.1, 46.2, 0, 30.7% for mammals respectively. When taking the total numbers into account for mixed species the rates were found to be 40.7, 5.9, 5.9, 47.5%.

Table 5. Definitions of the status and destiny patterns of orphans admitted to the rehabilitation center

Fate patterns	Description	Case number				TOTAL	
		Birds		Mammals			
		n	%	n	%	n	%
Release to wild	Releasable orphan, may successfully survive in the wild	45	42.9	3	23.1	48	40.7
Captivity	Non-releasable orphan, poor survivability in the wild	1	0.9	6	46.2	7	5.9
Euthanasia	Humanely assisted death, with worst prognosis	7	6.7	0	0	7	5.9
Died	Unassisted natural death	52	49.5	4	30.7	56	47.5
TOTAL		105	100	13	100	118	100

DISCUSSION

In this study which as explained in the materials and methods, the ratio of orphans was 18.4% of all the wild injured animals (640) brought to the Akurem. This ratio of orphans brought to any rehabilitation centre was more or less similar to that of the studies made in Australia (24.65%-Taylor-Brown A, et al., 2019), in South Africa (43% -Wimberger&Downs, 2010) and in Alabama USA (74%-Williamson& Lepczyk, 2017). As can be understood from the ratios, orphaned animals have occupied a significant position at the some different rehabilitation centers.

It was observed that the bigger proportion of the orphans admitted to the AKUREM was birds (89%) and the rest was mammals (11%). It was reported by Smith (2016) that the most of the orphans come to the rehabilitation center in the spring was birds rather than mammals. Similarly, statistics from the data for the year 2000, indicated that approximately 67% of casualties were birds, and 32% mammals (Kirkwood, 2003).

The reason for this seems that the habitats between human and birds are much closer compare to mammals and therefore birds seems to be much vulnerable than mammals. One can assume that the spring was the time of the year that the breeding season for birds started and it is most likely that the fledgling may fallen from their nests during spring (Smith, 2016).

On the contrary, more mammals (56.6%) were brought to wildlife rescue centers rather than birds in Australia (Taylor-Brown et al. 2019). However, in the BWRC (British Wildlife Rehabilitation Council) analysis showed that 50% of birds and 54% of mammals admissions were of offspring animals (Kirkwood 2003) in the UK. It is thought that wildlife rescue centers' habitat and biodiversity situation affects this ratio between birds and mammals. Among the orphaned mammals the highest rate belongs to red deer fawn with 53.8% Table 1. Red deer fawn can walk shortly after birth, and they won't begin to follow their mother until about one month of age. For this reason, their mother keeps them in a suitable place, for example in the bush, and goes to feed and returns soon. Spotted coats of the fawns serve as camouflage having no smell to help to avoid to be found by predators.

In this study, the orphan number was increased by the years, and the number of orphans adopted in 2019 was the highest (Table 2). This can be explained by the fact that the effectiveness of the operation of the center has increased over the years. On the other hand, this finding may also indicate that the social awareness to wild life is developing among the people by the time. It's common for humans to encounter baby birds and mammals during the birth or incubation season (spring and summer). In our study, the offspring were mostly admitted to center in July (45.8%) that was similarly reported by Wimberger and Down (2010). They reported that incubation of birds that hatch several times, such as pigeons, can also be admitted to the center in autumn in South Africa. This finding also supported by Kelly and Bland (2006). They reported that admissions for European sparrowhawks (*Accipiter nisus*) species were mostly seasonal, with most birds being admitted between July and September.

Many wild orphan animals (85.6%) brought to the AKUREM were from the urban areas while 14.4% were from rural. 94.3% of the orphans in urban areas were belongs to birds while only 15.4% were mammals. This can be explained by the fact that birds live closer to humans in urban areas (Table 3). Among the birds, common kestrel (24.8%), common swifts (21.9%) and pigeons (19.0%) were admitted distinctively more than the other species (Table 1). Those birds living in close association with humans were the most frequently admitted to rehabilitation centres. Some bird species such as pigeons live close to humans as they can meet a significant portion of their food requirements in urban areas (Marchesini, 2016). The reason why orphan birds are higher in urban areas may be due to the possibility of nesting in roof and roof eaves of the buildings. Wild birds can build their nests on trees, shrubs, under rocks and tree stumps, and in chimneys. As stated by Soulsbury and White (2015) urban wildlife has both positive and negative interactions with people. Since birds live close to humans, their offspring are easily found and easily collected, while mammals often avoid humans and tend to be encountered only when they clash with humans (Wimberger and Down, 2010).

In this study, 81.4% of orphans admitted to the center were identified as uninjured juvenils and most of them (81.9%) was orphan birds. Baby birds can spend days to weeks in the nest and they are fed by their parents during their early period of life. If the baby birds that fallen from their nest was healthy, they must be placed back in their nests by the rescuers. At the early ages the healthy baby birds may leave the nest and hop along the neighboring branches for gaining strength or flying exercise may fallen ground. In that case, people with not enough knowledge about wildlife find those animals, they think that those animals are orphan and unnecessarily take such animals to rehabilitation center. (Williamson&Lepczyk 2017). Normally those animals should be placed back to their parents care in their nests. If the fledging is looks healty should be left at a point where cats, dogs and other creatures cannot reach without being removed from their area (Miller, 2012).

After rescue and rehabilitation process, orphaned animal releasing success was 40.7% (Table 5). This finding was supported Grogan and Kelly (2013). It was reported by RSPCA's (Royal Society for the Prevention of Cruelty to Animals) wildlife centres as the typical of other wildlife centres in the UK, around

40 per cent of casualties are released. Releasing success was higher (42.9%) in birds than the mammals (23.1%). This finding is thought to be due to the arrival of many healthy uninjured juvenil birds. Kelly et all. (2011) have found the release rate of 31% in orphaned wood pigeons (Columba palumbus) and they have found the euthanased rate of 37%, the died rate was 30%. On the other hand our euthanased rate was only 6.7% and mortality rate was 49.5% in birds. The reason was that in some cases we hoped that they would get better rather than euthanased, but it didn't happen. Unfortunately, the death rate (47.5%) was more than our releasing rate (40.7%) for all the orphans.

The rate of captivity was found to be very high (46.2%) in unhealthy orphan mammals (Table 5). For some orphaned wild animals, life in captivity is safer. The prognosis for rescued orphans in captivity is not always positive. They needed specialized care with special care facilities, time and resource. Furthermore, they are especially susceptible to disease and other captivity related problems (imprinting). Hovewer, fledglings have a much better chance of survival in the wild than in captivity (Smith, 2016). We can say that the survival skills of orphaned birds thrown out by their families from their nests are poor, even if they seem physically healthy. This may be the reason for the mortality rate which was quite high. On the other hand, this high mortality rate also may be due to the fact that our center has been in operation recently and therefore our facilities are not sufficient. Insufficiency of wildlife veterinarians and rehabilitators and their lack of education in Turkey have previously been reported by Kandir&Aslan (2017). It is obvious that the high standard facilities, care programs, housing systems, hygiene, handling, nutrition and treatment practices are important factors for rehabilitation success (Dubois and Fraser, 2003).

Captivity rate for unhealthy orphan mammals was found very high (46.2%). For some orphaned wild animals, life in captivity is safer then release to nature. Because of some young animals raised by human, think themselves are human and they may develop a different instict from others. Animals that develop instincts in this way will not want to be with other members of their own species and will not be able to properly interact with them (Ruth, 2012). Regarding the fate of orphan mammals, 5 red deer fawn (*Cervus elaphus*) were sent to a semi-natural area belonging to DKMP after weaning (kept in captivity) and one feral horse (*Equus caballus*) yearling continues to be kept in captivity in the AKUREM. Of the birds, only one Imperial Eagle (*Aquila heliaca*) could not return to nature due to wings problem and remained in the center for student educational purposes.

CONCLUSIONS

This research is important as it is the first study in Turkey for orphaned wild animals to examine the acceptance reasons and fate patterns in the wildlife rehabilitation center. Every breeding season, a large number of wild orphan animals are taken to rehabilitation centres for treatment, care, raise and release. Therefore, the analysis of admission records of wild orphan animals to the rehabilitation center and the resulting actions can provide important information about wildlife rescue. Three important conclusions were reached in this research;

1. Social education and awareness activities on this subject will reduce the problem associated with orphan animals in the wildlife.
2. With the technical knowledge obtained from the researches on wildlife, the attractiveness of the research making will increase and as a result, the success of the rehabilitation centers will increase.
3. Increasing the number of such studies will be an opportunity for wildlife rehabilitation centers to measure their success and reach higher levels. In this context, it can be concluded that cooperation between countries is important in the partnership of states, universities and NGOs.

Conflict of Interest: The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Investigation of *Enterobius Vermicularis* Contamination in Parks and Public Transportation Vehicles in Eskişehir

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ABSTRACT

In this study, it was aimed to determine the prevalence of *E. vermicularis* in various public transportation vehicles and parks in the city center of Eskişehir. For the study, 7 public transportation vehicles and 6 parks were selected in the city center of Eskişehir. Diagnosis was made using cellophane tape method. A total of 192 samples were collected from children's parks and public parks, and 138 samples from public transportation vehicles. In 4.1% of the samples taken from the parks; *E. vermicularis* egg was found in 0.8% of the samples taken from public transportation vehicles. It has been observed that park and public transportation vehicles can be effective in *E. vermicularis* infection. It was identified that deficiencies in public health services, environmental and personal hygiene were identified and necessary measures were taken.

Keywords: *Enterobius vermicularis,* playground, public transport vehicles, Eskişehir

***

Eskişehir'de Park ve Toplu Taşıma Araçlarında *Enterobius vermicularis* Kontaminasyonunun Araştırılması

ÖZ

Bu çalışmada Eskişehir il merkezinde hizmet veren çeşitli toplu taşıma araçları ile parklarda *Enterobius vermicularis*'in yaygınlığını belirlemek amaçlanmıştır. Çalışma için Eskişehir il merkezinde bulunan 7 toplu taşıma aracı ile 6 park seçilmiştir. Teşhis selofan bant yöntemi kullanılarak yapılmıştır. Çocuk parkları ve genel parklardan toplam 192 örnek, toplu taşıma araçlarından toplam 138 örnek toplanmıştır. Parklardan alınan örneklerin %4,1'inde; toplu taşıma araçlarından alınan örneklerin %0,8'inde *E. vermicularis* yumurtasına rastlanmıştır. *E. vermicularis* enfeksiyonunda park ve toplu taşıma araçlarının etkili olabileceği gözlenmiştir. Halk sağlığı hizmetleri, çevresel ve kişisel hijyen konusundaki eksikliklerin belirlenerek önlemlerin alınmasının gerekliliği belirlenmiştir.

Anahtar Kelimeler: *Enterobius vermicularis,* oyun parkı, toplu taşıma araçları, Eskişehir

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GİRİŞ

Oksiyür ya da kıl kurdu olarak bilinen *Enterobius vermicularis*, insan ve şempazelerde özellikle kalın bağırsağın sekum ve rektum bölgesinde yerleşim gösteren nematodlardan biridir. Bu parazit krem rengindedir. Erkekleri 2-5 mm dişileri ise 8-13 mm uzunluğundadır. Tüm dünyada bulunmakla birlikte, ılıman bölgelerde daha fazla görülmektedir (Güralp 1981, Özcan ve ark. 2004). Bu parazitin yumurtalarının enfekte hale gelebilmesi için, özel ortamlara (toprak, su gibi) ihtiyacı yoktur. Bu sebeple *E. vermicularis* geniş kitleleri etkileyerek yaygın enfeksiyonlara neden olmaktadır (Unat 1979, Zahariou ve ark. 2007). *Enterobius vermicularis* enfeksiyonunun en önemli belirtisi anal kaşıntıdır. Anal kaşıntı yumurtlamadan önce dişi parazitlerin anüs dışına göçü ile ortaya çıkmaktadır. Diğer belirtiler arasında ise iştahsızlık, bulantı, salya akması, sinirlilik, zayıflama, korkulu rüya, uykuda işeme ve uykusuzluk bulunmaktadır (Merdivenci 1978, Wang ve ark. 2016).

E. vermicularis'in dünyadaki yaygınlığı %2-42 arasında değişmektedir (Giray ve Keskinoğlu 2006). Ekonomik durumu ve eğitim düzeyi düşük, gecekondu yapılaşması fazla, temiz içme suyu yetersiz ve kanalizasyon konusunda alt yapı eksiklileri olan toplumlar arasında sık görülmektedir (Altındiş ve ark. 2004, Giray ve Keskinoğlu 2006, Ariyarathenam ve ark. 2010). Ülkemizde doğu bölgelerinde batı bölgelerine göre daha fazla görülmektedir. Sosyoekonomik koşulların kötü olduğu batı bölgelerinde de sık karşılaşılmaktadır (Altındiş ve ark. 2004, Giray ve Keskinoğlu 2006).

Halk tarafından kullanılan otobüs durakları, toplu taşıma araçları, halka açık tuvaletler, halka açık parklar ve halkın toplandığı meydanlar parazitle enfekte olma olasılığı yüksek olan yerlerdir (Borges ve ark. 2009). Bu araştırmada Eskişehir il merkezinde hizmet veren çeşitli toplu taşıma araçları ile oyun parklarında *E. vermicularis*'in yaygınlığını belirlemek amaçlanmıştır.

MATERYAL ve METOT

Araştırmanın örnekleri Nisan-Haziran 2017 tarihleri arasında Eskişehir il merkezinde hizmet veren ve araştırmacılar tarafından rastgele seçilen 7 toplu taşıma aracı ile 6 parktan temin edilmiştir. Örneklerin toplanması için hava koşullarının uygun olduğu zamanlarda toplu taşıma araçlarına ve parklara gidilmiştir ve el temasının fazla olabileceği düşünülen kısımlara selofan bant uygulanmıştır. Çocuk parklarından toplam 192 örnek, toplu taşıma araçlarından toplam 138 örnek toplanmıştır. Toplanan örnekler ağzı kilitli plastik torbalar içine konularak laboratuvara getirilmiştir. Laboratuvara getirilen örnekler 10X ve 40X büyütmede ışık mikroskobu altında kıl kurdu yumurtaları yönünden değerlendirilmiştir. Örneklerin analizinde yüzdelik ve bağımsız örneklem t testi kullanılmıştır. İstatistiksel anlamlılık $p<0,05$ olarak kabul edilmiştir.

BULGULAR

Alınan örneklerin laboratuvarda değerlendirilmesi sonucu Tablo 1, Tablo 2 ve Tablo 3'te görülen değerler elde edilmiştir.

Table 1. Distribution of positive and negative values detected in the samples collected areas.
Tablo 1. Örneklerin toplandığı alanlarda saptanan pozitif ve negatif değerlerin dağılımı.

Örnek Alınan Alan		**Pozitif (yumurta var)**		**Negatif (yumurta yok)**		**Toplam**	
		n	**%**	**n**	**%**	**n**	**%**
Parklar (n=192)	Park 1	0	0,0	6	100,0	6	100,0
	Park 2	0	0,0	26	100,0	26	100,0
	Park 3	0	0,0	40	100,0	40	100,0
	Park 4	2	3,6	54	96,4	56	100,0
	Park 5	3	7,5	37	92,5	40	100,0
	Park 6	3	12,5	21	87,5	24	100,0
Toplu Taşıma Araçları (n=138)	Araç 1	0	0,0	18	100,0	18	100,0
	Araç 2	0	0,0	18	100,0	18	100,0
	Araç 3	0	0,0	20	100,0	20	100,0
	Araç 4	0	0,0	22	100,0	22	100,0
	Araç 5	0	0,0	20	100,0	20	100,0
	Araç 6	1	5,0	19	95,0	20	100,0
	Araç 7	0	0,0	20	100,0	20	100,0

Örneklerin toplandığı parklar incelendiğinde Park 1, Park 2 ve Park 3'de *E. vermicularis* yumurtası saptanmamıştır. Park 4'den toplanan örneklerin %3,6'sında, Park 5'den toplanan örneklerin %7,5'inde ve Park 6'dan toplanan örneklerin %12,5'inde *E. vermicularis* yumurtası saptanmıştır (Tablo 1).

Örneklerin toplandığı toplu taşıma araçları incelendiğinde Araç 1, Araç 2, Araç 3, Araç 4, Araç 5 ve Araç 7'de *E. vermicularis* yumurtası saptanmamıştır. Araç 6'dan toplanan örneklerin %5,0'inde *E. vermicularis* yumurtası saptanmıştır (Tablo 1).

Table 2. Positive and negative values detected in the samples collected areas.
Tablo 2. Örneklerin toplandığı alanlarda saptanan pozitif ve negatif değerler.

Örnek Alınan Alan	**Pozitif (yumurta var)**		**Negatif (yumurta yok)**		**Toplam**	
	n	**%**	**n**	**%**	**n**	**%**
Parklar (n=192)	8	4,1	184	95,9	192	100,0
Toplu Taşıma Araçları (n=138)	1	0,8	127	99,2	138	100,0

Örneklerin toplandığı alanlar incelendiğinde parkların %4,1'inde ve toplu taşıma araçlarının %0,8'inde *E. vermicularis* yumurtası saptanmıştır (Tablo 2). Parklarda bulunan *E. vermicularis* yumurtası toplu taşıma araçlarından yaklaşık 5 kat fazladır.

Table 3. Distribution of positive values detected in ınfected areas where samples are collected.
Tablo 3. Örneklerin toplandığı enfekte alanlarda saptanan pozitif değerlerin dağılımı.

Örnek Alınan Alan		**Pozitif**	
		n	**%**
Parklar (n=192)	Park 1	0	0,0
	Park 2	0	0,0
	Park 3	0	0,0
	Park 4	2	22,2
	Park 5	3	33,3
	Park 6	3	33,3
Toplam		**8**	**88,8**
Toplu Taşıma Araçları (n=138)	Araç 1	0	0,0
	Araç 2	0	0,0
	Araç 3	0	0,0
	Araç 4	0	0,0
	Araç 5	0	0,0
	Araç 6	1	11,2
	Araç 7	0	0,0
Toplam		**1**	**11,2**
Genel Toplam		**9**	**100,0**

Örneklerin toplandığı enfekte alanlar incelendiğinde saptanan *E. vermicularis* yumurtasının %88,8'inin parklarda, %11,2'sinin toplu taşıma araçlarında olduğu belirlenmiştir. Parklarda saptanan yumurtaların tamamının genel parklarda olduğu görülmüştür. Toplu taşıma araçlarında saptanan parazit yumurtalarının tamamının Araç 6'da olduğu görülmüştür (Tablo 3).

Parklar ve toplu taşıma araçlarından alınan numunelerde saptanan pozitif *E. vermicularis* yumurtalarının ortalamaları karşılaştırıldığında istatistiksel olarak anlamlı bir fark bulunamamıştır (p=0,112). Parklar ve toplu taşıma araçlarından alınan numunelerde saptanan negatif *E. vermicularis* yumurtalarının ortalamaları karşılaştırıldığında istatistiksel olarak anlamlı bir fark bulunamamıştır (p=0,165).

TARTIŞMA

Bu çalışmada toplanan veriler, genel toplumun rahatlıkla ulaşabildiği toplu taşıma araçları ve

parklardan örnek alınması aracılığıyla elde edilmiştir. Enterobiasis'in daha çok okul öncesi ve okul dönemi çocuklarda görülmesi nedeniyle (Giray ve Keskinoğlu 2006) literatürde yer alan çalışmaların çoğunlukla anaokulu (Cazorla ve ark. 2006, Artan ve ark. 2008, Chu 2012, Haddad ve ark. 2014, Anuar ve ark. 2016), ilkokul öğrencileri (Özcan ve ark. 2004, Giray ve Keskinoğlu 2006, Cazorla ve ark. 2006, Hazır ve ark. 2009, Çeliksöz ve ark. 2010, Chai 2015) üzerinde yapıldığı görülmüştür. Diğer bazı araştırmalarda, etkenin yetişkinlere de kolaylıkla bulaşabildiği (Kochan ve ark. 2015), bazı bölgelerde hem yetişkinler hem çocuklar (Köksal ve ark. 2010) hem de yaşlılar için büyük bir halk sağlığı problemi oluşturduğu ve sayılan tüm gruplarda ölüme neden olabileceği bildirilmiş, enfekte olan kişilerin tekrar tekrar enfeksiyon geçirebilmesinin çok yaygın olduğu da vurgulanmıştır (Rasti ve ark. 2012). Bu nedenle çocuklardan numune alınarak yapılan çalışmaların yanı sıra, topluma açık yerlerden numuneler alınması gereklidir. Bu çalışma içerik bakımından literatürde sıklıkla karşılaşılan çalışmalardan farklıdır.

Afyonkarahisar'da yapılan bir araştırmada oyun parklarından alınan örneklerde parazitin yumurtasına rastlanmamıştır. Toplu taşıma araçlarından alınan örneklerin %1,33'ünde parazit yumurtasına rastlanmıştır (Dedeoğlu ve Kozan 2011). Bu çalışmada ise parklardan alınan örneklerin %4,1'inde; toplu taşıma araçlarından alınan örneklerin %0,8'inde *E. vermicularis* yumurtasına rastlanmıştır. Araştırmamızda saptanan *E. vermicularis* yumurtasının Afyonkarahisar'da yapılan çalışmada saptanandan yaklaşık 3,5 kat daha fazla olduğu görülmüştür. Bu durum aralarında kilometre olarak çok fazla uzaklık olmayan bu şehirlerin bulunduğu bölgesel farklılıktan, nüfuslarından ve sahip olduğu üniversite öğrencisi potansiyelindeki farklılıktan kaynaklanabileceğini akla getirmektedir. Ayrıca son yıllarda Eskişehir'e olan göçünde Enterobiasis enfeksiyonunun artışına neden olabileceğini düşündürmektedir.

Hem Afyonkarahisar'da hem de bu araştırmada oyun parklarında *E. vermicularis* yumurtasına rastlanmamıştır. Parklarda olduğu saptanan *E. vermicularis* yumurtalarının tamamı genel parklarda bulunmaktadır. Bu durum çocukların yanı sıra yetişkinlere yönelik çalışmaların da arttırılmasının gerekli olduğunu göstermektedir. Brezilya'da yürütülen bir çalışmada otobüslerden alınan örneklerin %18,7'sinde ve parkta yer alan iki tuvaletten alınan örneklerin %6,25'inde *E. vermicularis* yumurtasına rastlanmıştır (Borges ve ark. 2009). Bu çalışmada saptanan değerler bizim elde ettiğimiz değerlerden çok yüksektir. Bu durum ülkelerin sahip olduğu halk sağlığı hizmetleri, çevre koşulları ve kişisel hijyen uygulamalarındaki farklılıklardan kaynaklanabilir. Yapılan iki çalışmada da topluma açık yerlerden alınan örnekler analiz edilmiştir. Bu çalışmalara benzer başka bir araştırmaya literatürde rastlanmamıştır.

SONUÇ

Bu araştırmada incelenen 320 örnekten toplam 9'unda *E. vermicularis* yumurtası saptanmıştır. Yumurta pozitif örneklerin sekiz tanesi parklarda ve bir tanesi de toplu taşıma araçlarında bulunmuştur. Parklardan alınan örneklerde saptanan *E. vermicularis* yumurtası toplu taşıma araçlarından alınan örneklerden yaklaşık 5 kat fazladır. Çocuk parklarında *E. vermicularis* yumurtası saptanmamıştır.

Ülkemizde *E. vermicularis*'e yönelik yapılan araştırmalar sıklıkla okullardan örnek toplanarak yapılmaktadır. Otobüs, otobüs durakları, parklar ve halk tarafından kullanılan tuvaletler gibi topluma açık yerlerden daha fazla örnek alınarak, kapsamı daha geniş araştırmalar yapılması *E. vermicularis*'in ülkemizdeki durumunu ve risk altındaki toplumu belirlemeye yardımcı olacaktır. *E. vermicularis*'in durumunun tespit edilmesi ülkemizde halk sağlığı hizmetleri, çevresel ve kişisel hijyen konusundaki eksiklikleri ortaya çıkartabilir. Bu konuda eksikliklere yönelik alınacak önlemler, enfeksiyonla mücadelede önemli rol oynayabilir.

Çıkar Çatışması: Yazarlar, çıkar çatışması olmadığını beyan eder.

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RESEARCH ARTICLE

Assessment of Morphological Features of Bull Semen Thawed at Various Temperatures and Periods of Time with CASA

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ABSTRACT

Thawing condition is one the most important factors affecting the re-animation of the spermatozoon in order to fertilise the oocyte. For that matter, we aimed to evaluate the morphological features of the head and midpiece of cryopreserved spermatozoa thawed at different temperatures and for various durations, with using CASA (Computer aided sperm analyser). Frozen semen samples belonging to the same batch, collected from three different bulls were grouped as; control group thawed for 20 seconds at 37 ℃; and experimental groups were thawed for 30, 40, 50 and 60 s at 25 ℃ and 37 ℃; for 10, 15, 20 and 25 s at 40 ℃; for 3, 6, 9, 12 s at 70 ℃. Morphometrical features of the samples were evaluated by using CASA system with nine repetitions. As a result, straws thawed at 25℃ for 40 s had the highest average length of head (6.22 ± 0.09 μm), and the width of mid-piece (0.68 ± 0.01 μm). It was concluded that the thawing temperature and duration has affected/altered the morphometry of the sperm head and midpiece, although the results were not statistically significant (p> 0.005).
Keywords: Bull sperm, CASA, morphometry, sperm head, sperm mid-piece

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Farklı Süre ve Sıcaklıklarda Çözdürülen Boğa Spermalarının Morfolojik Fonksiyonlarının CASA Cihazı ile Değerlendirmesi

ÖZ

Suni tohumlamada kullanılan dondurulmuş spermanın çözüm sonu parametrelerini belirleyen faktörlerin başında çözdürme koşulları gelmektedir. Yapılan araştırma ile Simental, Holstein ve Brown Swiss ırkı boğaların donmuş spermalarının, deneysel olarak belirlenen farklı sıcaklık ve sürelerde çözdürülmesi sonucu CASA cihazı ile spermatozoon başı ve orta kısmına ait parametreler bakımından değerlendirilmesi amaçlanmıştır. Çalışmada gruplar; kontrol grubu 37 ℃'de 20 saniye; deney grupları ise, 25 ℃'de 30, 40, 50 ve 60 sn.; 37 ℃'de 30, 40, 50 ve 60 sn.; 40 ℃'de 10, 15, 20 ve 25 sn.; 70 ℃'de 3, 6, 9, 12 sn. olarak belirlenmiştir. Üç farklı ırk boğaya ait, tek ejakülasyonda elde edilen dondurulmuş boğa spermaları, 9 tekrarda CASA parametreleri açısından değerlendirilmiştir. 25 ℃'de 40 sn.'de çözdürülen spermaların baş uzunluklarının (6.22 ± 0.09), orta kısım genişliğinin (0.68 ± 0.01) diğer sıcaklık ve sürelere göre daha yüksek ortalamaya sahip olduğu saptanmıştır. Çözdürme sıcaklık ve süresinin spermatozoon baş ve orta kısmına dair parametreleri değiştirdiği ancak elde edilen sonuçların istatiksel olarak anlamlı olmadığı belirlenmiştir (p> 0.005).
Anahtar Kelimeler: Boğa spermatozoonu, CASA, morfoloji, spermatozoon başı, spermatozoon orta kısmı

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INTRODUCTION

In general, the length and width of the sperm head varies depending on the changes in the size and organization of the acrosome and nuclei (Mortimer, 2018, Gu et al. 2019). The relationship of sperm head and midpiece morphometry with fertility has been clearly established (Gil et al., 2009; Valverde et al., 2016; Yaniz et al., 2015), indicating that males with high fertility rates produce longer and conical spermatozoa (Beletti et al. 2005; Gravance et al. 2010). However, cryopreservation and thawing procedure causes acrosome, midpiece and tail abnormalities that are primarily originated by plasma membrane damage, resulting in rendering sperm cells with impaired fertilization ability. This indicates the presence of variation both in the membrane properties of individuals and their responses to freeze-thawing (Valverde et al. 2016) conditions and osmotic stress is related differences in the plasma membrane volume and shrinkage.

Changes in the temperature during the freezing and thawing of the sperm reduce the rate of motile spermatozoa and cause ultra-structural, biochemical and functional damage (Senger 1980). The fertilization ability of spermatozoa after thawing is greatly affected by the thawing temperature and duration (Senger 1980, İleri and Ak 1993, Nur et al. 2003). Subtle differences in shape, volume or area between spermatozoa are likely to be responsible for differences in water inlet and outlet microfluidic channel velocity throughout the plasmalemma during cryopreservation as well as the thawing process (Tekin and Daşkın 2019, Nur et al. 2003), which may be the source of morphologically different sperm subpopulations (Nunez-Martinez et al. 2007). Thus, leads to sperm chromatin changes during these processes (Karabinus et al. 1997). These changes are responsible for a decrease in the surface area of the sperm head, which may be caused by excessive condensation of spermatozoon nucleochromatin (Royere et al. 1998). Changes in chromatin have been associated with a reduced fertility in bulls (Sailer et al. 1996; Ostermeier et al., 2001; Januskauskas et al. 2003). As a result of the chromatin structure alterations and the decrease at the surface area of the sperm head, abnormal morphological structure may occur in the sperm head. Abnormal chromatin structure and head related morphological abnormalities of the spermatozoon are responsible for the loss of fertility in bulls (Saacke and White 1972; Gravance et al. 1998).

The midpiece morphology has a key importance for the mobilization of the spermatozoon due to phospholipid hydroperoxide glutamate peroxide and tyrosine phosphoryl proteins, which both have a primary role in sperm motility (Shivaji et al. 2009) that generates the ATP synthase beta subunit. Mitochondria are the source of adenosine triphosphate (ATP) in the midpiece of the sperm. It transmits energy to axonemia located in the principal part that produces the driving force of sperm (Piomboni et al. 2012, Gu et al. 2019). Therefore, the lengths of the sperm midpiece and the principal part can be critical in determining the sperm swimming rate and fertilization rate (Firman et al. 2010, Bennison et al. 2015; Gu et al. 2019). In house mice and primates, the length of the sperm midpiece was largely considered a reasonable predictor of sperm swimming rate/velocity (Anderson and Dixson 2002, Firman et al. 2010, Gu et al. 2019). With regard to sperm competition, beneficial changes in sperm morphology can increase swimming speed of the sperm cells, which is the main determinant of fertilization success (Ramm et al. 2005, Fisher et al. 2016, Gu et al. 2019). Interestingly, Non-Return Rates (NRR) in cows inseminated with the cryopreserved semen in which mid-length was measured, resulted with up to a 5% reduction in NRR in those with a longer midpiece (Shahani et al. 2010). Moreover, alterations in midpiece has a negative impact on the normal product of cellular respiration, thus creates Reactive Oxygen Species (ROS) that subsequently accumulates in the sperm midpiece and mitochondria (Desai et al. 2010).

Since the fertilising ability of sperm is highly affected by environmental conditions, the success of artificial insemination depends on providing the optimum conditions (Leticia, et al., 2013). While routine laboratory studies use scientific guidelines for thawing temperature and time values; indeed field conditions are usually different than those of the laboratory. The alterations in the morphometrical feauters of the sperm that could occur during the different thawing conditions can directly affect the result of insemination. Owing to the recent development of digitalized microscopic evaluation of spermatozoa with CASA (Computer Assisted Semen Analysis) technology, morphometric measurement of a spermatozoon became optimized and thus, introduced new possibilities in the spermatology assessment (Garcia-Herreros et al. 2007, Yaniz et al. 2015).

In this study, it was aimed to evaluate the morphological alterations in the head and midpiece of the spermatozoa subjected to different thawing time and temperatures in order to reflect various possible field conditions prior to artificial insemination practice.

MATERIALS and METHODS

Selection of Semen

For this study, a total of 153 commercially frozen semen straws from three different bull breeds

(Holstein, Simmental and Brown Swiss) were used. All the semen samples were selected from a single semen batch to eliminate individual differences, diluted, packed in 0,25 ml/straw, frozen and stored at -196℃ under the commercial standard cryopreservation process by Supergenetics® company, Konya/Turkey. Only the frozen semen samples were accepted with the following criteria met; motility with 70%, morphologically normal spermatozoa rate and viability with 80 % were used.

Table 1. Thawing temperatures and durations of the groups

Groups	Thawing temperature and times
Control	37 ℃ 20 s
Group 1	25 ℃ 30 s
Group 2	25 ℃ 40 s
Group 3	25 ℃ 50 s
Group 4	25 ℃ 60 s
Group 5	37 ℃ 30 s
Group 6	37 ℃ 40 s
Group 7	37 ℃ 50 s
Group 8	37 ℃ 60 s
Group 9	40 ℃ 10 s
Group 10	40 ℃ 15 s
Group 11	40 ℃ 20 s
Group 12	40 ℃ 25 s
Group 13	70 ℃ 3 s
Group 14	70 ℃ 6 s
Group 15	70 ℃ 9 s
Group 16	70 ℃ 12 s

Thawing of the Semen

Frozen semen straws from Simental, Holstein and Brown Swiss were divided into 17 groups according to the thawing time and temperature. Each semen straw (0.25 ml) was thawed at the temperatures and times given in the groups (Table-1). The straws were thawed in a water bath (Memmert, MiniTube®, Germany), taken to the Eppendorf tubes (1.5 ml.) previously warmed at heating table (Leica, HI 1220, Germany) then immediately used for morphological staining.

Sperm Preparation and Morphological Staining

To examine the morphology, smear samples were taken and dried in the air at least for 30 s with previously cleaned slide to eliminate the foreign particles during analysis. Then, smear samples were covered with fixation solution for 1 minutes, and excessive part was drained. Thereafter, the fixed smears placed horizontally onto staining tray, and covered with Sperm Blue stain (Microptics®, Spain) for 3 minutes. Once stained, all the slides were permanently sealed with Eukitt® mounting medium and a coverslip.

Spermatozoa Morphometry

The measurement of spermatozoa head and midpiece morphometric analysis were done with SCA morphological software (Microptics®, Spain). A phase-contrast microscope (Nikon, Eclipse 50i) with a camera attachment (Basler, Scout Specific digital Basler camera with 1280×1024 resolution, 60 frame per sec.) were used under x60 magnification objective. SCA® Morphology module were used for automatic morphometry analysis under bright-field microscopy. Spermatozoa were randomly selected and captured from different slide fields until 100 spermatozoa were reached. The selection of those spermatozoa with foreign particles and overlapped ones were discarded manually. The dimensions of the head and midpiece were manually selected, measured simultaneously by the software system and recorded as μm unit into a separate excel file.

Statistical Analyses

Descriptive statistics for the data were calculated and shown as "Arithmetic Mean ± Standard Error". For each variable, the group effect is examined with the help of mixed models; in the model created, repetitions of semen measurements are random; degrees, duration basic effects are included as fixed effects. In cases where the interaction term was found meaningful, simple effects analysis with Bonferroni correction was applied. For all statistical analysis, $p < 0.05$ criterion was used. The analyzes were carried out through the Stata 12 / MP4 (License No: 50120500264) statistical package program.

RESULTS

The parameters related to the head of sperm, thawed at different times and temperatures are given in Table-2 while the parameters related to the mid-piece are given in Table-3. When the parameters were evaluated statistically, no significant difference was found ($p > 0.005$).

The lowest head width was obtained as 2.89 ± 0.05 μm in semen thawed for 60 s at 37 ℃ ($p > 0.005$). The head widths of sperm, which were thawed at 70 ℃ for both 6, 9 and 12 s, was determined as 2.96 ± 0.06 μm. Sperm thawed for 3 s at the same temperature were found to have a head width of 2.91 ± 0.05 μm.

The lowest head length measurement was found to be 6.04 ± 0.09 μm in group thawed at 70 ℃ for 3 s. It was determined that the head perimeter at the same temperature and time had the lowest value with 13.03 ± 0.19 μm. The head area was the highest as 17.42 ± 0.49 μm^2 in sperms thawed for 12 s at 70 ℃. Sperm in the group thawed at 25°C for 50 s have the lowest head area, 16.54 ± 0.43 μm^2 ($p > 0.005$).

When the midpiece widths were evaluated, the semen thawed at 25℃ 40 s were found to have the highest value of 0.68 ± 0.01 μm, and the lowest value of 0.6 ± 0.03 μm at 25 ℃ 50 s ($p > 0.005$). It was determined that the semen thawed at 25 ℃ 40 s in the middle part area had the numerically highest with 5.26 ± 1.58 μm^2.

Table 2: The morphometrical features related to the head of sperm, thawed at different temperatures and time periods

Groups (n=9)	**Arithmetic mean ± Standard error**			
	Head Length (μm) (p=0.930)	**Head Width** (μm) (p=0.994)	**Head Area** (μm^2) (p=0.993)	**Head Perimeter** (μm) (p= 0.997)
Control (37℃ 20 s)	6.15 ± 0.07	2.92 ± 0.04	16.88 ± 0.38	13.28 ± 0.17
37℃ 30 s	6.21 ± 0.11	2.95 ± 0.06	17.27 ± 0.50	13.36 ± 0.24
37℃ 40 s	6.11 ± 0.08	2.90 ± 0.05	16.70 ± 0.39	13.21 ± 0.15
37℃ 50 s	6.16 ± 0.12	2.93 ± 0.06	16.90 ± 0.64	13.22 ± 0.23
37℃ 60 s	6.07 ± 0.09	2.89 ± 0.05	16.65 ± 0.42	13.15 ± 0.21
25℃ 30 s	6.10 ± 0.06	2.93 ± 0.03	16.69 ± 0.41	13.18 ± 0.12
25℃ 40 s	6.22 ± 0.09	2.95 ± 0.06	17.33 ± 0.53	13.38 ± 0.18
25℃ 50 s	6.11 ± 0.08	2.92 ± 0.05	16.54 ± 0.43	13.23 ± 0.18
25℃ 60 s	6.15 ± 0.08	2.93 ± 0.05	17.11 ± 0.48	13.31 ± 0.17
40℃ 10 s	6.15 ± 0.06	2.93 ± 0.04	16.93 ± 0.33	13.23 ± 0.14
40℃ 15 s	6.13 ± 0.07	2.92 ± 0.05	16.86 ± 0.39	13.32 ± 0.15
40℃ 20 s	6.15 ± 0.09	2.96 ± 0.07	17.09 ± 0.49	13.32 ± 0.22
40℃ 25 s	6.18 ± 0.09	2.94 ± 0.05	17.16 ± 0.50	13.37 ± 0.23
70℃ 3 s	6.04 ± 0.09	2.91 ± 0.05	16.78 ± 0.44	13.03 ± 0.19
70℃ 6 s	6.12 ± 0.07	2.96 ± 0.06	17.01 ± 0.43	13.29 ± 0.16
70℃ 9 s	6.21 ± 0.09	2.96 ± 0.06	17.33 ± 0.48	13.39 ± 0.19
70℃ 12 s	6.30 ± 0.09	2.96 ± 0.06	17.42 ± 0.49	13.43 ± 0.22

Table 3: The morphometrical features related to the midpiece of sperm. thawed at different temperatures and time periods.

Groups (n=9)	Arithmetic mean ± Standard error	
	Midpiece Width (μm) (p=0.514)	**Midpiece Area** (μm²) (p=0.381)
Control (37℃ 20 s)	0.64 ± 0.02	3.53 ± 0.12
37℃ 30 s	0.65 ± 0.01	3.81 ± 0.08
37℃ 40 s	0.67 ± 0.01	3.65 ± 0.10
37℃ 50 s	0.65 ± 0.02	3.61 ± 0.18
37℃ 60 s	0.65 ± 0.01	3.76 ± 0.10
25℃ 30 s	0.64 ± 0.02	3.48 ± 0.18
25℃ 40 s	0.68 ± 0.01	5.26 ± 1.58
25℃ 50 s	0.60 ± 0.03	3.34 ± 0.12
25℃ 60 s	0.66 ± 0.01	3.73 ± 0.15
40℃ 10 s	0.65 ± 0.02	3.60 ± 0.14
40℃ 15 s	0.63 ± 0.01	3.62 ± 0.05
40℃ 20 s	0.66 ± 0.03	3.52 ± 0.17
40℃ 25 s	0.67 ± 0.01	3.66 ± 0.17
70℃ 3 s	0.65 ± 0.02	3.79 ± 0.12
70℃ 6 s	0.65 ± 0.03	3.45 ± 0.18
70℃ 9 s	0.65 ± 0.01	3.70 ± 0.12
70℃ 12 s	0.64 ± 0.01	3.52 ± 0.08

DISCUSSION

Routine assessment of sperm, including the assessment of normal spermatozoa morphology, has long been used to assess the effects of the semen freezing process and thawing procedures. Abnormal spermatozoa morphology is an important indicator of decreased fertility in stallions and bulls. Spermatozoa head abnormalities have been associated with early embryonic loss, decreased embryo quality, and reduced capacity to bind to the ova (Maroto-Moralesa et al. 2010). Studies investigating chromatin condensation and the morphology of spermatozoa suggest that abnormal chromatin condensation may be affected by or lead to morphological abnormalities. The change in sperm head parameters revealed that it was associated with fertility potential and abnormal chromatin structure in bulls (Sailer et al. 1996). In addition, changes in spermatozoon chromatin structure after scrotal isolation of bulls were found to be parallel to an increase in morphologically abnormal spermatozoa percentage (Karabinus et al. 1997). Chromatin condensation in epididymal cat spermatozoa assessed by aniline blue and acridine orange staining was determined to be significantly higher than spermatozoa with tail defects only in spermatozoa with head abnormalities (Hingst et al. 1995). Abnormal chromatin condensation was observed in about 95% of human spermatozoa with some abnormalities. The percentage of spermatozoa showing normal chromatin condensation was found to be lower than those with abnormalities in the spermatozoa head (Dadoune et al. 1998).

The present study showed unexpected correlation between sperm thawing temperature and time on sperm morphometric parameters even if it is not statistically significant. The control group (37 ℃ 20 s) and 70 ℃ 12 s head area determined as 16.88 ± 0.38 μm²; and 17.42 ± 0.49 μm² respectively (Table 2). It is known that spermatozoon morphology is also negatively affected at this temperature. In parallel with the studies mentioned above, the change in the head area draws attention. Even though the thawing temperature is set for 70 ℃ in 12 s perfectly, the cooling time from 70 ℃ to heating stage temperature should be taking into consideration. In line with above mentioned statement, our preliminary result for intact membrane rate conversely showed the highest abnormality. Based on this, it is determined that the changes occurring per sperm also show negativities related to morphology. The head width of sperm thawed in 70 ℃ 6, 9 and 12 s was found to be the same as 2.96 ± 0.06 μm. The same temperature value was found to be 2.91 ± 0.05 μm in semen thawed for 3 s (Table 2). When the parameters on the head perimeter is taken into account, the lowest value

was determined as 13.03 ± 0.19 μm² in the semen thawed for 3 s at 70 (Table 2). The lowest value of the head length ratio of the sperms that were kept for 3 s at 70 °C was determined as 6.04 ± 0.09 μm (Table 2), but no significant difference was found between the parameters (p> 0.005). However, this results could be interpret as the timing and temperature is take a importance place for optimised osmotic swelling at right ionic equilibrium. Gravance et al. (2009) compared the length and width of the head before and after semen cryopreservation, it was determined that the head length before cryopreservation was 8.67 μm, while it decreased to 8.45 μm after freezing and thawing. The head width was decreased from 4.55 μm to 4.39 μm. Higher thawing temperatures should provide even faster heating, but are not usually prefered due to the perceived risk of cell injury above 37 °C (Calamera et al. 2010).

Sperm are exposed to changes in lipid composition of the sperm plasma membrane (Buhr et al. 1989), externalization of phosphatidylserine, physical and chemical stress (Schiller et al. 2000), externalization of phosphatidylserine (Glander and Schaller 1999) and thus reduction in head size (Gravance et al. 1998) during freezing. Human spermatozoa has also been reported to be particularly sensitive to lipid peroxidation caused by the oxygen radical (Jones et al. 1979, Alvarez et al. 1987), membrane phospholipid damage (Alvarez and Storey 1995, Aitken 1995). ATP produced by thermal flagellum for maintaining sperm motility (Calamera et al. 1982) and ATP loss due to membrane damage (Calamera et al. 2010). In addition to membrane-bound cryo-damage, oxygen radicals have been found to damage peripheral axonemia and nuclear DNA (Sailer et al. 1995). The production of superoxide anion (O_2^-) has been shown to increase during thawing in bovine sperm (Chatterjee and Gagnon 2001). This may partially explain why DNA damage has increased immediately after it has been resolvedChatterjee and Gagnon (2001) have determined that the potential for increased motility recovery observed after thawing at 40 °C will be with a faster recovery rate of sperm enzymatic antioxidant activity. During thawing, there are two processes that terminate the degree of cell damage: [1] the amount of oxygen radical production and [2] recovery rate of enzymatic antioxidant activity. As principle, the higher the temperature rises, the faster the recovery of enzymatic antioxidant activity occurs. Therefore, it has been reported that after thawing at 40 °C, the increase in the production of oxygen-radical that occurs during thawing can be neutralized more efficiently than at 37 °C. When sperm DNA integrity and ATP content were evaluated to detect sperm damage due to oxygen-free radical origin at different thawing temperatures tested, the thawing at 40 °C had no observable determinative effect on DNA integrity. No remarkable difference in ATP content was observed between semen thawed at 40 °C and thawed at 37 °C. In the present study, the head length parameters of the control group, which thawed in 37 °C 20 s, and the sperms thawed in 40 °C 10 s, were found to be very close to each other with the values of 6.15 ± 0.07 μm and 6.15 ± 0.06 μm, respectively. In length, perimeter and area of the head parameters, 37 °C 30 s (6.21 ± 0.11 μm /13.36 ± 0.24 μm /17.27 ± 0.5 μm) and 40 °C 25 s (6.18 ± 0.09 μm /13.37 ± 0.23 μm /17,16 ± 0,5 μm) have the highest values among the same temperatures. In this study, morphometrical values of sperm head were similar between 37 °C and 40 °C. It was observed that the width of the midpiece at 37 °C 40 s and 40°C 25 s were uniform as 0,67 ± 0,01 μm. Based on these parameters, 37 °C and 40 °C temperature values appear to have a similar effect on sperm morphology.

In mammals, the variability in total sperm length has been stated to occur mainly due to differences in the length of the flagellum and the mid-piece (Cummins and Woodall 1985). The spermatozoa mid-piece contains a dense helix array mitochondria that provides energy to push the cell that determines the flagellar beat frequency (Cardullo and Baltz 1991). The size of the mid-piece of the spermatozoa can be important in determining the outcome of sperm competition. Comparative studies between vertebrates showed that the sperm size correlated positively with swimming speed (Fitzpatrick et al., Lüpold et al. 2009). Studies show that increases in the mid-piece volume can turn into higher swimming speed and thus provide an advantage in sperm competition (Anderson and Dixson 2002). As a contradiction to this hypothesis, a study in red deers (Cervus elaphas) revealed that there was a negative correlation between sperm mid-piece length and sperm swimming speed. Spermatozoa, which have long heads and shorter mid-piece, swim faster. However, no relation was found between sperm velocity and flagellum or total sperm lengths (Malo et al. 2006). This highlights the need for investigation on these relationships in other mammals. In general, spermatozoa with longer tails have been found to have a tendency to reach the ovum faster while swimming in the female genital tract and to swim faster than those with a shorter tail (Gomendio and Roldan 1991). In the present study, semen thawed in 25 °C 40 s were determined to have the numerically greatest midpiece width with a value of 0.68 ± 0.01 μm as well as midpiece area with 5.26 ± 1.58 μm² (Table 3). The group with the lowest mid-piece area was found to be 3.34 ± 0.12 μm² in the sperm thawed at 25 °C for 50 s.

In conclusion, when the head and mid-piece parameters of the sperm thawed at different times and temperatures were evaluated, the changing time and temperature caused different results in the

parameters. The thawing conditions of semen are a factor that directly affects spermatozoa. Since changes in time and temperature affect the morphology of spermatozoa, changes occur in the mid-piece and head sizes. These changes have positive or negative effects on the fertilization ability of spermatozoa. When performing insemination in field conditions, it has been observed that paying attention to the dissolution time and temperature of the semen is important for the positive functioning of the insemination process and obtaining pregnancies. Any setbacks that occur during this process affect spermatozoa negatively and decrease their fertilization abilities. Especially in high temperature values, it was seen in the parameters related to the head and mid-piece that morphology was negatively affected. It should be noted that the cause of unsuccessful insemination may be due to changes in temperature and time that will occur during the thawing of the semen.

Conflict of Interest: The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

The Relationship Between Milk Fatty Acid Profile and Expression Levels of *SCD*, *FASN* and *SREBPF1* Genes in Damascus Dairy Goats

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ABSTRACT

In this study, the relationship between the expression levels of *SCD, FASN, SREBPF1* genes and milk fatty acid profiles in goat milks with low (LSFA) and high (HSFA) saturated fatty acid content was investigated and correlated. In HSFA group, *SCD, FASN* and *SREBPF1* genes were approximately 7, 9 and 4 folds more expressed than LSFA in milk somatic cells, respectively (P<0.01). Also, positive correlations were determined between *SCD* and *FASN* (0.907; P<0.001), *SCD* and *SREBPF1* (0.628; P<0.001), *FASN* and *SREBPF1* (0.720; P <0.001) genes. Positive and important correlation was found between C6:0 (Caproic acid) and *SCD* (0.468; P<0.05) and *SREBPF1* (0.388; P<0.05) genes. On the other hand, positive correlations were found between all of these three genes and C8:0 (Capric acid) and C10:0 (Caprylic acid). In milk samples, C14:0 (Myristic acid) and *SREBPF1* genes were correlated positively (0.469; P<0.05). Moreover, positive correlation was found between the odour index and *SCD* (0.553; P<0.01), *FASN* (0.444; P<0.05), *SREBPF1* (0.499, P<0.05) genes. The results showed that *SCD, FASN* and *SREBPF1* genes have important effects on the milk fatty acid profile and milk quality of goats and these genes may be candidate in selection applications in goats.

Keywords: Gene expression, Goat, Milk fatty acids, Somatic cells

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Damascus Keçilerinde Süt Yağ Asidi Profili ile *SCD*, *FASN* ve *SREBPF1* Genlerinin Ekspresyon Seviyeleri Arasındaki İlişki

ÖZ

Bu çalışmada, doymuş yağ asidi içeriği düşük (LSFA) ve yüksek (HSFA) olan keçi sütlerinde *SCD, FASN, SREBPF1* genlerinin ekspresyon düzeyleri ile süt yağ asidi profilleri ve aralarındaki ilişki araştırılmıştır. HSFA grubunda süt somatik hücrelerinde *SCD, FASN* ve *SREBPF1* genleri, LSFA grubuna göre yaklaşık sırasıyla 7, 9 ve 4 kat daha fazla ifade edilmiştir (P<0,01). Ayrıca, *SCD* ve *FASN* (0,907; P<0,001), *SCD* ve *SREBPF1* (0,628; P<0,001), *FASN* ve *SREBPF1* (0,720; P <0,001) genleri arasında pozitif korelasyonlar tespit edilmiştir. C6:0 (Kaproik asit) ile *SCD* (0,468; P<0,05) ve *SREBPF1* (0,388; P<0,05) genleri arasında pozitif ve önemli korelasyonlar bulunmuştur. Bununla birlikte, bu üç genin tamamı ile C8:0 (Kaprik asit) ve C10:0 (Kaprilik asit) yağ asitleri arasında pozitif korelasyonlar belirlenmiştir. Süt örneklerinde C14:0 (Miristik asit) ve *SREBPF1* geni arasında pozitif korelasyon gözlenmiştir (0,469; P<0,05). Ayrıca, koku indeksi ile *SCD* (0,553; P<0,01), *FASN* (0,444; P<0,05), *SREBPF1* (0,499, P<0,05) genleri arasında pozitif korelasyon tespit edilmiştir. Sonuçlar, *SCD, FASN* ve *SREBPF1* genlerinin keçilerin süt yağ asidi profili ve süt kalitesi üzerinde önemli etkileri olduğunu ve bu genlerin keçilerde seleksiyon uygulamalarında aday olabileceğini göstermiştir.

Anahtar Kelimeler: Gen ekspresyonu, Keçi, Süt yağ asitleri, Somatik hücre

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INTRODUCTION

The basic principle of animal breeding is to obtain healthy populations with superior characteristics in terms of yield and quality. With this principle, sustainable breeding studies continue in animal husbandry. Researchers have focused on characters such as meat yield, milk yield and milk quality in ruminants, which are important farm animals (Akcapınar and Ozbeyaz 1999, Ozkan and Yakan 2017). Increasing awareness of nutrition raises the demand for healthy and quality food. The world population is increasing day by day. Currently, the population of around 8 billion people is expected to be around 10 billion by 2050 (Anonymous 2020a). Increasing population and consumption awareness requires higher quality production in animal foods such as eggs, meat, milk and dairy products (Haile et al. 2016, Balthazar et al. 2017).

The World Health Organization (WHO) states that the saturated/unsaturated fatty acids and the n6/n3 ratios should be less than 1.0 and 4.0, respectively (WHO, 2013). Studies show that goat milk has more positive values in terms of these values than cow and sheep milk (Ceballos et al. 2009, Silanikove et al. 2010, Yakan et al. 2019). On the other hand, goat milk is more digestible than cow's milk. The goat milk's fat globules are smaller and also milk is richer in terms of short and medium chain fatty acids (Kompan and Komprej 2012, Yurchenko et al. 2018). Goat breeding is an increasingly important form of livestock for some dairy products such as cheese and ice cream (Yurchenko et al. 2018). It is estimated that there are more than 1000 goat breeds in the world and the total number of goats is around 1 billion today (Crisa et al. 2016, Anonymous 2020b). Today, the number of goats increased approximately 2-folds compared to 10 years ago and it is reported that more than 10 million goats exist in Turkey (Anonymous 2020a). In addition to pure and native breeds such as the Hairy goat, Angora goat, cultural breeds such as the Saanen, German Alaca Noble goat, Toggenburg and Damascus goat, and hybrid breeds such as the Kilis, Akkeçi, Bornova goat constitute the Turkish goat population. Damascus goat, due to its high milk yield and fertility properties, is an important animal breeding in countries such as Turkey, Syria, Lebanon, Israel and Cyprus (Keskin 2002, Yakan et al. 2019).

While quality in animal products has been associated with hygiene and cheating formerly, today it is evaluated in terms of parameters such as composition, processing and technology of the product (Raynal-Ljutovac et al. 2005, Yakan et al. 2019). Milk fatty acid composition is one of the most important factors affecting the quality of milk and dairy products. The amounts and rates of saturated and unsaturated fatty acids in milk composition considerably affect milk quality (Yakan et al. 2019). It has been reported that the consumption of saturated fatty acids triggers cardiovascular diseases; however polyunsaturated fatty acids (PUFA) are protective against cardiovascular diseases (Simopoulos et al. 2008). On the other hand, it has been stated that subacute ruminal acidosis may be estimated in ruminants with the fatty acid profile in milk, and milk fatty acids may be used as biomarkers for detecting subacute ruminal acidosis (Colman et al. 2015, Yurchenko et al. 2018).

In goats, milk secretion type is apocrine. Therefore, the number and content of somatic cells in milk are different. The somatic cells that provide the natural defense mechanism of the mammary gland are composed of leukocytes, polymorphous nuclear cells, and largely epithelial cells. In ruminants, somatic cell count is one of the most important parameter that gives information about mammary health and animal physiology. Molecular activity in milk somatic cells is reported to be an important marker for understanding about biological activity in the mammary gland (Murrieta et al. 2006, Feng et al. 2007). In molecular studies related to mammary health and activity, it is possible to study from milk somatic cells without the need invasive methods such as biopsy in tissues (Feng et al. 2007, Jacobs et al. 2013). In addition to giving ideas about mammary health, somatic cells reflect the biological activity in molecular pathways such as lipogenesis in mammary tissue (Murrieta et al. 2006).

Although there are a lot of researches about the molecular activities of genes in the lipogenesis pathway in non-ruminant species, what is known about the relationship between these genes and milk fat synthesis in ruminants, especially goats, is quite limited (Yao et al. 2017). The mammary gland in lactation is one of the major organs where lipid synthesis takes place (Rudolph et al. 2010). It has been reported that *SCD* (Stearoyl-CoA Desaturase), along with many factors in goats, is particularly effective in the formation of milk fatty acid composition (Kompan and Komprej 2012). *SCD* is highly responsible for fatty acid elongation in milk (Kompan and Komprej 2012). *FASN* (Fatty Acid Synthase) and *SREBPF1* (Sterol Regulatory Element-Binding Transcription Factor 1) are another important regulators of lipogenic pathway. In this study, it is aimed to investigate the expression levels of *SCD, FASN* and *SREBPF1* genes in the goat milk with low and high saturated fatty acid content and to reveal the relationship between the expression levels of these genes and the milk fatty acid profile in goat milk.

MATERIALS and METHODS

Animal Materials

In the study, 20 milk samples were taken from 2-3 years old Damascus goats, which are in the 4th month of lactation. Goats were under semi-intensive

feeding. Approximately 100 ml milk collected to nuclease free and sterile tubes from each goat from the morning milking (50 ml volume falcons were used for sample collection). Samples were CMT (California Mastitis Test) negative and animals were healthy. Then, milk samples were quickly transferred to the laboratory for analyzes under cold chain.

Cream and Somatic Cell Collection

The samples were centrifuged at 1800 xg for 15 minutes at + 4 °C. After centrifugation, samples were kept at – 20 °C for cream layer for 10 min. The cream layer of samples was collected to new tubes with the help of a spatula. Cream samples were kept at – 20 °C until fatty acid analyzes.

After cream layer was removed, the supernatants in falcon tubes were discarded and the pellets at the bottom of falcons were transferred to 15 ml, sterile and nuclease-free falcon tubes. Cell suspensions in the falcon tubes were completed with PBS to 15 ml. Samples were centrifuged at 1800 xg for 15 minutes at +4 °C. Then, supernatants of samples were poured out; 1 ml TRI Reagent (Sigma-Aldrich, USA) solution was added to the cell pellets of samples. Total RNA isolation was made from the cell pellets homogenized in TRI Reagent.

Fatty Acid Analysis

Approximately 500 µl cream from each sample was used for fatty acid analysis. The creams were homogenized with 2 ml of 2N methanolic KOH for 4 minutes at room temperature. Thereafter, 4 ml of n-Heptane (Merck, USA) was added to the homogenate. After homogenization for 2 minutes at room temperature, samples were centrifuged at 200 xg for 5 minutes to phase separation. Methyl esters collected in the upper phase were transferred to vials with a volume of 1.5 ml and fatty acids were determined by HP Innowax column (60 m length, 0.25 mm i.d. x 0.25 µm film) in Gas Chromatography (HP Agilent 6890, USA). Injector temperature was set at 250 °C and detector temperature at 270 °C. Helium was used as the carrier gas. Injection was splitless with a total injection volume of 1 µl and injector was washed three times with n-Heptan. Oven temperature was programmed initially at 90 °C for 3 min and was increased to 250 °C with a 3 °C/min ramp rate. The determined peaks on chromatogram were verified by comparison of retention times of internal standards (FAME Mix, Supelco, USA)

According to the results of fatty acid contents of milks, the samples were divided into two groups as Low Saturated Fatty Acid Content (LSFA) and High Saturated Fatty Acid Content (HSFA). The groups were created by ordering the samples from large to small according to the SFA contents. The first 10 samples constitute LSFA group, while the last 10 samples formed HSFA group.

Total RNA Isolation and cDNA Synthesis

Total RNA isolation was performed from milk somatic cells according to the TRI-Reagent (Sigma-Aldrich, USA) kit protocol (Rio et al. 2010). After chloroform-isopropyl alcohol-ethyl alcohol steps, the RNA pellets were obtained from the samples. Depending on their size, the RNA pellets were diluted with 30-100 µl of nuclease-free water and checked for quality.

The purity (A260/280) and concentration of the RNA isolated from the samples were checked in the nucleic acid meter (Merinton, SMA-1000 UV Spectrophotometer). Also, 28S and 18S rRNA subunits were evaluated in 1% agarose gel for RNA quality controls (100 V and 25 min).

The DNase treatment was performed to samples for possible genomic DNA contamination (DNase I, RNase free, Thermo Scientific, USA, Cat no: EN0525). According to the Revertaid First Strand cDNA Synthesis kit (Thermo-Scientific, USA) protocol, cDNA synthesis was performed. In the thermal cycler (Bio-Rad T100, USA), the reaction was carried out for 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C. After reaction, the final volume of the samples were completed to 200 µL with nuclease free water and kept at – 80 °C until gene expression analyses were performed.

Quantitative Real-Time PCR Analysis

Amplification of genes were performed using 10 µl of each cDNA sample with the kit containing SYBR Green I dye (Power SYBR® Green PCR Master, ThermoFisher Scientific, USA, Cat no: 4367659). The reaction was arranged 10 minutes at 95 °C, followed by 15 seconds at 95 °C, 60 seconds at 60 °C, and 40 cycles in qPCR (Bio-Rad CFX-96 Touch Real time PCR, USA). Each cDNA sample was studied as duplicate and *ACTB* gene was used as reference gene. The forward and reverse sequences of the primers used in the amplification of genes are shown in Table 1.

Table 1. Forward and reverse sequences of primers of studied genes

GENES	FORWARD AND REVERSE PRIMER SEQUENCES*	PRODUCT LENGTH
FASN	F: 5'-GCACACAATATGGACCCCCA-3'	183
	R: 5'-CATGCTGTAGCCTACGAGGG-3'	
SCD	F: 5'-ATCGCCCTTACGACAAGACC-3'	186
	R: 5'-CATAAGCCAGACCGATGGCA-3'	
SREBPF1	F: 5'-AACATCTGTTGGAGCGAGCA-3'	134
	R: 5'- TCCAGCCATATCCGAACAGC-3'	
ACTB	F: 5'-TGGATCGAGCATCCCCAAAG-3'	169
	R: 5'-ACTGGCCCCTTCTCCTTAGA-3'	

*: All primer sequences were designed in this study by the authors via Primer-BLAST (NCBI).

Statistical Analysis

SPSS package program (Version 23.0) was used to calculate the obtained data. Student-t test was used to measure the difference between the groups. Spearman correlation coefficient was used to calculate the correlation between parameters. Odour (Yakan et al. 2019), thrombogenic and atherogenic indexes were calculated using fatty acid parameters (Ulbricht and Southgate 1991). On the other hand, the $2^{-\Delta\Delta Ct}$ method was used for gene expression calculations, and results were given as fold changes (Livak and Schimitgen 2001). $P<0.05$ was accepted as significant.

RESULTS

The saturated fatty acid content was 64.92±0.90(%) in the LSFA group, while HSFA group saturated fatty acid content was 71.27±0.45(%), ($P<0.001$). Between groups, variable levels of significance in terms of fatty acids were determined (Table 2).

Table 2. Milk fatty acid profiles of LSFA and HSFA groups (Mean±SEM)

Fatty acids (%)	LSFA	HSFA	P
C4:0 (Butyric Acid)	1.62±0.07	1.47±0.15	-
C6:0 (Caproic Acid)	2.06±0.08	2.16±0.12	-
C8:0 (Caprylic Acid)	2.42±0.11	2.68±0.14	-
C10:0 (Capric Acid)	7.60±0,38	9.28±0.33	**
C11:0 (Undecanoic Acid)	0.23±0.02	0.19±0.02	-
C12:0 (Lauric Acid)	2.95±0.17	3.68±0.17	-
C14:0 (Myristic Acid)	8.63±0.30	10.63±0.47	**
C14:1 (Miristoleic Acid)	0.44±0.04	0.64±0.31	-
C15:0 (Pentadecylic Acid)	1.52±0.09	1.37±0.04	-
C15:1 (Pentadecenoic acid)	0.38±0.02	0.31±0.01	*
C16:0 (Palmitic Acid)	24.43±0.69	26.7±0.56	*
C16:1 (Palmitoleic acid)	0.97±0.11	0.82±0.10	-
C17:0 (Margaric acid)	1.02±0.04	0.87±0.05	**
C17:1 (Heptadecenoic Acid)	0.49±0.03	0.38±0.01	*
C18:0 (Stearic Acid)	11.27±0.47	10.87±0.48	-
C18:1 (Oleic Acid)	26.48±1.13	21.18±0.50	***
C18:2 n6 (Linoleic Acid)	1.46±0.25	1.29±0.20	-
C18:3 n3 (Alphalinolenic Acid)	1.57±0.17	1.68±0.17	-
C20:0 (Arachidic Acid)	0.71±0.08	0.89±0.20	-
C20:1 (Gondoic Acid)	1.42±0.08	0.98±0.04	***
C20:2 n6 (Eicosadienoic Acid)	0.48±0.08	0.41±0.09	-
C20:3 n3 (Eicosatrienoic Acid)	0.18±0.02	0.18±0.03	-
C20:3 n6 (Dihomo-g –linolenic Acid)	0.13±0.02	0.14±0.02	-
C20:4 n6 (Arachidonic Acid)	0.18±0.01	0.16±0.01	-
C22:0 (Behenic Acid)	0.38±0.01	0.33±0.01	**
C22:1 (Erucic Acid)	0.22±0.03	0.16±0.01	-
C24:0 (Lignoceric Acid)	0.15±0.02	0.19±0.04	-
C24:1 (Nervonic Acid)	0.82±0.12	0.64±0.04	-

*: P<0.05; **:P<0.01; ***:P<0.001; -:P>0.05

Differences were found in some parameters determined in samples grouped according to saturated fatty acid content (Table 3). While the nutritional value was lower in LSFA than HSFA group (P<0.01), atherogenic and thrombogenic index values were higher (P<0.001) (Table 3).

Table 3. Some parameters were determined according to the fatty acid compositions in the groups (Mean±SEM)

PARAMETERS	LSFA	HSFA	P
ΣSFA	64.92±0.90	71.27±0.45	***
ΣMUFA	29.79±1.07	24.03±0.56	***
ΣPUFA	3.87±0.35	3.71±0.28	-
ΣUFA	33.66±0.85	27.74±0.44	***
Σn6	2.18±0.25	1.87±0.22	-
Σn3	1.70±0.19	1.84±0.17	-
Σn6/n3	1.38±0.17	1.12±0.19	-
OI	13.70±0.60	15.59±0.69	-
NV	1.56±0.08	1.21±0.05	**
AI	1.46±0.07	2.07±0.09	***
TI	1.27±0.04	1.60±0.03	***

SFA: Saturated fatty acids; **MUFA:** Monounsaturated fatty acids; **PUFA:** Polyunsaturated Fatty acids; **UFA:** Unsaturated fatty acids; **OI**: Odour index; **NV:** Nutritional Value; **AI:** Atherogenic index; **TI:** Thrombogenic index
*: P<0.05; **:P<0.01; ***:P<0.001; -:P>0.05
OI= (C4:0+C6:0+C8:0+C10:0); **NV=** (C18:0+C18:1)/C16:0;
AI = (C12:0+(4*C14:0)+C18:0)/ΣUFA
TI= (C14:0+C16:0+C18:0)/((0.5*C18:1)+(0.5*ΣMUFA)+(0.5*Σn6)+(3*Σn3)+(Σn3/Σn6))

Correlation values between milk fatty acid parameters such as SFA, MUFA, PUFA, and UFA in milk are given in Table 4.

Table 4. Correlations between some parameters in milk

PARAMETERS	ΣMUFA	ΣPUFA	ΣUFA	Σn6	Σn3	Σn6/n3	OI	NV	AI	TI
ΣSFA	-0.937***	0.023	-0.997***	-0.119	0.332	-0.364	0.623**	-0.820***	0.929***	0.889***
ΣMUFA		-0.244	0.932***	-0.014	-0.523**	0.383*	-0.657**	0.815***	-0.892***	-0.789***
ΣPUFA			0.005	0.811***	0.582**	0.212	0.183	-0.194	-0.018	-0.053
ΣUFA				0.156	-0.341	0.398*	-0.591**	0.826***	-0.929***	-0.886***
Σn6					0.056	0.683***	0.147	0.003	-0.198	-0.005
Σn6/n3							-0.023	0.341	-0.442*	-0.114
OI								-0.361	0.480*	0.411*
NV									-0.756***	0.808***
AI										0.847***

:P<0.05;:P<0.01; ***:P<0.001

RNA samples had appropriate purity (A260/280=1.87±0.03) and concentration (283.07±42.37 ng/µL). *SCD*, *FASN* and *SREBPF1* genes were expressed approximately 6 (6.35; P<0.05), 9 (9.17; P<0.05) and 4 (4.26; P<0.05) folds more in the HSFA group compared to the LSFA group, respectively (Figure 1).

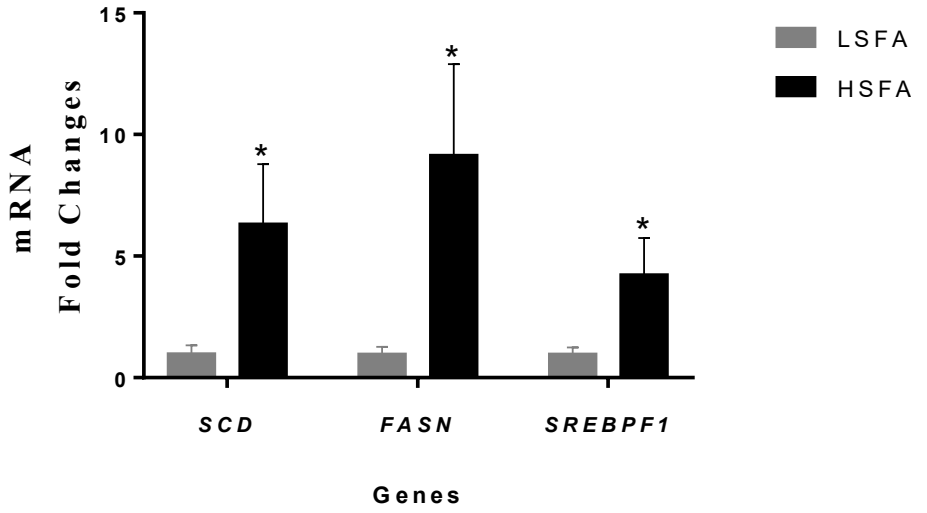

Figure 1. mRNA fold changes of genes in LSFA and HSFA groups (*: P<0.05)

Some correlations were determined between *SCD, FASN, SREBPF1* genes and fatty acid parameters. The correlation results showed that there were important, positive and high correlations between genes (Between *SCD* and *FASN*: 0.907: P<0.001; between *SCD* and *SREBPF1*: 0.628; P<0.001; between *FASN* and *SREBPF1*: 0.720: P<0.001). Also, with variable levels of significance, positive and negative correlations were found between genes and fatty acids (Table 5).

While there was a positive correlation between saturated fatty acid ratio and *SCD* (0.444; P<0.05), *FASN* (0.344; P>0.05) and *SREBPF1* (0.405; P<0.05), a negative correlation was found between the monounsaturated fatty acid ratio and the expression levels of *SCD* (-0.540; P<0.01), *FASN* (-0.439; P<0.05) and *SREBPF1* (-0.471; P<0.05). In addition, there was a positive correlation between *SCD* gene and polyunsaturated fatty acid ratio (0.426; P<0.05) and negative correlation between the unsaturated fatty acid ratio and *SCD* (-0.433; P<0.01) and *SREBPF1* (-0.414; P<0.05) were found. But then, a positive correlation was found between the *SCD* and n3 ratio. Odour index was found correlated with *SCD*, *FASN* and *SREBPF1* genes (0.553; 0.444; 0.499, respectively, P<0.05). A positive correlation was found between *SREBPF1*, a lipogenic transcription factor, and the atherogenic index (0.398; P<0.05).

Table 5. Correlations between genes and fatty acid parameters

PARAMETERS&	***SCD***	***FASN***	***SREBPF1***
C6:0 (Caproic Acid)	0.468*	0.370	0.388*
C8:0 (Caprylic Acid)	0.573**	0.457*	0.466*
C10:0 (Capric Acid)	0.492*	0.400*	0.508*
C14:0 (Myristic Acid)	0.197	0.241	0.469*
C17:1 (Heptadecenoic Acid)	-0.325	-0.254	-0.432*
C18:1 (Oleic Acid)	-0.466*	-0.377*	-0.478*
C18:3 n3 (Alphalinolenic Acid)	0.460*	0.420*	0.232
C20:1 (Gondoic Acid)	-0.385*	-0.284	-0.490*
C20:2 n6 (Eicosadienoic Acid)	0.519*	0.339	0.253
ΣSFA	0.444*	0.344	0.405*

ΣMUFA	-0.540**	-0.439*	-0.471*
ΣPUFA	0.426*	0.259	0.211
ΣUFA	-0.433*	-0.355	-0.414*
Σn6	0.301	0.099	0.113
Σn3	0.391*	0.356	0.211
Σn6/n3	-0.083	-0.217	-0.087
OI	0.553**	0.444*	0.499*
NV	-0.277	-0.140	-0.202
AI	0.287	0.247	0.398*
TI	0.229	0.090	0.223

&: Statistically significant correlations were showed in table
*****: P<0.05; ******: P<0.01

DISCUSSION

In recent years, with the increasing demand for goat milk, goat husbandry has gained importance and the level of breeding has increased (Novotna et al. 2019). In this study, *SCD, FASN* and *SREBPF1* genes expression levels and fatty acid profiles of goats that divided into two groups according to milk SFA contents were investigated.

Saturated fatty acids in the diets are associated with cardiovascular diseases (Siri-Tarino et al. 2010). Investigation of the factors that play roles in the formation of variation in dietary fatty acids has become an important goal. An important part of milk fatty acids in ruminants are saturated fatty acids (Haile et al. 2016). One of the most important parameters in the formation of milk quality is fatty acid profile (Chilliard et al. 2003). Numerous candidate genes and transcription factors in lipogenic pathways regulate the formation of milk fatty acid qualities and quantities via production and secretion of fatty acids (Moioli et al. 2007, Bionaz and Loor 2008).

In HSFA group, while C10:0, C14:0 and C16:0 fatty acids ratios were more than LSFA group (P<0.01), MUFAs as C15:1, C17:1, C18:1 and C20:1 and C22:0 fatty acids ratio were significantly lower (P<0.05). According to milk fatty acid composition, no significant difference was found between the groups in terms of other unsaturated fatty acids (Table 2). In accordance with the literature, while the atherogenic and thrombogenic index values were found to be high in the HSFA group, the unsaturated fatty acid ratio and thus the nutritional value were found lower (Haile et al. 2016, Idamokoro et al. 2019) (Table 3). As expected, a negative correlation was found between the monounsaturated fatty acid ratio and the odour, atherogenic and thrombogenic indexes. In addition, it was determined that there was a positive correlation between the nutritional value and the unsaturated fatty acid ratio and it was understood that this correlation probably occurred due to MUFAs.

In HSFA group *SCD, FASN and SREBPF1*, primary lipogenic genes, were expressed more than 4 folds compared to the LSFA group and these results showed that these genes played an important role in the synthesis of saturated fatty acids in goat mammary gland (Figure 1).

SCD has a role as the key regulator in de novo fatty acid synthesis in mammary gland together with *ACC* and *FASN* (Bernard et al. 2008). It is reported that the main function of *SCD* in the mammary gland plays a role in the conversion of long chain fatty acids, especially C16:0 and C18:0, to C16:1 and C18:1, respectively. *SCD* has a significant effect on the fluidity of milk as well (Feng and et al. 2007). In a study it is reported that the *SCD* gene is more expressed during the lactation period in goat mammary tissue compared to the dry period, and increased *SCD* expression led to increase of the MUFA content and fat storage in the cell (Yao et al. 2017). In the same study, it is stated that concentration of oleic acid and synthesis of triacylglycerol are decreased with silencing of *SCD* gene. On the other hand, it is reported in some studies that that animals with high *SCD* activity in milk may be selected for high quality milk production that have high UFA content such as oleic acid (Feng et al. 2007, Jacobs et al. 2013). However, in this study, while HSFA groups' *SCD* gene expression was approximately 6 folds more than LSFA groups (Figure 1), UFA contents of HSFA groups' were lower than LSFA groups (Table 5). In addition, there was no difference in oleic acid levels between the groups. It is thought that lactation period and animal race may be caused these results. Since, mammary gland molecular activity and composition of milk change in different periods of lactation. On the other hand, it is reported that the milk fatty acid composition of three different goat breeds were

different under similar environmental conditions (Idamokoro et al. 2019).

Abnormal *SCD* expression in humans is known to be associated with obesity and its complications (Huang et al. 2015, Southam et al. 2015). In cattle and goats, it is reported that the polymorphism in *SCD* gene has led to change of milk fatty acid composition and therefore milk quality characteristics have changed (Conte et al. 2010, Alim et al. 2012). It is reported in a study, the over-expression of the *SCD* gene causes significant upregulation of some lipogenic genes especially as *FASN* and *PPARG* in lactating goats (Yao et al. 2017). In this study, it is determined that there is a very high correlation between *SCD* and *FASN* gene (0.907; P<0.001). *SREBPF1*, which is responsible for the activity of a large number of lipogenic genes such as *SCD* and *FASN* in mammals, shows a high level of positive correlation with both *SCD* (0.628; P<0.001) and *FASN* (0.720; P<0.001). *SREBPF1*, a lipogenic transcription factor, is a trigger of *SCD* and *FASN* genes and it regulates milk fatty acid profiles with activation in mammary epithelial cells in lactating ruminants. *SREBPF1* is required to trigger *SCD* expression (Huang et al. 2015, Souhtam et al. 2015). Disorders in milk fatty acid synthesis have been reported as a result of abnormal *SREBPF1* activity (Xu et al. 2016). It is reported that *SREBPF1* gene expression increased approximately 2 times in cattle, rats and humans, while it increased up to 30 times in goats due to mammary gland physiology in the period from pregnancy to lactation (Xu et al. 2016). *SREBPF1* activity is well defined in humans, rodents and cattle, but what is known about *SREBPF1* in goats is limited.

In a study investigating the lipogenic activity in goat breast epithelial cells, *SREBPF1* is found to trigger *FASN* gene expression (Xu et al. 2016). In another study, it is reported that *FASN* is a key metabolic regulator that has a primary role in long chain fatty acid synthesis (Han et al. 2018). *FASN* gene which is expressed in large numbers in tissues in mammals has shown that it is one of the major genes in goat mammary gland (Haile et al. 2016). In this study on Damascus goats, the positive correlation between the genes of which the expression levels were investigated compatible with the other studies (Xu et al. 2016, Haile et al. 2016, Han et al. 2018). In addition, a significant positive correlation was determined between *FASN* and C18:3 n3. In selection studies, *FASN* gene expression levels should be taken into consideration to obtain high quality milk according to milk fatty acid profile, especially in terms of C18:3 n3. Short and medium chain fatty acids are fatty acids between C4:0-C14:0. In goats, it is reported that approximately half of C16:0 with these fatty acids are de novo synthesized in mammary epithelial cells (Haile et al. 2016). In this study, no correlation is found between C16:0 and *SCD*, *FASN* and *SREBPF1* genes. However, a positive correlation is found between C14:0 used in the calculation of atherogenic and thrombogenic indexes and *SREBPF1* (0.469; P<0.05). With these results, it is thought that there is a relationship between milk with high atherogenic and thrombogenic indexes and *SREBPF1*.

Significant positive correlations were found between target genes of study (*SCD*, *FASN*, *SREBPF1*) and C6:0, C8:0, and C10:0 fatty acids (Table 5). *SCD*, *FASN* and *SREBPF1* genes have important effects on the synthesis of these fatty acids used in the calculation of the odour index. Capric and caprylic acids have been used in medical therapy for metabolic-related digestive disorders such as hyperlipoproteinemia, premature infant feeding, epilepsy, cystic fibrosis, and coronary heart diseases (Kompan and Komprej 2012). Considering this situation, it is thought that the expression levels of these genes in the mammary epithelial cells may give important ideas in selection applications on milk quality in goats.

CONCLUSION

In conclusion, the expression levels of *SCD*, *FASN* and *SREBPF1* genes, which are important in the regulation of energy metabolism, are investigated in milk with low and high saturated fatty acid content and their relationship with fatty acid profile in this study. It is thought that successful results may be obtained by investigating these genes in the selection applications on milk quality in ruminants. Also, more studies at the molecular levels are needed to investigate the importance of fatty acid profile in the formation of milk quality.

Conflict of Interest: The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Equine Adipose Tissue Derived Mesenchymal Stem Cells and Their Multilineage Differentiation

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ABSTRACT

Mesenchymal stem cells (MSCs) have become a potent source for cell therapy due their inherent properties of self-renewal and ability to undergo unlimited divisions in the field of regenerative medicine. In this study, equine adipose tissue stem cells (EASCs) have been evaluated on the basis of their morphology, proliferation potential and multilineage differentiation capabilities for their future usage in cell therapy. The cells were further differentiated into osteogenic and adipogenic cell lineages as well as into adipose derived neurospheres to ensure the stemness of propagated cells. In our study, we further successfully cultured the neurosphere derived cells which were cultured from neurospheres after enzymatic dissociation. The study exhibits that EASCs offer a suitable choice for cellular regenerative therapy in equines. Nonetheless, there is a still need to molecular characterization of EASCs in order to establish a standard practice in equine medicine.
Keywords: Equine, Morphology, Multilineage differentiation, Neurosphere, Stem cells

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At Yağ Dokusu Kaynaklı Mezenkimal Kök Hücrelerin İzolasyonu ve Çoklu Farklılaştırması

ÖZ

Mezenkimal kök hücreler kendilerini yenileyebilme ve sınırsız bölünebilme kabiliyetlerinin vasıtasıyla rejeneratif tıp alanında etkili bir kaynak haline gelmiştir. Bu çalışmada at yağ dokusu kaynaklı mezenkimal kök hücreler (EASCs) gelecekte hücresel tedavilerde kullanılabilirliği adına, morfolojileri ve çoklu farklılaşma kabiliyetleri bakımından değerlendirmeye alınmıştır. Elde edilen hücrelerin kök hücre özelliğini kesinleştirmek için hücreler osteojenik ve adipojenik farklılaştırmaya tabii tutulmuş ve nörosfer farklılaştırması yapılmıştır. Bunun yanı sıra, çalışmada elde edilen nörosferlerin enzimatik olarak çözülümünden sonra elde edilen nörosfer kaynaklı hücreler kültüre edilmiştir. Çalışma at yağ dokusu kaynaklı kök hücrelerin atlarda rejeneratif tıp için uygun bir seçim olabileceğini öne sürmektedir. Fakat at hekimliğinde pratik uygulama için bir standardın oluşturulmasından önce daha detaylı moleküler karakterizasyon çalışmalarına ihtiyaç duyulmaktadır.
Anahtar Kelimeler: At, Morfoloji, Çoklu farklılaştırma, Nörosfer, Kök hücre

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INTRODUCTION

Stem cell research has become an important tool for modern science in the field of gene targeting, cloning, drug research as well as regenerative medicine. Stem cells are known for their unique and promising adequacies such as capability of self-renewal and ability to differentiate into various types of somatic cells (Biehl and Russell 2009). Besides, stem cells have the ability to migrate into the inflammation sites (Laflamme et al. 2007) and facilitate cellular regeneration (Rompolas et al. 2012) and could be source of treatment and cure to various debilitating and notorious diseases (Laflamme et al. 2007, Chen et al. 2018, di Domenico et al., 2019). Broadly, stem cells are classified as totipotent, pluripotent, multipotent, oligopotent and unipotent stem cells with the addition of re-programmed induced pluripotent stem cells (Zakrzewski et al. 2019). Although embryonic stem cells (ESCs) have higher cellular potency (Zakrzewski et al. 2019), MSCs have several benefits over embryonic cells (ESCs), as serious ethical concerns exist while using ESCs and MSCs have lesser immunogenecity (Macrin et al. 2017). Multipotent stem cells are being frequently used in stem cell research as they are easy to obtain and have enough potency to differentiate into many cell types (Mirzaei et al. 2018). The adult stem cells are considered as multipotent stem cells and they could be procured from many tissues of an individual such as bone marrow, muscles, adipose tissue, etc. (Quimby and Dow 2015). The International Society of Cellular Therapy has set certain criteria to declare stem cells as mesenchymal stem cells which include (a) adherence capability to plastic, (b) expression of CD105, CD73, and CD90, (c) absence of expression of CD45, CD 34, CD14 or CD11b, CD79 α or CD19, and HLA class II, and (c) ability to differentiate into different cell lineages like osteocytes, adipocytes, and chondrocytes (Dominici et al. 2006). EASCs are reported to have exhibited expression of mesenchymal markers (CD29, CD44 and CD90) and not the major histocompatibility complex II (MHC-II) and CD34 (hematopoietic marker), hence fulfilling the criteria of MSCs (Alipour et al. 2015).

In animals, application of stem cells is getting more important and gaining momentum day by day. In horse, stem cell therapy for cases like tendinitis (Rivera et al. 2020), laminitis (Angelone et al. 2017) has brought about a new dimension in veterinary medicine. Autologous and allogenic applications of equine adipose tissue derived stem cells (EASCs) have become a focus for therapeutic purposes. There are several promising uses of EASCs for tendinitis which resulted in improvement and nearly full recovery; inspiring many researchers for the use of stem cells (Pacini et al. 2007, Crovace et al. 2010, Rivera et al. 2020). The safety of using stem cells for therapeutic approaches is a concern as they might induce cancer or inflamation. For this reason use of stem cells in equine had been investigated by researchers and their results propose that it is safe and easy to use stem cells in equines (Barberini et al. 2018).

In adipose tissues, presence of stem cell number is almost 35 times higher than the stem cells available in bone marrow. It has been noted that one gram of adipose tissue contains 5000 adipose tissue stem cells approximately (Huang et al. 2013). Considering this fact, in the present study EASCs have been isolated, identified, propagated and differentiated. We also aimed to devise a method to isolate viable, feasible and efficient stem cells which could be used further for *in-vitro* and *in-vivo* researches as well as for clinical trials.

MATERIALS and METHODS

Collection of Adipose Tissue and Isolation of EASCs

The adipose tissues were collected at postmortem from a 3-4 years old horse brought to Research and clinical center of Afyon Kocatepe University (AKU), Turkey (Record#107, 16/06/15). Approximately, 8 grams of subcutaneous fat tissue was collected from the gluteal region of horse under optimum sterile conditions in 50 ml centrifuge tube (VWR, USA) containing sterile HBSS (Sigma, USA) with 1% penicillin-streptomycin (Gibco, UK) and 0.1% amphotericin-B (Biowest, France). Soon after, the tissue was transported to Histology and Embryology laboratory, Faculty of Veterinary Medicine, AKU, Turkey for stem cell isolation and further studies. Harvested adipose tissues were placed in a 10 mm petri dish, washed four times with sterile HBSS containing 1% penicillin-streptomycin and 0.1% amphotericin-B and cut into small pieces (about 1 cm^3 size). The tissues were minced into very small pieces with the help of scissors before enzymatic digestion. Later, samples were transferred into a 50 ml centrifuge tube and about 5 ml collagenase-IA (0.01%) (Gibco, USA) was added and incubated into a shaking water bath at 37 °C for 2 hours. During enzymatic digestion, the tube was stirred by turning upside down at 15 minutes interval. After the enzymatic dissociation, the digestion mixture was strained with the help of 70 µm cell strainers (Grenier Bio-One GmBH, Germany) and collected sample was centrifuged at 300 g for 10 minutes. Following the centrifuge, the supernatant was discarded and the pellet was suspended in low glucose Dulbecco's Minimum Essentials Medium (LG-DMEM) (Sigma, USA) containing of 10% Fetal Bovine Serum (FBS) (Biowest, South American Origin), 1% penicillin-streptomycin (Gibco, UK), 200 mM L-Glutamine (Gibco, UK) and 0.1% amphotericin-B (Biochrom GmbH, Germany). Afterwards, cells were seeded into 25cm^2 cell culture flasks and incubated in a

humidified atmosphere with 5% CO_2 at 37 ºC. Medium was changed every 3rd day and cells were passaged until they reached the required confluence. Cells from 3rd passages were used for differentiation studies.

Colony Forming Units Assay

To evaluate the colony forming properties of the isolated stem cells; harvested cells from passage three and five (P3 and P5) were seeded at different densities of 250 cells/cm², 500 cells/cm², 1000 cells/ cm², in 6-well plates and continued to culture for 15 days (3 replicates each). The culture medium was replenished every 3 days with aforementioned medium. At the end of the 15th day; cells of the colonies were fixed with 4% paraformaldehyde (Merck, Germany) and stained with crystal violet (Pre-Med, Turkey). Colonies having at least 16-20 cells were took into consideration for counting (Lange-Consiglio et al. 2013). The microscopic images were obtained using low objectives under stereo microscope and the captured images were analyzed by CS-photoshop.

Doubling Time Calculation of EASCs

Cells were harvested from primary culture were seeded at 25 cm² flask at a density of 20,000 cells / cm² and cultured under 5% CO_2 in humidified incubator at 37 °C. Complete renewal of medium was performed with every three days interval till cells reach 80-90% confluence. After treatment with 0.05% trypsin-EDTA (Sigma, USA) passaging was performed and harvested cells were counted by Neubauer chamber by Trypan blue (Sigma-Aldrich, USA) dye exclusion method. This process was continued from P1 till P7. There were three sets of cultures in each passage. Cell culture time was calculated for each passage and doubling time was calculated by using following formula (Roth and Tuggle 2015).

$$\text{Doubling time} = \frac{\text{duration} * \log(2)}{\log(\text{Final concentration}) - \log(\text{Initial concentration})}$$

Growth Curve Determination of EASCs

To generate the growth curve and to observe the behavioral pattern of cell proliferation, EASCs were seeded at the density of 8000 cells per cm² into four-well cell culture plates (Thermo Scientific, Denmark), at passage 3, 5 and 7 (P3, P5 and P7). Cells were cultured for 14 days and in every 2 days interval cells were trypsinized and counted by using Neubauer chamber and Trypan blue dye. The cell culture medium was changed every third day of culture. This process was repeated for three sets for each of the mentioned passages.

Neurospheres generation

Stem cells from P3 and P5 were considered for neurospheres generation. To perform the neurospheres generation, four-well cell culture plates were coated with 0.01% Poly-L-Lysine (Sigma, USA). The cells were seeded in the plates at a density of 50,000 cells/cm². Medium containing DMEM/F-12 (Gibco, UK), 1% Penicillin-Streptomycin (Gibco, UK), 0.1% Amphotericin-B (Biochrom GmbH, Germany), 1% Insulin- Transferrin- Selenium (Gibco, USA), 20 ng/ml EGF (Sigma, USA) and 20 ng/ml FGF (R&D Systems, USA) was used to generate neurospheres. Plates were cultured in 95% humidity and 5% CO_2 supply conditions at 38.5 °C. Half of the differentiation medium was changed with great care to avoid aspirating neurospheres, in every two days. The neurospheres were photographed at 24th, 48th, 96th and 120th hours post induction under inverted microscope using DP-25 software. There were 3 replicates for each.

Culture of Neurospheres into Neurospheres Derived Cells

Neurospheres were collected into a 15 ml centrifuge tube carefully along with culture medium from the dishes. By flushing 2 ml of serum free medium into the wells; remaining neurospheres were harvested completely. Neurospheres that were collected into the centrifuge tube were centrifuged at 1000 rpm for 3 minutes and supernatant was discarded. After that the cell pellet was resuspended with 1 ml of trypsin-EDTA and incubated at 37 ºC for 1 minute. Afterwards, 1 ml of FBS containing culture medium was added into the tube to stop the enzymatic activity of trypsin-EDTA. The final mixture was centrifuged at 1000 rpm for 3 mins. Cell pellet was resuspended in 2 ml of serum containing culture medium after discarding supernatant. Then, cell suspensions were re-plated on poly-D-lysine (Company, Country) coated 6-well culture plates with 2ml serum containing medium in each well used for EASCs culture. Adipose neurospheres derived cells were grown under normal culture condition at 37 ºC and 5% CO_2 (De Oliveira Carvalho 2012) where medium was changed every 2 days. After reaching confluence, cells were harvested using trypsin-EDTA.

Osteogenic Differentiation of MSCs

After third passage, MSCs were collected and seeded into a 24-well cell culture plate at 29000 cells/well with the help of differentiation medium containing 10% FBS (Biowest, South America), 1% penicillin-streptomycin (Gibco, UK), 0.1% amphotericin-B (Biochrom GmbH, Germany), 200 mM L-Glutamine (Gibco, UK), 0.1µM dexamethasone (Sigma, Belgium), 0.05 mM ascorbic acid (Dr. Ehrenstorfer GMbH, Germany) and 10 mM ß-glycerophosphate (Gibco, UK) in α-MEM (Lonza, Belgium). Medium was changed with every three days interval and condition was regularly checked under inverted microscope. To ensure the differentiation, cells were stained with Alizarin Red S at 14th and 21th day of

differentiation. Photographs were taken using DP-25 software under inverted microscope.

Adipogenic Differentiation of EASCs

After third passage, EASCs were collected and seeded into 24 well cell culture plate at 29000 cells/well using adipogenic induction medium containing LG-DMEM, 10% FBS, 1% Penicillin-Streptomycin (Gibco, UK), 0.1% amphotericin-B (Biochrom GmbH, Germany), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma, Germany), 1μM dexamethasone, 10 μg/ml insulin (Gibco, USA) and 100 μM indomethacin (Cayman Chemical Company, USA). Additionally, at Day 7 and 15 of differentiation, EASCs were cultured in adipogenic maintenance medium composed of LG-DMEM, 10% FBS, 0.05% Pen/Strep and 10 μg/ml insulin. Medium was changed by three days interval and status of cells was checked under inverted microscope according to the cell culture manual (Cat# SCR020, Chemicon® International). To evaluate the differentiation, cells were stained with Oil red O at 14th and 19th day for identification of adipogenic character. Photographs were taken using DP-25 software under inverted microscope.

RESULTS

Morphology of Equine Adipose Tissue Derived MSCs

In the culture flask, after the isolation, cells exhibited adherence to plastic surface. In subsequent passages, cells were observed for good proliferation potential and were observed to reach 90-95 % confluence in the P2, P3 and P4 within 3-4 days. EASCs were observed to have spindle shaped of fibroblast like morphology under inverted microscope, a characteristic feature of stem cells. After, initial culture, EASCs from P2 onward exhibited homogenous fibroblast-like morphology (Fig. 1).

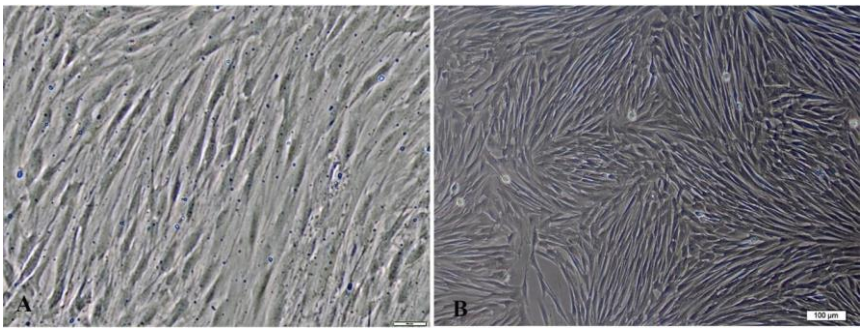

Figure 1. . EASCs dominantly exhibited fibroblast-like morphology starting from P2 and onwards. **A:** EASCs at P2; **B:** EASCs at P6.

Colony Forming Unit Assay

EASCs were evaluated for numbers of colony formation in relation with different seeding densities at the beginning of culture and at different passages (P3 and P5). At both passages, it was observed that numbers of colonies were observed to have significant increase which was proportional to increase in seeding density of EASCs (Fig. 2A-F). However, it was found that EASCs from P3 exhibited more colonies compared to P5 which was observed after staining with crystal violet stain.

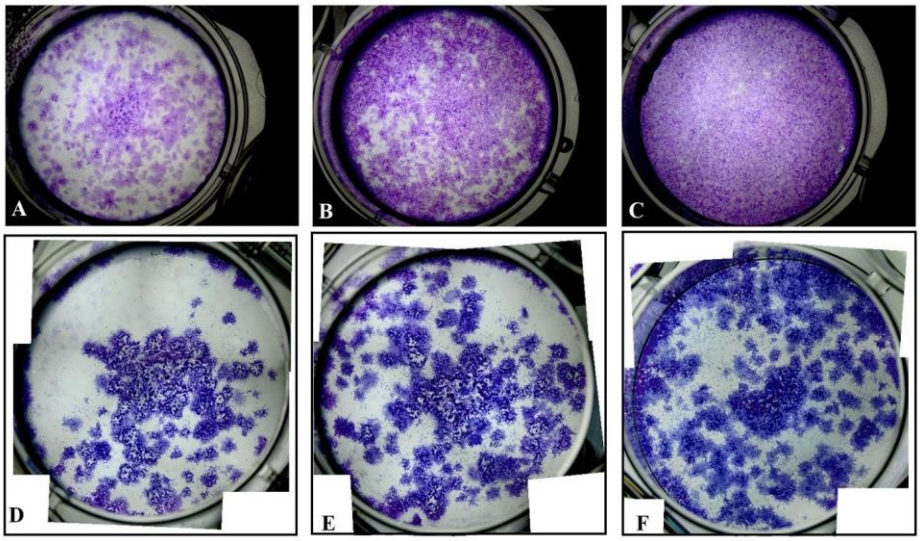

Figure 2. Colony forming unit assay showed that in both P3 **(A,B,C)** and P5 **(D,E,F)**; colony numbers increased depending upon seeding densities. For both passages concentration of 250 cells/cm² **(A,D)** resulted with least colonies while 500 cells/cm² density **(B,E)** demonstrated better results but 1000 cells/ cm² **(C,F)** had highest number of colonies. It was visible that P3 cells gave rise much more colonies compared to P5 cellsfor all concentrations.

Doubling Time Analysis

During doubling time calculation, it was observed that EASCs exhibited significantly highest proliferation rates with minimal doubling time at P5 followed by P6 and P4 when compared to other passages ($p<0.05$). However, no significant difference was observed among P4, P5 and P6 (Fig. 3).

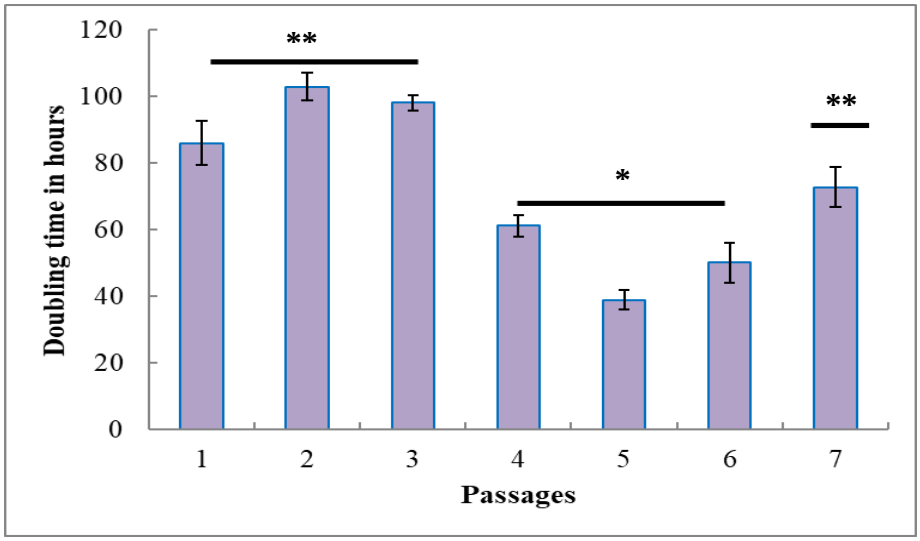

Figure 3. Doubling time analysis of EASCs at different passages (P1-P7). Results are presented as mean ± standard deviation. Significance is shown by differences in * ($p<0.05$).

Growth Curve Analysis

The growth curve study for EASCs showed that the initial phase is was stand for 2-4 days for all recorded passages (P3, P5 and P7). The subsequent log phase was noticed for 4-10 days in and then declined. From the growth pattern it was revealed that the cells of P5 have got the longer stationary phase revealing higher potentiality for proliferation. With the subsequent advancement of passaging, cells had slower and lesser prolificacy (Fig. 4).

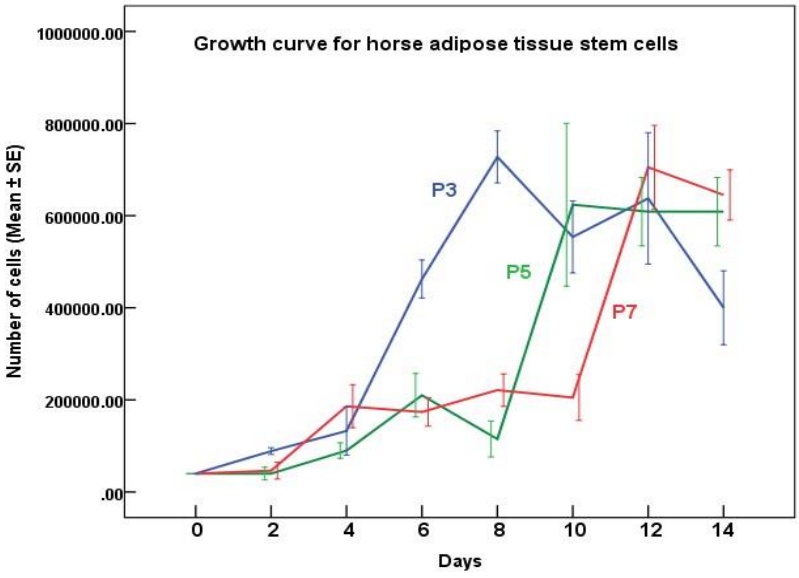

Figure 4. Growth curve analysis of EASCs at P3, P5 and P7. The study showed that P5 have got the longer stationary phase and the cells demonstrated slower growth by the advancing passages

Neurospheres Generation

As a part of multilineage differentiation studies, neurospheres were generated on lysine coated plates with define medium (Fig. 5). For EASCs at P5, neurospheres were observed as spheroid, three-dimensional (3-D) structures that usually formed by the 48th h of culture but took about 4 days to become fully matured and free floating spheroids. After the 5th day, the spheres were observed to disintegrate. Neurospheres formation was found to be comparatively slower at P3 when compared to P5 and P7. It was noticed that with the advancement of time, the 3-D structure of neurospheres become degraded gradually and disappeared finally.

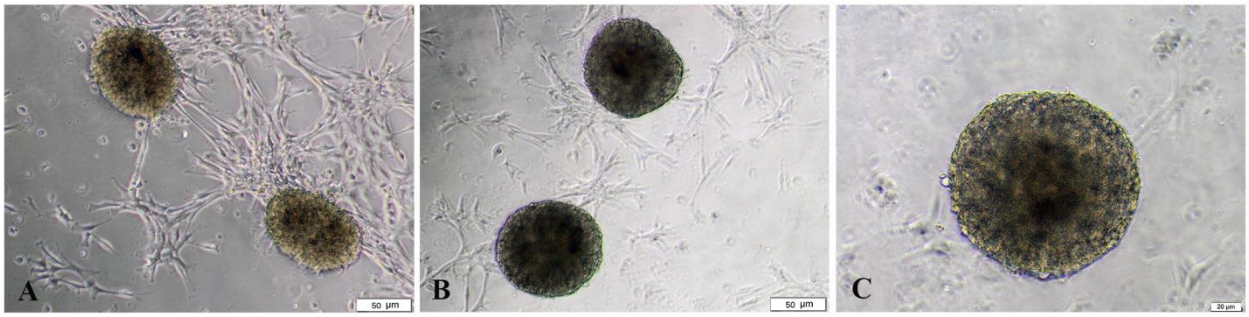

Figure 5. Neurospheres were successfully generated under the influence of EGF and FGF. Bar: 50 μm **(A, B)**; 20 μm **(C)**.

Culture of Neurospheres into Neurospheres Derived Cells

The generated neurospheres were collected and treated with trypsin-EDTA. After the trypsinization, cells were re-plated on poly-D-lysine coated plates with culture medium containing serum. The disintegrated cells were found to be reattached with culture surface and began to grow within 22 h (Fig. 6). After 72 hours of culture, it was observed that neurospheres derived cells started to grow from periphery of neurospheres and became confluent within couple of days during monolayer cell expansion. The cells observed to have fibroblast like morphology meanwhile, center of neurospheres was observed to have dark appearance. The cells were cultured till P5 and were observed to have good proliferation potential throughout the cultured time.

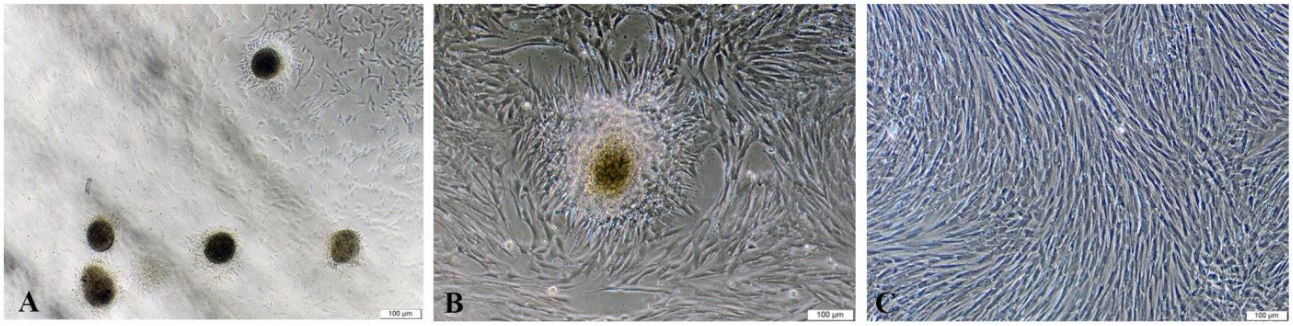

Figure 6. The EASCs neuropsheres were able to regenerate neurosphere derived cells. After the seeding, cells started to grow from periphery of neurospheres **(A, B)**. These cells exhibited fibroblast-like morphology **(C)**.

Osteogenic Differentiation Potential

EASCs were observed to have good osteognic potential after 21 days of culture in induction medium *in-vitro*. The characteristic osteogenic differentiation was observed by extracellular calcium deposition stained orange red with Alizarin Red-S staining while the control sample remained unaffacted (Fig. 7).

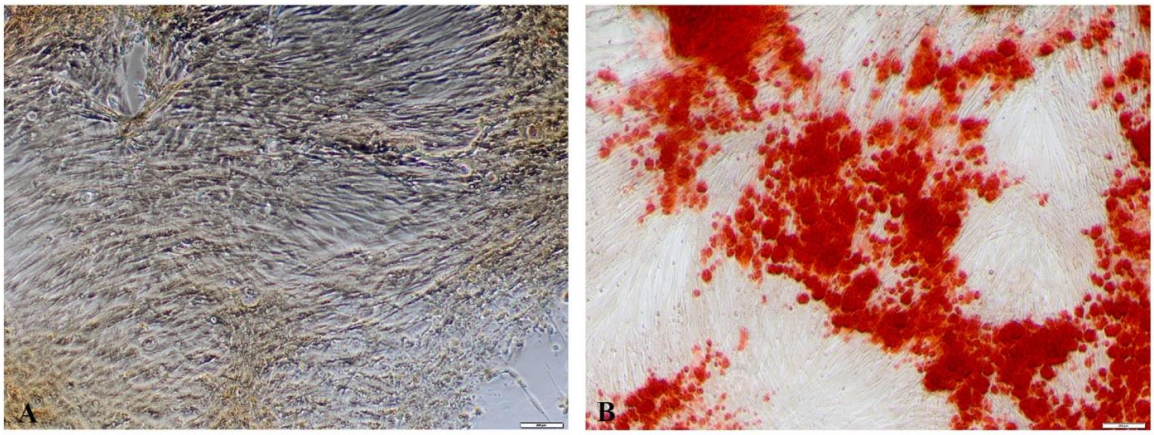

Figure 7. During the multi-lineage differentiation studies, EASCs were subjected with osteogenic differentiation. After 21 days of differentiation, calcium deposits were demonstrated with alizarin red-s staining **(B)**. The control sample was unaffacted by the stain **(A).**

Adipogenic differentiation Potential

The adipogenic differentiation of EASCs was confirmed by Oil red-O staining. Nuclei stained in black after hematoxylin staining and the accumulation of lipid droplets in the cytoplasm of the cells stained in bright red colour at 14th and 19th days of the differentiation study. Meanwhile the control sample remained unaffacted after staining (Fig. 8).

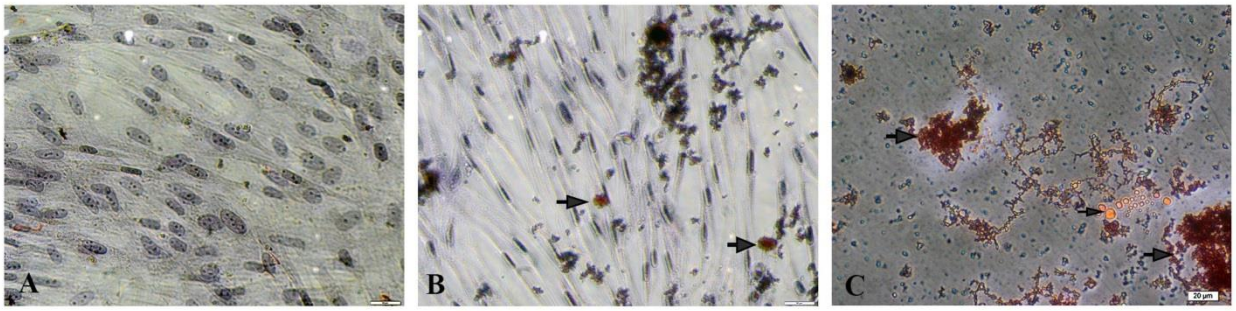

Figure 8. EASCs were also subjected to adipogenic differentiation for 14 and 19 days. To demonstrate the adipogenic differentiation, Oil Red-O stainings were performed at both time intervals. The oil droplets were in red for both day 14 samples **(B)** and day 21 samples **(C)**. There was no staining in the control group **(A)**. **Arrow:** Oil droplets.

DISCUSSION

Adipose tissues are gaining more popularity among researches for stem cell therapies and applications due to ease of isolation and high proliferation potential. In consistent with previous findings from human adipose stem cells (De Coppi et al. 2007) and from equine (Lange-Consiglio et al. 2012), EASCs exhibited fibroblast morphology and strong adherent properties. During primary culture at initial seeding, EASCs were observed to less homogenous in morphology and showed a combination of branched spindle shaped fibroblast like cells and ovoid or circular cells similar to previous reports (Dominici et al. 2006, Iacono et al. 2012, Zuk et al. 2001). At P0, the aggregated cells with nodular or globular structures were recognized as budding nest. This particular phenotype criterion has already been reported by Alipour et al. (2015) and Tepliashin et al. (2005) who suggested that this accumulation give rise to populate special type of cells with uniform morphology.

The growth curve and doubling time analysis showed that EASCs possess high proliferation potential. Although, we couldn't perform comparative study for specific proliferation potential of EASCs with any other stem cell, in a comparative study, Burk et al. (2013) reported that adipose derived stem cells express higher proliferation potential when compared to bone marrow derived stem cells. The EASCs are reported as multipotent stem cells by several researches (Dixon-Shanies et al. 1975, Gimble and Guilak 2003) and EASCs getting more importance day by day for application in regenerative cell therapy (De Mattos Carvalho et al. 2011, Vidal et al. 2007).

It is reported that to be established as a stem cell, cultured cells should be able to differentiate into multiple lineages such as adipogenic, osteogenic or neurogenic lineages (De Schauwer et al. 2011, Dominici et al. 2006). Cells are reported to exhibit some phenotypic, metabolic and genetic changes during differentiation process which could be assessed by expression of specific markers (De Schauwer et al. 2011). However, such changes can also be detected by staining of the differentiated cells with tissue specific staining agents. EASCs were successfully differentiated into osteogenic lineage which was analyzed by Alizarin Red-S staining. Adipogenic differentiation was proved by Oil red-O to detect intracellular lipid droplets ensuring that the cells differentiated into adipogenic lineage. These findings and procedures were similar to previous studies in this regard (Koç et al. 2008, Moonesi Rad et al. 2019, Fink and Zachar 2011, Nawaz et al. 2020, Ozden-Akkaya et al. 2019).

Furthermore, neurospheres generation from adipose tissue make provision for the treatment of alleviation of neurogenic disorder or diseases that may need proper administration media and process. Researchers had reported similar results from different sources of various species such as human (Lee et al. 2011), bovine (Ozden-Akkaya et al. 2019), canine (Altunbaş et al. 2016) in the context of neurosphere generation under the influence of EGF and FGF, supporting our findings. Although equine stem cells of different sources like equine umbilical cord (Martino et al. 2014), equine amniotic membrane (Lange-Consiglio et al. 2012) known to be able to differentiate into neurogenic cells; there were no reports found on neurospheres generation for equine. This study shows that EASCs are able to form neurospheres. Moreover, it was reported (De Oliveira Carvalho. 2012) that neurospheres derived cells have that potential capability to propagate and regenerate into neuron like cells having neuronal marker β-tubulin-III and amyloid precursor protein. The yielding of monolayer plastic adherent fibroblast like cells from neurospheres (Girard et al. 2011) is one of the criteria for evaluation of neurospheres that was also confirmed in this study. Although we were unable to

prove molecular characteristics of the cultured cells; neurospheres gave rise to fibroblast-like cells which were adherent to the plastic surface and demonstrate a good proliferation rate while the cells were able to undergo sub-culture for many passages.

CONCLUSIONS

The morphologic criteria of the isolated and proliferated mesenchymal stem cells and its differentiation into multiple cell lineages confirmed their identity as multipotent stem cells. These findings also favors the regenerative therapy with MSCs that are able to migrate and take residence to the injured or application sites with propagation of desired cells of the target tissues. Also, a small amount of adipose tissue is able to give large amount of cells for the therapy. However, more detailed and in-depth investigations are important to establish stem cells proliferation and differentiation within the spatial distribution of adipose tissue in animal body to understand detailed molecular and biological system in extensive clinical uses.

Conflict of Interest: The authors declare that they have no conflict of interest

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RESEARCH ARTICLE

Effects of Oxytocin Administration on Doppler Indices of Mammary Artery in Postpartum Ewes

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ABSTRACT

Oxytocin hormone, which is frequently used in order to hasten uterine involution process and to ensure milk ejection, is also effective in regulation of vascular tone. Doppler ultrasonography is a highly practical technique that allows the evaluation of blood flow characteristics of tissues and organs. In this study, it was aimed to investigate the hemodynamic changes of mammary artery caused by oxytocin administration in postpartum ewes. The study was conducted with 20 primiparous Kivircik ewes which lambed singleton. The ewes were randomly divided into oxytocin and control groups (n=10 for each group). A dose of 10 IU/sheep oxytocin and 1 ml/sheep saline solution were administered intramuscularly bid on the first 3 days postpartum to oxytocin and control groups, respectively. The pulsed Doppler ultrasonography is used to measure pulsatility and resistance indices (PI and RI, respectively) of external pudendal artery. Although oxytocin group tended to have relatively higher PI and RI values, significant differences were detected only in PI values of the first and second days postpartum ($p<0.001$ and $p<0.05$, respectively) between groups. It has been concluded that oxytocin administration increased the resistance of mammary artery in early postpartum ewes.

Keywords: Doppler ultrasonography, Ewes, Oxytocin

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Postpartum Dönemdeki Koyunlarda Oksitosin Uygulamasının Meme Arteri Doppler İndeksleri Üzerine Etkileri

ÖZ

Doğum sonrası uterus involüsyonunu hızlandırmak ve sütün indirilmesini sağlamak amacıyla sıklıkla uygulanan oksitosin hormonu vasküler tonusun düzenlenmesinde de etkilidir. Doppler ultrasonografi, doku ve organlara ait kan akımı özelliklerinin değerlendirilebilmesine olanak sağlayan oldukça pratik bir tekniktir. Bu çalışmada postpartum dönemdeki koyunlara uygulanan oksitosin hormonunun meme kan akımında oluşturduğu hemodinamik değişikliklerin incelenmesi amaçlandı. Çalışma tekiz kuzulamış olan 20 adet primipar Kıvırcık ırkı koyun ile gerçekleştirildi. Koyunlar randomize şekilde oksitosin ve kontrol gruplarına ayrıldı (her grup için n=10). Oksitosin ve kontrol gruplarına sırasıyla 10 IU/koyun dozda oksitosin ve 1 ml/koyun dozda serum fizyolojik doğum sonrası ilk üç gün boyunca günde 2 kez kas içi yoldan uygulandı. Puls Doppler ultrasonografi tekniği kullanılarak eksternal pudendal artere ait pulsatilite ve rezistans indeksleri (sırasıyla PI ve RI) ölçüldü. Oksitosin grubu daha yüksek PI ve RI değerlerine sahip olma eğiliminde olmasına karşın gruplar arasında anlamlı farklılık sadece birinci ve ikinci postpartum günlerin PI değerlerinde saptandı (sırasıyla $p<0,001$ ve $p<0,05$). Postpartum erken dönemdeki koyunlara uygulanan oksitosin hormonunun, meme arterinin direncini arttırdığı sonucuna ulaşıldı.

Anahtar Kelimeler: Doppler ultrasonografi, Koyun, Oksitosin

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GİRİŞ

Koyunlarda meme dokusunun vaskülarizasyonu eksternal iliak arterin kolu olan eksternal pudendal arter tarafından sağlanır. Bu damarın uzantısı meme arteri olarak tanımlanır (Adam ve ark. 2016). Memenin fonksiyonel ve fizyolojik aktivitesi dokuya gerçekleşen kan akımı ile yakından ilişkilidir (Kensinger ve ark. 1983, Piccione ve ark. 2004). Ruminantlarda meme kan akım özelliklerinin incelendiği önceki yıllara ait çalışmalar invaziv yöntemleri barındırıyorken (Gorewit ve ark. 1989, Reynolds ve ark. 1968) günümüzde vasküler fonksiyon araştırmaları Doppler ultrasonografi sayesinde kolaylıkla yapılabilmektedir (Piccione ve ark. 2004).

Doppler ultrasonografi, doku ve organlara ait hemodinamik özelliklerin değerlendirilebilmesine olanak sağlayan, noninvaziv, pratik ve oldukça etkin bir ön tanı aracıdır. Ses dalgalarının hareket halindeki eritrositler tarafından farklı oranlarda geri gönderilmesi sonucu oluşan Doppler kayması, spektral mod veya renk kodları üzerinden monitöre aktarılır. Doppler ultrasonografi ile kan akımının haritası, yönü ve hızı belirlenebilmekte ve kan perfüzyonu hakkında bilgi edinilebilmektedir. Bir spektral teknik olan puls Doppler ultrasonografi, incelenecek doku veya organa ait çeşitli kan akım parametrelerinin kalitatif ve kantitatif değerlendirilmesine olanak sağlar (Erdoğan 2018, Meinecke-Tillmann 2017; Petridis ve ark. 2017). Bu parametrelerden pulsatilite ve rezistans indeksleri (sırasıyla PI ve RI), arteriyel kan akım hızı dalga formlarının yarı kantitatif değerlendirilmesinde sıklıkla kullanılır. PI puls dalga formu sönümlemesinin derecesini tanımlar. RI vasküler yatağın direnç göstergesidir (Meinecke-Tillmann 2017). Dokunun hemodinamiği ile yakından ilişkili olan bu indeksleri başka deyişle tanımlamak gerekirse: artan RI incelenen arterde direnç artışına ilaveten kan perfüzyonunun azaldığını gösterirken artan PI ise ölçüm alınan noktanın daha distalinde perfüzyonun azaldığını yansıtır (Ginther 2007). PI ve RI, açıdan bağımsız parametreler oluşları sebebiyle özellikle jinekolojik değerlendirmeler için önem taşır (Erdoğan 2018).

Oksitosin, hem bir nörohipofiz hormonu hem de merkezi nöromodülatör özellikte peptit yapılı bir hormondur. Oksitosin reprodüktif ve maternal davranışların gelişiminde rol oynar ve nöroendokrin yolla süt alveollerindeki myoepitel hücreleri uyararak sütün indirilmesini kolaylaştırır. (Cumbers ve ark. 2007, Landgraf ve Neumann 2004). Doğum sonrası myometriyal kontraksiyonları arttırmak ve sütün indirilmesini hızlandırmak amacıyla da uygulanan oksitosin hormonu, myoepitelyal hücre farklılaşması, proliferasyonu ve kontraksiyonunu arttıran bir etkiye sahiptir. (Rizzo ve ark. 2012, Thibonnier ve ark. 1999).

Oksitosin bu bilinen etkilerinin yanı sıra vasküler tonusun düzenlenmesinde de etkilidir. Yapılan çalışmalarda oksitosinin farklı tür ve dokularda vazodilatasyon ya da vazokonstriksiyon gibi birbirine zıt etkilere sahip olduğu görülür (Gutkowska ve ark. 2000, Petersson 2002, Petersson ve ark. 1996, Rizzo ve ark. 2012, Thibonnier ve ark. 1999, Vedernikov ve ark. 2006). İnsan vasküler endotelyal hücreleri, uterus ve meme oksitosin reseptörleri ile aynı yapıya sahip olan oksitosin reseptörleri eksprese eder. Bu endotelyal oksitosin reseptörlerinin vazodilatör bir cevap oluşturduğu rapor edilmiştir (Thibonnier ve ark. 1999). Ratlarda gerçekleştirilen bir çalışmada oksitosin uygulaması sonrası, kan basıncının uzun süreliğine düşük seyrettiği bildirilmiştir (Petersson ve ark. 1996). Köpeklerde intravenöz oksitosin enjeksiyonu sonrası meydana gelen vazodilatasyon, kan basıncında azalma ve kalp atım sayısında artma ile ilişkilendirilmiştir (Nakano ve Fisher 1963). Oksitosin enjeksiyonu plazma atriyal natriüretik peptit (ANP) salınımını indükleyerek sodyum ve potasyum ekskresyonunu arttırır (Haanwickel ve ark. 1995). Oksitosinin kardiyovasküler etki mekanizmasının incelendiği bir çalışmada kalp ve büyük damar yapıları içerisinde oksitosin mRNA'ları tespit edilmiştir. Renal elektrolit ekskresyonundaki artışın vasopressinden bağımsız meydana geldiği; oksitosinin etkisiyle lokal olarak üretilen ANP'nin damar basıncının azalmasına sebep olabileceği bildirilmiştir (Gutkowska ve ark. 2000). Bahsedilen vazodilatör etkilerin aksini belirten çalışmalar da mevcuttur. Ratlarda oksitosine ait vazokonstriktif etkinin bölgesel duyarlılık gösterdiği bulunmuştur. Oksitosin uterin arter üzerinde kuvvetli konstrüksiyona sebep olurken bu etki femoral arterde ve aortada giderek azalmış, karotid arterlerde ise bulunmamıştır (Vedernikov ve ark. 2006). Oksitosinin vazokonstriktör ya da vazodilatör etkilerinin seksüel siklus dönemine veya uygulama dozuna göre farklılık arz edebileceği bildirilmiştir. Birbirinden farklı olan bu etkiler oksitosin reseptörlerinin hem endotelyal hem vasküler düz kas hücrelerinde bulunmasından kaynaklanır (Petersson 2002, Prosser ve ark. 1996).

Çiftlik hayvanlarında meme kan akımının incelendiği çeşitli araştırmalar mevcuttur. Dişi kuzularda beslenmenin meme bezi gelişimi üzerine etkisi (Dantas ve ark. 2017), gebelik toksemisinin laktogeneziste meydana getirdiği değişiklikler (Barbagianni ve ark. 2015), emziren ve makinalı sağım uygulanan koyunlarda meme kan akımı (Piccione ve ark. 2004), koyunlarda meme involüsyonu süreci (Petridis ve ark. 2014), mastitisli keçilerde meme kan akımı (Santos ve ark. 2015), ineklerde meme veni kan akımı ile California Mastitis Test (CMT) skorları arasındaki ilişki (Rişvanlı ve ark. 2018) ve yine ineklerde oksitosin uygulamasının meme veni hemodinamiği üzerine etkisi (Rizzo ve ark. 2012) puls

Doppler ultrasonografi ile incelenmiştir. Koyunlarda ise günlük oksitosin enjeksiyonunun süt verimi ve laktasyon uzunluğunda meydana getirdiği değişiklikler (Zamiri ve ark. 2001) araştırılmış olmakla birlikte meme kan akımı üzerine etkisini değerlendiren bir çalışmaya rastlanmamıştır. Bu çalışmada koyunlarda doğum sonrası uygulanan oksitosinin eksternal pudendal arter PI ve RI parametreleri üzerindeki etkisinin incelenmesi amaçlanmıştır.

MATERYAL VE METOT

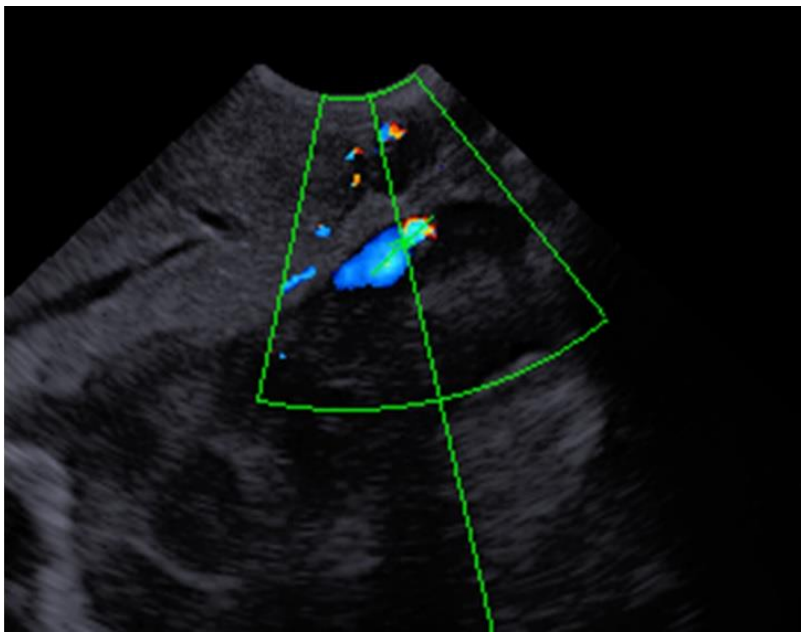
Çalışmada klinik olarak sağlıklı olduğu bilinen primipar 20 adet Kıvırcık ırkı koyun kullanıldı. Hayvanların yaş ortalaması 2,15±0,23 olup vücut kondüsyon skoru literatür bilgiye uyumlu şekilde 3,125±0,32/5 olarak belirlendi (Russel ve ark. 1969). Tüm koyunların normal yolla, müdahalesiz şekilde tek kuzu yavruladığı ve yavru zarlarını doğumu izleyen ilk 6 saat içerisinde attığı görüldü. Çalışma materyali ağılda kuzuları ile birlikte barındırıldı, çalışma süresince yavrularını emzirdi ve klinik mastitis bulgusuna rastlanmadı. Koyunlar Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı tarafından gereksinimleri doğrultusunda hazırlanan rasyon ile beslendi. Doğum sonrası koyunlara yiyebilecekleri kadar kaliteli kuru çayır otu ve hayvan başına 0,5kg/gün koyun süt yemi (%18,72 ham protein, 2493 Mcal/kg metabolik enerji) verildi. Su ve yalama taşına ad libitum erişim sağlandı. Tanımlanan tüm klinik uygulamalar İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi Birim Etik Kurulu tarafından onaylanmıştır (rapor no: 2019/27).

Koyunlar randomize şekilde oksitosin ve kontrol gruplarına ayrıldı. Oksitosin grubuna (n=10) doğum sonrası ilk 3 gün boyunca 12 saat ara ile 10 İÜ/koyun dozda kas içi oksitosin hormonu (Oksitosin, Vetaş, Türkiye) uygulandı. Kontrol grubuna ise (n=10) çalışma grubu ile eş zamanlı olarak 1 ml/koyun dozda kas içi %0,09 NaCl solüsyonu enjekte edildi. Ultrasonografik muayeneler doğum sonrası ilk 3 gün boyunca günde bir kez, oksitosin ya da plasebo uygulamasından sonraki 10. dk'da gerçekleştirildi.

Ultrasonografik muayeneler ayakta zaptedilerek, minimum stres düzeyinde gerçekleştirildi. Meme arterine ait hemodinamik ölçümler, eş zamanlı B-mod ultrasonografi cihazının (Esaote Pie Medical MyLab Five Vet, Esaote Pie Medical, İtalya) puls Doppler tekniği ile gerçekleştirildi (6,6 MHz mikrokonveks prob, 60 mm derinlik). Memeyi besleyen eksternal pudendal arteri belirlemek için prob meme lobunun kaudaline, meme-abdomen sınırına konumlandırıldı. Ardından renkli Doppler modu ile pudendal arter görüntülendi. Optimal renk akımı sağlandığında puls Doppler ile spektral mod aktifleştirildi ve örnekleme yapıldı (Şekil 1). İnsonasyon açısının 60°'den küçük olmasına özen gösterildi. Örnekleme sırasında hayvanların sakin ve hareketsiz olmasına dikkat edilerek, en az üç ardışık artefaktsız trase sonrası PI, RI değerleri otomatik olarak kaydedildi. İşlem sırasıyla her iki meme lobu için tekrarlandı, grupların karşılaştırılmasında iki ölçümün ortalaması kullanıldı.

İstatistiksel Analizler

İstatistiksel analizler için SPSS 20.0 paket veri programı kullanıldı. Oksitosin ve kontrol gruplarına ait PI ve RI ortalama değerleri arasında farklılık olup olmadığı Student t testi ile incelendi. İstatistiksel farklılık için $p<0,05$ anlamlı kabul edildi.

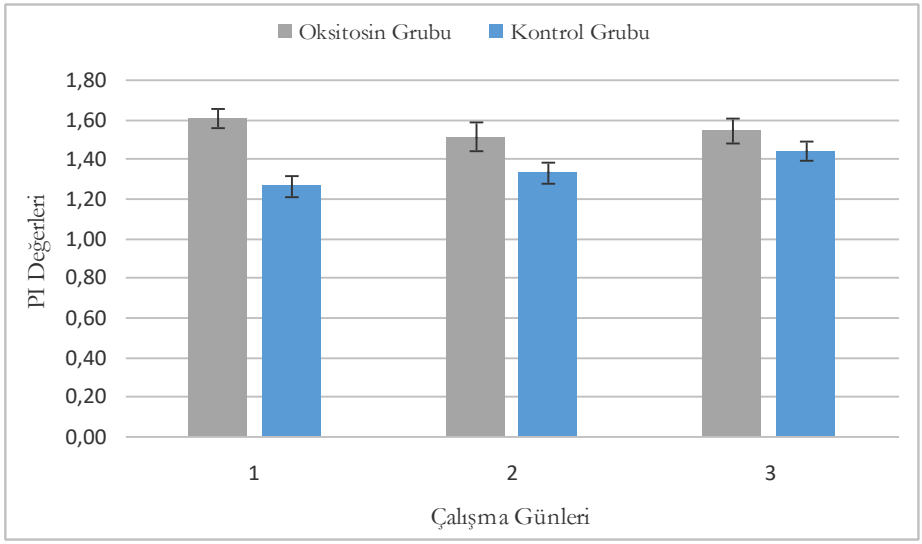


Şekil 1. Eksternal pudendal arterin puls Doppler ultrasonografi ile görüntüsü
Figure 1. Image of external pudendal artery by pulse Doppler ultrasonography

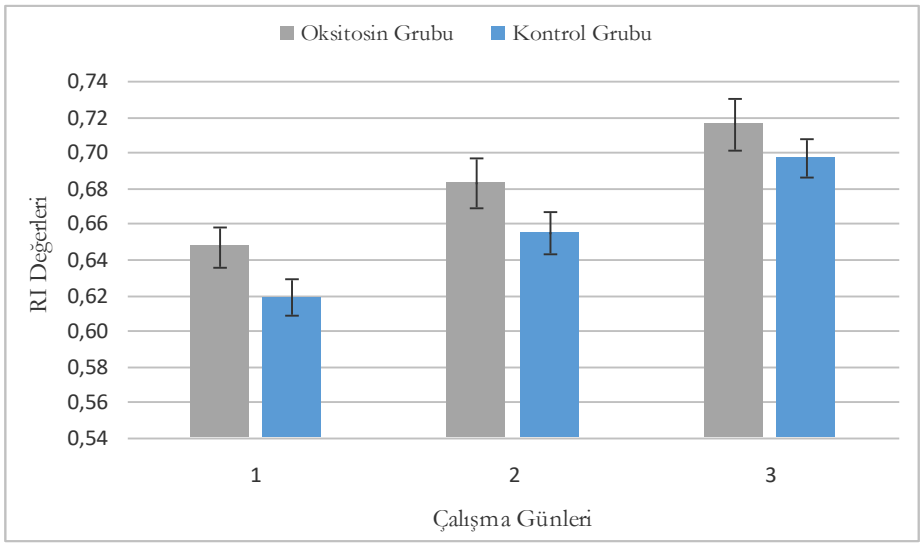
BULGULAR

Meme arterine ait hemodinamik parametreler incelendiğinde oksitosin uygulanan grubun kontrol grubundan matematiksel olarak daha yüksek PI ve RI değerlerine sahip olduğu görüldü. Gruplar arasındaki PI değerleri ortalaması farkı, postpartum birinci ve ikinci günde anlamlı bulundu (sırasıyla $p<0,001$ ve $p<0,05$). Oksitosin uygulanan grupta en yüksek PI değeri (1,61±0,05) çalışmanın ilk gününde saptandı. Kontrol grubunda ise bu indeks, çalışma ilerledikçe matematiksel bir artış gösterdi (Şekil 2).

Günlük ortalama RI değerleri incelendiğinde gruplar arasında istatistiksel farklılık saptanmadı (Şekil 3). Bununla birlikte RI değerleri her iki grupta da artan bir seyir gösterdi.



Şekil 2. Oksitosin ve kontrol gruplarına ait ortalama PI değerleri
Figure 2. Mean PI values of oxytocin and control groups



Şekil 3. Oksitosin ve kontrol grubuna ait ortalama RI değerleri
Figure 3. Mean RI values of oxytocin and control groups

TARTIŞMA ve SONUÇ

Sütçü işletmelerde memeden sütün indirilmesini sağlamada, mastititis olgularının tanı ve sağaltımında oksitosin hormunu sıklıkla uygulanır (Macuhova ve ark. 2004). Koyunlarda da laktasyon sürecini süt verimi yönünden yönetmek için oksitosin uygulaması yapılmakla birlikte (Zamiri ve ark. 2001) doğum sonrası yavru zarlarının atılımını kolaylaştırmak ve involüsyon sürecini hızlandırmak amacıyla da oksitosine başvurulur (Kuru ve ark. 2016). Uterus ve meme dokusunun yanı sıra endotelyal doku ve merkezi sinir sistemi dokusunda da reseptörlere sahip olan oksitosinin seksüel ve maternal davranışları uyarıcı etkilerine ilaveten kardiyovasküler fonksiyonları olduğu da belirlenmiştir (Gutkowska ve ark. 2000, Thibonnier ve ark. 1999). Bu kardiyovasküler etki, incelenen damar yapısına göre vazodilatasyon ya da vazokonstriksiyon ile sonuçlanır (Petersson 2002, Prosser ve ark. 1996, Rizzo ve ark. 2012). Sunulan çalışmada elde edilen indeks verileri, oksitosinin meme arterinde direnç arttırıcı etkisini ortaya koydu. Çalışmada eksternal pudendal artere ait çap verileri değerlendirilmemiş olmakla birlikte damar direncindeki artışın vazokonstriktif etkiyi işaret ettiği düşünülmektedir. Oksitosin uygulanan grupta gözlenen istatistiksel öneme sahip PI artışı (Şekil 2), oksitosinin meme damarı direncinde ve basıncında artışa, distal perfüzyonda ise azalmaya sebep olduğunu tanımlamaktadır.

Koyun meme dokusuna ait kan akım özelliklerinin incelendiği araştırma sayısı kısıtlıdır. Dişi kuzularda beslenmenin meme bezi gelişimi üzerine etkisinin incelendiği bir çalışmada gruplar arasında vücut gelişimi farklılıkları oluşmasına rağmen meme arterine ait PI ve RI değerleri benzer bulunmuştur (Dantas ve ark. 2017). Gebeliğin özellikle son dönemlerinden itibaren laktogenezis için gelişen meme dokusunu besleyen eksternal pudendal arter genişler ve dokuya kan akımı artar. Gelişen meme dokusunda meydana gelen hemodinamik değişiklikler çeşitli Doppler ultrasonografi indeksleri ve parametreleri ile takip edilebilir (Barbagianni ve ark. 2015). Makineli sağım uygulanan koyunlarda meme arterine ait sistolik ve diyastolik akım hızlarının sağım sonrası arttığı tespit edilmiş; bu durum meme içi basıncın sağıma bağlı olarak düşmesi ile ilişkilendirilmiştir. Emziren koyunlarda memenin kuzular tarafından devamlı uyarılmasının bir sonucu olarak sabah ve akşam ölçümleri arasında farklılık oluşmadığı bildirilmiştir. Kuru dönemde ise kan akım hızı, meme bezinin inaktif olmasından ötürü daha düşük gözlenmiştir (Piccione ve ark. 2004). Sunulan çalışmada koyunların kuzuları ile birlikte barındırılarak emzirmenin kısıtlanmaması, meme içinde biriken süt miktarında bireysel farklılıklar oluşturabilmekle birlikte çalışmanın tekiz kuzu yavrulamış primipar hayvanlarda ve postpartum ilk 3 gün içinde gerçekleştirilmesi ile birörneklik sağlanmaya çalışıldı. Her iki grupta da çalışma ilerledikçe artan RI değerleri (Şekil 3), meme arteri direncinde artışı ve akım hızında azalmayı işaret etti. Bu durum kuzuların sık aralıklarla memeyi uyarması neticesinde memenin makineli sağımda meydana geldiği gibi tam boşalmadan tekrar süt üretimine devam etmesi ve dolayısıyla meme içi basıncın artması ile açıklanabilir. Meme bezinin involüsyon sürecinde ise PI ve RI değerlerinin arttığı bildirilmiştir. Ancak bu durum boşaltılmayan sütün meme içinde birikerek meme içi basıncı artmasından ziyade, süt sekresyonundaki azalmaya bağlı olarak dokunun kanlanma ihtiyacının azalmasının bir sonucu olduğuna dikkat çekilmiştir (Petridis ve ark. 2014). Laktasyondaki ineklere uygulanan oksitosinin meme veninde vazodilatasyona sebep olduğu bildirilmiştir. Ancak oksitosinin incelenen damar yapısına göre farklı etkileri olabileceği ve hormonun myoepitelyal hücreler üzerindeki etkisi neticesinde artan arteriyel kan akımının bir sonucu olarak da venöz vazodilatasyon şekillenebileceğine dikkat çekilmiştir (Rizzo ve ark. 2012). Sunulan çalışmada emzirmeye bağlı olarak süt üretiminin devam etmesine ilaveten uygulanan oksitosinin süt alveollerinde oluşturduğu kontraktil etkiler neticesinde meme dokusuna ait kan akımında değişikliklerin gözlenebileceği sonucuna varılmıştır. Ayrıca oksitosinin damar endotel ve kas hücreleri üzerindeki olası lokal vazokonstriktif etkisi, oksitosin grubunda gözlenen direnç artışı ile ilişkilendirilebilir. Oksitosinin damar tonusu üzerindeki bu karşıt etkilerinin çalışmalar arasındaki metot farklılığından kaynaklanmış olabileceği düşünülmektedir. Elde edilecek verilerin çalışmanın gerçekleştirildiği dönem (seksüel siklus, gebelik, laktasyon, meme involüsyonu), hayvanların yavruları ile ya da ayrı barındırılması, emzirme, sağım, hastalık durumu, incelenen damar tipi gibi faktörlerden etkileneceği göz önünde bulundurulmalıdır.

Doppler ultrasonografi ile ruminantlarda meme hastalıkları ve gebelik toksemisi gibi olgularda belirgin değişimler gözlenmiştir (Barbagianni ve ark. 2015, Rişvanlı ve ark. 2018, Santos ve ark. 2015). Sunulan çalışmada sağlıklı koyunlara ait Doppler indekslerinin, meme kan akımını etkileyebilecek çeşitli hastalıkların ayırıcı tanısında yol gösterici olacağı düşünülebilir.

Yapılan bu çalışma ile doğum sonrası oksitosin uygulamasının, koyunların meme arteri direncini arttırdığı sonucuna ulaşıldı. Oksitosinin koyunlarda meme arteri tonusu üzerindeki etkilerini ortaya koymaya yönelik yeni çalışmalara ihtiyaç duyulmaktadır.

Conflict of Interest: The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Research on The Antioxidant Efficiacy of Olive (*Olea Europaea* L.) Leaf Using by *in vitro* Methods

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ABSTRACT

In this study, antioxidant properties of olive (*Olea europaea* L.) leaves containing oleuropein were investigated in *n*-hexane, ethyl acetate, and methanol based extracts obtained from dried olive leaves at different concentration by various *in vitro* methods. Percentage yields of the *O. europaea* were found for the *n*-hexane extract 13.21%, for ethyl acetate extract 26.15% and for methanol extract 34.59%, respectively. Total phenolic substance content (85.27±15.03%), linoleic acid reduction (89.52±9.77%) and reduction capacity (1.49±0.03) were the highest in the methanol extract. Finally, the DPPH radical scavenging efficiency (72.93±0.42%), reduction of iron (II) ions (50.53±5.53%) and superoxide radical scavenging activity (72.93±0.42%). were the highest in the ethyl acetate extract. In conclusion, it was seen that total phenolic substances, linoleic acid reduction and reduction capacity of the methanol extract of the antioxidant activity of the *O. europaea* was more active when equated with *n*-hexane and ethyl acetate extracts. Also, it was found out that the ethyl acetate extract was more effective in DPPH radical scavenging, iron reduction, linoleic acid reduction and superoxide radical scavenging activity. According to the data obtained, it is thought that olive leaf might be evaluated as a natural and cheap alternative antioxidant in different fields such as food, cosmetic, pharmaceutical industry and animal nutrition.

Keywords: Antioxidant activity, *Olea europaea* L., Oleaceae, Olive, Total phenolic substance

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Zeytin (*Olea Europea L.*) Yaprağının Antioksidan Etkilerinin *in vitro* Yöntemlerle Araştırılması

ÖZ

Bu çalışmada pek çok sekonder metabolitin yanında major madde olarak oleuropein içeren zeytin (*Olea europaea* L.) yaprağından elde edilen edilen *n*-hekzan, etil asetat ve metanol ekstrelerin farklı konsantrasyonlardaki antioksidan aktiviteleri çeşitli *in vitro* yöntemlerle incelenmiştir. Zeytin yaprağının *n*-hekzan, etil asetat ve metanollü ekstrelerinin yüzde verimleri sırasıyla %13.21, 26.15 ve %34.59 olarak bulunmuştur. Toplam fenolik madde içeriği (85.27±15.03%), linoleik asit indirgeme (89.52±9.77%) ve indirgenme kapasitesi (1.49±0.03) methanol ekstresinde en yüksek idi. Son olarak, DPPH radikali süpürücü aktivitesi (72.93±0.42%), demir (II) iyonlarını indirgeme (50.53±5.53%) ve süperoksit radikali giderme aktivitesi (72.00±1.35%) etil asetat ekstresinde en yüksekti. Sonuç olarak zeytin yaprağının metanollü ekstresinde toplam fenolik madde, linoleik asit indirgeme ve indirgenme kapasitesi diğer ekstrelere göre daha aktif bulunmuştur. DPPH radikali tutma, demir (II) şelatlama ve süperoksit radikali giderme aktivitesinde ise etil asetat ekstresinin daha etkili olduğu tespit edilmiştir. Elde edilen veriler doğrultusunda, zeytin yaprağının gıda, kozmetik, ilaç endüstrisi ve hayvan besleme gibi alanlarda doğal alternatif bir antioksidan olarak değerlendirilebileceği düşünülmektedir.

Anahtar Kelimeler: Antioksidan aktivite, *Olea europaea* L., Oleaceae, Toplam fenolik madde, Zeytin

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INTRODUCTION

Olive and olive products have important place from in terms of Turkey economy, in which ranks 4th in the field of olive cultivation among the world countries (Keser and Bilal 2010). In Turkey, olive leaves remaining after harvesting the fruits are generally to be destroyed by burned due to they are waste. Investigations for evaluation of the biological activity have revealed that leaves of this plant to possess antibacterial, antifungal and antioxidant properties (Benavente-García et al. 2000, Ferreira et al. 2007, Lee et al. 2010). Olive, a valuable plant in terms of nutritional value, contains water (50%), lipid (22%), protein (1.6%), sugar (19.1%), cellulose (5.8%), minerals, hydrocarbons, tocopherols and polyphenols (Kiritsakis 1998). Olive leaf also contains high functional value phenolic compounds (tyrosol, coumaric acid, hydroxytyrosol, gallic acid, caffeic acid, ferulic acid), flavonoids (luteolin, quercetin, catechin, apigenin) and secoiridoids (oleuropein, ligstrocyte) (Dekanski et al. 2009, Talhaoui et al. 2015). Oleuropein, which causes bitter and acrid aroma of olive, is the main component of olive tree (Silva et al. 2006) and has antioxidant, antimicrobial, antiviral, anti-inflammatory, antiatrogenic, hypolipidemic, antiaging, and parkinson, alzheimer, skin, heart and cancer-protective effects (Ferreira et al. 2007, Omar 2010, Barbora et al. 2014).

Olive leaf extracts are used primarily in animal nutrition and biomass energy. In addition, it is assumed that presence of excessive level of cardiotonic substance oleanolic acid in olive leaves may prove it beneficial for hypertesnive associated cardiac arrests (Somove et al. 2003).

Leaves of olive usage in the food, cosmetics and pharmaceutical industry is becoming increasingly common (Spinelli et al. 2010, Rodrigues et al. 2015, Souilem et al. 2017). Although there are studies to reveal different biological effects of olive, which have been reported to have anti-inflammatory, antiallergic, antibacterial, antimycotic, immunoregulator, antidiabetic and hypolipemic effects (Omar 2010), researches on *in vitro* or *in vivo* effects of olive leaves are quite insufficient.

The objective of this study is to investigate the antioxidant properties of n-hexane, ethyl acetate and methanol based extracts obtained from olive leaves using *in vitro* methods.

MATERIALS and METHODS

Plant material

Olea europaea L. (Oleaceae) leaves were acquired from Selçuk district of İzmir province, Turkey in December 2017. The leaf samples after collection were first verified a by Prof. Dr. Esra Akkol, Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Turkey and were deposited in the Herbarium of same Faculty.

Preparation of extracts

Leaves (400 g) of *O. europea* were grinded after comlete shade-driying. The grounded powder was later extracted with 2000 mL n-hexane, ethyl acetate and methanol each, for 5 days at room temperature. The collected extracts were first strained and later vaporized under low pressure at 40°C till there were completely dried. The percentage yields of each extract were calculated and were found to be 13.21% for *n*-hexane, 26.15% for ethyl acetate, and 34.59% for methanol, respectively.

HPLC analysis

The high-performance liquid chromatography (HPLC) analysis was conducted by using 1200 series HPLC system by Agilent Technologies (Palo Alto, CA, USA) according to method as described by Ansari et al. (2006). The system consisted of a quaternary pump, in-line degasser, column thermostat, and ultraviolet detector. Agilent Chemstation software was used for the data processing and acquisition. The chromatographic separation of the fractions was performed by a LiChrospher® 100 RP C-18 (film thickness, 5 μm; 250 × 4.0 mm) (Supelco Inc., Bellefonte, PA, USA) column using an isocratic elution of HPLC-grade water adjusted to pH 2.5 with *o*-phosphoric acid:acetonitrile (80:20 vol/vol). Detection wavelength was 280 nm, and the flow rate was optimized at 1 mL/minute. The stock solution (1 mg/mL) of oleuropein (Chromadex, Irvine, CA, USA) was prepared by dissolving it in HPLC-graded water and was used as reference. The calibration curve was achieved by diluting the stock seven times, and the equation was designed. The methanol extract was solubilized in methanol, while ethyl acetate extract was liquefied in a small quantity of ethyl acetate and then diluted with isopropanol to the desired volume. Oleuropein and the all other extracts were filtered by using a membrane filter (pore size, 0.45 μm). The reference compound and diluted extracts were inoculated for evaluation of their oleuropein concentration.

In vitro antioxidant property tests

Estimation of total phenolic substances

The overall phenolic substances of olive leaf extracts were measured using Folin-Ciocalteu's phenol reagent (FCR) as described by Singleton and Rossi (1965). The principle of this method rely on the formation of a blue colored compound as a result of the phenolic substances reducing FCR. The absorbance of blue compound is recordedat 760 nm. The total phenolic substances of olive leaf extracts are presented in terms of mg gallic acid equivalents (GAE) per g of extract.

The gallic acid was used as standard solution. 0.5 mL of test extracts solution was stirred along with 0.5 mL FCR and incubated for 3 minutes. Then, 2% Na_2CO_3

was added and stored at room temperature for 2 h. After incubation period, the absorbance of reaction mixture was recorded at 760 nm against distilled water by using as blank. The results were computed by using the standard calibration curve of gallic acid (62.5-1000 µg/mL**)** and were demonstrated in terms of gallic acid equivalents (GAE µg/g).

Evaluation of DPPH radical scavenging activity

The DPPH scavenging activity of olive leaf extracts was established by using the technique of Blois (1958). The method works on the principle of removing DPPH, which is a stable free radical and has a dark purple colour. When the DPPH radical is scavenged, the colour of the reaction mixture shifts from purple to yellow and reduces absorbance at 517 nm.

Briefly, 0.5 mL olive leaf extracts (100-250-500-1000 µg/mL) were supplemented with DPPH (2 mL, 0.1 mM). Each mixture was placed in the dark for 30 min and the absorbance was recorded at 517 nm against ethanol using as blank. *A* blank is reffered as measure of the absorbance of the control reaction which contains all reagents except test compounds. *A* sample is measure of the absorbance of the olive leaf extracts only. The percentage of inhibition was calculated against blank.

Inhibition % = (*A* blank - *A* sample/ *A* blank) × 100

Antioxidant activity with ferric thiocyanate method in linoleic acid system

Antioxidant activity in a linoleic acid emulsion system was evaluated using the ferric thiocyanate method (Pan et al. 2007). According this method, peroxides, an oxidation product of linoleic acid, oxidize Fe^{2+} to Fe^{3+}. As a result, a decrease in absorbance reflects increased level of antioxidant activity.

The linoleic acid emulsion was developedby adding 77.5 µL of linoleic acid, 87.5 mg of Tween 20 and 25 ml of phosphate buffer (0.04 M, pH 7.0). Then the mixture was homogenized. Variable concentrations (100-250-500-1000 µg/mL) of olive leaf extracts (0.5 mL) were added into linoleic acid emulsion (1.25 mL, 0.2 M, pH 7.0) and phosphate buffer (0.75 mL, 0.04 M, pH 7.0). The blend was incubated at 37°C which accelerated the peroxidation process. The control composed of linoleic acid and phosphate buffer without adding extracts. After an interval of 24 h each, the readings were recorded. The mixture 0.05 mL of misture was removed and mixed thoroughly with 2.35 mL of 75% ethanol, 0.05 mL of 30% ammonium thiocyanate and after 3 minutes with 0.05 mL of 20 mM ferrous chloride in 3.5% HCl. After blendig with all component, the mixture was set out for 5 minutes at room temperature. The levels of peroxidation were calculated by measuring the absorbance at 500 nm.

The percentage of inhibition was determined against control by using following formula:

Inhibition % = 1-(*A* sample/ maximum control) × 100

Determination of iron (II) ions chelating activity

Iron (II) ions chelating activity of olive leaf extracts was performed conferring to protocol described by Dinis et al. (1994). This method works on the principle of inhibition in the formation of ferrous iron-ferrozine complex of chelating agents in the test tube. The decrease in red colour was determined by decrease in absorbance of the ferrous iron-ferrozine complex at 562 nm.

Concisely, altered concentrations of olive leaf extracts (100-1000 µg/mL) in 0.5 mL were mixed with a solution of 0.6 mM ferrous chloride (0.05 mL) and kept for 30 min. Later, the mixture was supplemented with ferrozine (5 mM, 0.1 mL). After 10 min, absorbance of the test tubes and EDTA as standards (50-250 µg/mL) were recorded spectrophotometrically at 562 nm. The control consisted of distilled water only without $FeCl_2$ and ferrozine. The percentageinhibition of iron chelating activity was recorded with following equation:

Inhibition % = 1-(*A* control - *A* sample/ *A* control) x 100

Measure of superoxide radical scavenging activity

The superoxide radical scavenging activity works on the principle that the superoxide radical produced by the NADH (nicotinamide adenine dinucleotide)/PMS (phenazine methosulfate)/O_2 complex reduces nitro blue tetrazolium (NBT) from yellow to purple-coloured formazone (Nishimikiet al. 1972). The decrease of the absorbance values indicates consumption of superoxide radical anions.

According to method, 0.5 mL NBT (156*µ*M) and NADH (468 *µ*M) in the sodium phosphate buffer (20 mM, pH 7.4) were added to different concentrations of extract solutions (0.5 mL, 100-250-500-1000 µg/mL) in phosphate buffer. The reaction began by supplementation of PMS (50µL, 60 µM) into the reaction mixture and incubated at room temperature for 5 minutes. Then, the absorbance was monitored at 560nm against the corresponding distilled water as control.

The percentage of inhibition was calculated by using following formula:

Inhibition % = (*A* control - *A* sample/ *A* control) × 100

Ferric ions reducing antioxidant power

Reducing capacity of olive leaf extracts was determined according to the developed method Oyaizu (1986). The method is based on Fe^{+3} ions are reduced to Fe^{+2} ions. When ferric chloride ($FeCl_3$) is added, the absorbance of the complex compound formed in Prussian blue colour is measured at 700 nm. The highest absorption values mean the highest reduction capacity for extracts.

A 0.5 mL solution of extracts (100-250-500-1000 µg/mL) was mixed with 1.25 mL of phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide. After incubating at 50°C for 20 minutes, the reaction mixture was further supplemented with 1.25 mL of 10% trichloroacetic acid and vortexed. The reaction mixture was centrifuged at 2500 rpm for 10 minutes. Later, 1.25 mL supernatant was collected and mixed with equal volume of distilled water and 0.25 mL $FeCl_3$ (0.1 %). It was kept for 10 minutes at room temperature and the absorbances were evaluated at 700 nm against distilled water as blank.

Statistical analysis

Antioxidant activity assays was conducted in triplicate. The results obtained were evaluated by SPSS 13.0 and given as mean ± standard error values. The statistical differences among groups were analysed through One way ANOVA analysis. Duncan test was used as a posthoc test. Statistical significance of all data was determined as $P < 0.05$ level.

RESULTS

The results obtained with the n-hexane, ethyl acetate and methanol extracts of the leaves of O. europea in the antioxidant activity tests are given in Table 1-6.

The olive leaf extracts were examined for their total phenolic substance amounts (Table 1) according to acquire from standard gallic acid curve graph (Figure 1). The highest phenolic content of methanolic extract was 85.27±15.03 mg GAE in 1000 µg/mL while the lowest value was 0.18±1.32 µg GAE/g extract in n-hexane extract (500 µg/mL).

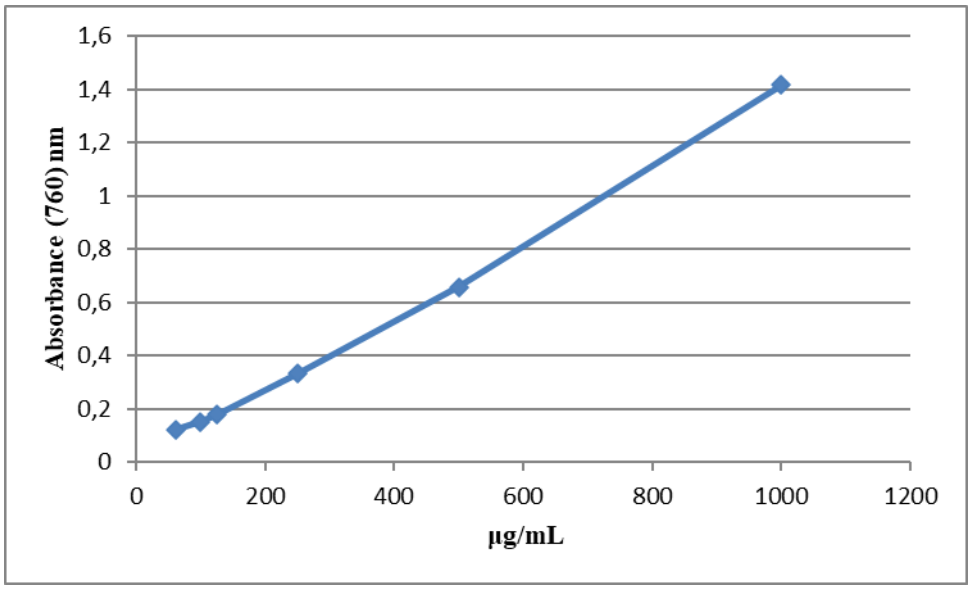

Figure 1. Standard gallic acid curve graphic

Table 1. Total phenolic substances concentration of the extracts obtained from O. europea leaves

Extracts	Total phenolic substances (µg GAE/g ± SEM)			
	100µg mL^{-1}	250µg mL^{-1}	500µg mL^{-1}	1000µg mL^{-1}
***n*-Hexane**	0,4177±2,34^{d}	2,79±0,71^{d}	0,18±1,32^{d}	12,53±3,82^{c,d}
Ethyl acetate	13,49±8,22^{c,d}	14,91±3,59^{c,d}	19,89±2,29^{c,d}	33,67±4,12^{b,c}
Methanol	11,58±0,63^{c,d}	26,78±3,14^{c}	48,39±1,42^{b}	85,27±15,03^{a}

^{a,b,c,d} Different letters in the same column represent the statistically significant difference between the mean values P<0,001
SEM: Standard error meaning

The percentages of DPPH inhibition of olive leaf extracts at different concentrations are shown in Table 2. According to the results, DPPH radical scavenging activity was found to be highest in ethyl acetate extract (72.93 ± 0.42%) at a concentration of 500 µg/mL, the lowest value in n-hexane extract (100 µg/mL) was 13.77 ± 1.23%.

Table 2. DPPH radical scavenging activity of the extracts obtained from *O. europea* leaves

Extracts	DPPH radical scavenging activity (Inhibition % ± SEM)			
	100µg mL^{-1}	250µg mL^{-1}	500µg mL^{-1}	1000µg mL^{-1}
***n*-Hexane**	13,77±1,23^{f}	43,67±1,04^{e}	64,10±4,95^{a,b,c}	71,17±2,93^{a}
Ethyl acetate	42,37±3,34^{e}	61,50±0,79^{b,c,d}	68,40±0,67^{a,b}	72,93±0,42^{a}
Methanol	55,77±1,58^{c,d}	55,67±2,83^{c,d}	54,43±5,17^{d}	60,53±4,33^{b,c,d}

a,b,c,d,e,f Different letters in the same column represent the statistically significant difference between the mean values P<0,001
SEM: Standard error meaning

As shown in Table 3, the highest value linoleic acid reduction was obtained as 89.52±9.77% in methanolic extract at 250 µg/mL concentration, the lowest value as 61.19±4.05% in ethyl acetate extract at 100 µg/mL concentration.

Table 3. Antioxidant activity in linoleic acid system of the extracts obtained from *O. europea* leaves

Extracts	Antioxidant activity in linoleic acid system (Inhibition % ± SEM)			
	100µg mL^{-1}	250µg mL^{-1}	500µg mL^{-1}	1000µg mL^{-1}
***n*-Hexane**	76,19±2,03^{a,b,c}	76,19±5,61^{a,b,c}	75,24±1,26^{a,b,c}	71,67±1,26^{b,c}
Ethyl acetate	61,19±4,05^{c}	63,09±0,48^{c}	65,24±6,67^{c}	86,67±7,63^{a,b}
Methanol	83,57±2,70^{a,b}	89,52±9,77^{a}	74,79±3,93^{a,b,c}	67,67±2,34^{c}

^{a,b,c} Different letters in the same line represent the statistically significant difference between the mean values P<0,001
SEM: Standard error meaning

The effects of various amounts of olive leaf extracts on reduction of iron (II) ions are given in Table 4. While it was found 50.53±5.53% in ethyl acetate extract at 1000 µg/mL concentration, the lowest value was 8.16±0.43% in n-hexane extract 100 µg/mL concentration.

Table 4. Iron (II) ions chelating activity of of the extracts obtained from *O. europea* leaves

Extracts	Iron (II) ions chelating activity (Inhibition % ± SEM)			
	100µg mL^{-1}	250µg mL^{-1}	500µg mL^{-1}	1000µg mL^{-1}
***n*-Hexane**	8,16±0,43^{e}	8,95±0,14^{d,e}	9,05±0,20^{d,e}	8,85±0,18^{d,e}
Ethyl acetate	14,87±1,22^{c,d,e}	16,91±5,22^{b,c,d,e}	25,62±5,90^{b}	50,53±5,53^{a}
Methanol	11,61±0,24^{d,e}	15,33±0,20^{c,d,e}	17,70±0,33^{b,c,d}	23,26±0,31^{b,c}

^{a,b,c,d,e,} Different letters in the same column represent the statistically significant difference between the mean values P<0,001
SEM: Standard error meaning

As shown in Table 5, the superoxide radical scavenging activity was detected 72.00±1.35% in ethyl acetate extract (250 µg/mL), the lowest value as 13.76±2.24% in n-hexane extract at 500 µg/mL concentration.

Table 5. Superoxide radical scavenging activity of the extracts obtained from *O. europea* leaves

Extracts	Superoxide radical removal activity (Inhibition % ± SEM)			
	100µg mL^{-1}	250µg mL^{-1}	500µg mL^{-1}	1000µg mL^{-1}
***n*-Hexane**	13,76±2,24^{f}	31,53±1,97^{e}	39,96±3,52^{d}	51,02±0,46^{c}
Ethyl acetate	70,78±2,99^{a}	72,00±1,35^{a}	63,88±1,65^{a}	67,29±2,23^{a}
Methanol	59,02±2,51^{a,b}	56,90±0,85^{b}	67,02±1,47^{a}	68,82±0,65^{a}

^{a,b,c,d,e,f} Different letters in the same column represent the statistically significant difference between the mean values P<0,001
SEM: Standard error meaning

Finally, according to the results while the highest value in the reduction capacity of extracts was found 1.49±0.03 in methanol extract at 1000 µg/mL concentration and the lowest value was in ethyl acetate extract (100 µg/mL) (Table 6).

Table 6. Reduction capacity of the extracts obtained from *O. europea* leaves

Extracts	Reduction capacity (Absorbance ± SEM)			
	100µg mL^{-1}	250µg mL^{-1}	500µg mL^{-1}	1000µg mL^{-1}
***n*-Hexane**	0,25±0,01^{e,f}	0,23±0,02^{e,f}	0,24±0,01^{e,f}	0,28±0,00^{d,e}
Ethyl acetate	0,19±0,01^{f}	0,25±0,01^{e,f}	0,21±0,00^{d}	0,44±0,01^{c}
Methanol	0,33±0,00^{d}	0,54±0,01^{b}	0,90±0,04^{a}	1,49±0,03^{a}

^{a,b,c,d,e,f} Different letters in the same column represent the statistically significant difference between the mean values P<0,001
SEM: Standard error meaning

To determine the oleuropein concentration in the extracts, HPLC analysis was used. In order to determine the oleurpeins's concentration in all extracts, calibration curve and the equation were first proved by using oleuropein as reference compound. Retention time was recorded as 9.1 ± 3 min for the oleuropein. The reference oleuropein and the all extracts were inoculated respectively to confirm if the detected major peak is oleuropein or not. The repetitive increase in the area of the same peak confirmed the idea that the major peak was oleuropein in the methanol extract. Thus, it has been measured that 1 g of methanol extract contains 24.12 ± 2.87 mg of oleuropein.

DISCUSSION and CONCLUSSION

In the current study, it was recorded that the yield of extraction of olive leaves of the Memecik cultivar was 34.59% in methanol, 26.15% in ethyl acetate and 13.21% in *n*-hexane extracts. Orak et al. (2019) reported the yield of olive leaf extracts varied from 24.26-29.87% and also the yield of methanol extract of Memecik type was found at the level of 27.11%. In addition, the oleuropein in methanol extract was determined as 24.12 ± 2.87 mg / g in this study. The result obtained for oleuropein in the methanol extract is in accordance with previous report which state that oleuropein, the main phenolic component for all genotypes, range from 21.0 to 98.0 mg /g in the methanolic extract (Orak et al. 2019). Orak et al. (2019) reported that oleuropein found at 38.2±1.9 for the methanol extract of Memecik type olive leaf. Moreover, hydroxytyrosol, verbascoside, luteolin 7-*O*-glucoside and luteolin 4′-*O*-glucoside have been widely contained in olive leaves (Talhaoui et al. 2015).

The maximum amount of total phenolic substances was determined as 85.27 ± 15.03 in methanol extract (1000 µg / mL), the lowermost value was found as 0.18 ± 1.32 mg gallic acid (GAE) / g in *n*-hexane extract (500 µg / mL). This value in the methanol extract was found to be lower than 209 ± 3.4 mg GAE/g reported by Orak et al. (2019) for Memecik cultivar olive leaf. Orak et al. (2019) described that total phenolic substances was found as a 110-268 mg GAE/g in the methanol extract of leaves of other *Olea* species growing in Turkey. These differences stem from cultivated conditions, natural habitats of the olive tree.

In this study, the highest value in the DPPH radical scavenging activity was measured 72.93±0.42% for ethyl acetate extract at 500 µg/mL concentration and the lowest value was 13.77±1.23% in *n*-hexane (100 µg/mL). Addition, it was found in this study that when the concentration of extracts in methanol, ethyl acetate and *n*-hexane groups decreased, DPPH radical scavenging activity was found to decreasing accordingly. Hayes et al. (2009), olive leaf methanol extract DPPH radical scavenging activity increased from 100 ppm to 400 ppm and this increase reached 94% at 1000 ppm. Our results were consistent with these reported results. Stupans et al. (2002) reported that this activity was due to hydroxytyrosin and oleuropein, a radical scavenging agent in olive leaves. Non-enzymatic lipid peroxidation caused by free radicals begins with lipid peroxidation of H atoms containing an electron from conjugated double bonds in fatty acids and the fatty acids in the membrane are converted to lipid free radicals. These radicals are unstable and are converted to oxygen by lipid peroxide radical (LOO·) and then by electron from other fatty acids to lipid peroxides (LOOH). Malondialdehydes are orginated as a result of peroxidation of fatty acids while chaining continues (Halliwel and Gutterigde 1990). In our study, the highest value linoleic acid reduction was found as 89.52±9.77% in methanol extract at 250 µg/mL concentration, the lowest value as 61.19±4.05% in ethyl acetate extract at 100 µg/mL concentration. Danahaliloğlu et al. (2018) found that the % inhibition values in the methanolic extract of the Halhalı type olive leaf ranged between 72.89-41.32%. The highest value in reduction of iron (II) ions was found as 50.53±5.53% in ethyl acetate at 1000

µg/mL concentration, the lowest value as 8.16±0.43% in *n*-hexane 100 µg/mL concentration. Hayes et al. (2009) reported that iron chelating activity was found in methanolic extracts as ellagic acid>sesamol> olive leaf extract> lutein, respectively.

It is known that superoxide dismutase (SOD) is a key antioxidant enzyme and catalyses the dismutation of the superoxide (O_2^-) radical into eithermolecular oxygen (O_2) or hydrogen peroxide (H_2O_2). SOD has three isozymes as Mn-SOD, Fe-SOD and CuZn-SOD, which localized in different cell compartments of olive leaf (Corpas et al. 2006; Halliwell et al. 2000). According to our findings, the highest superoxide radical scavenging activity was found 72.00±1.35% in ethyl acetate extract (250 µg/mL), and was measured the lowest value as 13.76±2.24% in n-hexane extract (500 µg/mL). Lee and Lee (2010) reported that SOD activity was determined at 18% in oleuropein of olive leaves, 67% in routine, 83% in caffeic acid and 19% in their mixtures. Finally, the highest reduction capacity was found 1.49 ± 0.03 in methanol extract (1000 µg / mL) and the lowest value was 0.19 ± 0.01 in ethyl acetate extract (100 µg /mL) in our study. Danahaliloğlu et al. (2018) reported that FRAP values for methanol extracts of olive leaves of Halhali type were between 0.96-0.11.

Various antioxidant activity differences observed in the olive leaf extracts are thought to be due to the level of polyphenolic compounds passing into the solvent used and the difference in their chemical structure. Considering the extracts according to polarity, it was found that phenolic substances dissolved in methanol extract alone or together synergistically reduce free radicals, chelate metal ions, prevent lipid peroxidation and have metal-reducing capacities.

As a result, it was determined that the methanolic extract of olive leaf was found to be more active than other extracts in terms of total phenolic substances, linoleic acid reduction and reduction capacity. Considering this study data, where the antioxidant activities of olive leaves in different extracts were determined, it was concluded that olive leaf might be regarded as a natural alternative antioxidant source in food, cosmetics, pharmaceutical and animal nutrition fields.

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Conflict of Interest: The authors declare that they have no conflict of interest

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