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Research Article

An Examination of the Relationships Between Live Weight and Body Measurements in Karacabey Merino Sheep Through the Path Analysis Approach

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Abstract

Direct and indirect effects of some of the body measurements on Karacabey Merino Sheep's live weights were estimated using a path analysis in this study. When setting the path model, live weight, back length, body length, shin girth, chest depth, rump width and chest girth measurements of 249 Karacabey Merino sheep of which ages varied between 1 and 4 years were utilized. According to the two-equation causal path model, the body measurement which had the highest direct effect on live weight was chest girth, and it was also shown that chest depth and rump width had indirect effects. It was found that the model expressed a good fit according to the goodness of fit criteria. Consequently, it was revealed that the body measurement which had the greatest contribution to the model set for live weight estimation was chest girth and causal relations between the independent variables variable could be shown with the path analysis.

Keywords: *Path analysis, Path diagram, Karacabey Merino sheep, Live weight*

Karacabey Merinosu Koyunlarinda Canlı Ağırlık İle Vücut Ölçüleri Arasındaki İlişkilerin Path Analizi Yaklaşımı İle İncelenmesi

Özet

Bu çalışmada Karacabey Merinosu koyunlarının canlı ağırlıkları üzerine, bazı vücut ölçülerinin doğrudan ve dolaylı etkileri path analizi kullanılarak tahmin edilmiştir. Path modeli kurulurken, 1-4 yaş arası 249 baş Karacabey Merinosu koyununun canlı ağırlık, sırt uzunluğu, vücut uzunluğu, incik çevresi, göğüs derinliği, sağrı genişliği ve göğüs çevresi ölçümlerinden yararlanılmıştır. İki denklemli nedensel path modeline göre canlı ağırlık üzerinde doğrudan etkisi en yüksek vücut ölçüsünün göğüs çevresi olduğu belirlenmiş, göğüs derinliği ve sağrı genişliğinin dolaylı etkileri de gösterilmiştir. Modelin uyum iyiliği kriterlerine göre iyi uyumu ifade ettiği de tespit edilmiştir. Sonuç olarak; canlı ağırlık tahmini için oluşturulan modele en yüksek katkıyı sağlayan ölçünün göğüs çevresi olduğu, ayrıca path analizi ile bağımlı değişkene ek olarak bağımsız değişkenler arasındaki nedensel ilişkilerin de gösterilebileceği ortaya konmuştur.

Anahtar sözcükler: *Path analizi, Path diyagramı, Karacabey Merinosu koyunları, Canlı ağırlık*

INTRODUCTION

Increasing the yield in sheep breeding just as in other stock breeding is possible through enhancing livestock's genetic structures and environmental conditions and the studies of breeding and selection. Meat yield is one of the primary yields that is not only of financial importance but also meets human's needs [1-3]. For increasing the meat yield in sheep, it is necessary to identify the sheep breed to be selected, the area in which sheep will be bred, and the rangeland and climate conditions optimally [2,4]. Yet, reasons such as tendency towards breeding of low-yield sheep breeds, sheep feeding in low-quality rangelands and early slaughter affect sheep breeding in Turkey in a negative way [5].

Influencing the meat yield, live weight is considered an important criterion in livestock choice in breeding. So much time and labor force is spent for weighing the live weight in rural areas and at small-scale business where measuring equipment is not used frequently [6,7]. Therefore,

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livestock's body measurements are often utilized for live weight estimation [8,9]. On the other hand, the relationship between live weight and morphological measures presents a complex structure. While the degree and direction of the relationship can be determined with correlation analysis in this case, regression analysis can be used when estimating live weight with body measurements. However, effects among variables in the correlation and regression analysis cannot be sufficiently explained as they can be also shaped by a third variable [10]. With developments in the area of computers in recent years, multivariate methods, which have been developed as alternatives to situations where correlation and regression analyses fall insufficient in analyzing relationships among complex variable structures, have become applicable. One of those methods is path analysis. Path analysis is utilized when identifying the causal (direct or indirect) and non-causal (unanalyzed and spurious) effects of one variable on another and breaking variables' correlation coefficients into the components of those variables [6,11]. Path analysis is a subset of structural equation models and presents itself as a methodology complementary to multiple regression model which helps determining the explanatory variable that affects the response variable more [10,12].

The purpose of this study was to identify direct and indirect effects between live weights and several body measurements of Karacabey Merino sheep bred at Karacabey Agricultural Enterprise and to evaluate the model set with different goodness of fit criteria.

MATERIAL and METHODS

The livestock material of the research was 249 Karacabey Merino sheep at the age of 1-4 years bred at Karacabey Agricultural Enterprise. Maintanance and feeding of animals housed in enterprise conditions were carried out on a routine programme of the enterprise. In the study, live weight (the body weight that was taken using a digital scale [kg]), back length (the vertical distance between the base of neck and the waist axis [cm]), body length (the distance between the point of shoulder and the pin bone [cm]), shin girth (the circumference of mid metacarpus [cm]), chest depth (the vertical distance from sternum to withers [cm]), rump width (the distance between the outer edges of Tuber ichii [cm]), chest girth (the circumference of the chest [cm]) and withers height (the distance from the surface of the platform to the withers [cm]) measurements obtained from the sheep after shearing were used.

Descriptive statistics were calculated first for all the variables. Pearson's correlation coefficient was utilized to determine the degree and direction of the relationships among the variables. To identify the variables to be included in the path analysis, preliminary assessments were performed with the multiple regression analysis. Path analysis was conducted and a path diagram was created to interpret direct and indirect relationships among the variables of the model.

The path coefficients were calculated with the help of standardized regression coefficients as follows:

The multiple linear regression model that is constituted by independent variables X_i and the error term e and the dependent variable Y explained by them is expressed as

$$Y = b_0 + b_1X_1 + b_2X_2 + \ldots.. + b_kX_k + e \quad (1)$$

In this model,

b_0: regression constant,

b_i: partial regression coefficients.

The linear regression equation for standardized variables is formed as in Equation 2.

$$Y = P_{YX_1}X_1 + P_{YX_2}X_2 + ... + P_{YX_k}X_k + P_{YX_e}X_e \quad (2)$$

Path coefficient (P) is defined as the standardized regression coefficient which shows the direct effect of the independent variables in the path model on the dependent variable, and expressed as the effect which the change of 1 standard deviation in the explanatory variable creates on the dependent variable. In multiple regression analysis, the beta coefficients between the independent variables and the dependent variable are also denoted by P, because they are defined as path coefficients at the same time.

$$P_{YX_k} = b_k \frac{S_{X_k}}{S_Y} \quad (3)$$

Here,

P_{YX_k} : path coefficient,

S_{X_k} : standard deviation of the independent variable ,

S_Y : standard deviation of the dependent variable Y, and

b_k : partial regression coefficient [10].

In the path analysis, one or more dependent variables are analyzed through each independent variable; in other words, more than one multiple regression analyses can be performed at the same time [13]. By this means, models with two or more equations as well as one-equation models can be set. In *Fig. 1a,* equation of the one-equation path model is written as

$$y = a + b_1x_1 + b_2x_2 + b_3x_3 \quad (4)$$

In *Fig. 1b,* equation of the two-equation path model is written as

$$x_3 = a + b_1x_1 + b_2x_2 \quad (5)$$

$$y = a + b_1x_1 + b_2x_2 + b_3x_3 \quad (6)$$

In the path model, direct effect is utilized when identifying the direct effect between two variables and indirect effect

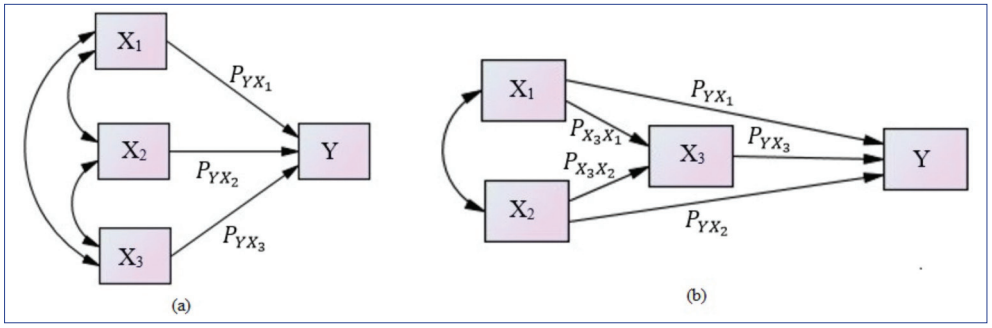

Fig 1. Path models (a) One-equation path model (b) Two-equations path model

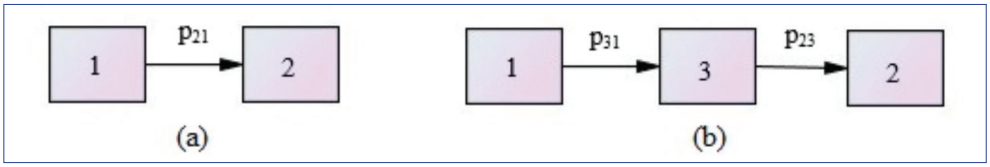

Fig 2. Causal relation types in path diagram (a) Direct effect (b) Indirect effect

is the effect of a variable on another variable through one or more variables.

Direct effect is described as the path coefficient between two variables as shown in *Fig. 2a*. Indirect effect can be calculated by multiplying the path coefficients on the path between the variables. So in *Fig. 2b* the indirect effect between Variable 1 and 2 can be calculated by multiplying the path coefficients and [11].

Only the causal (direct and indirect) effects were emphasized for the path model in this study. The fit of model was evaluated in accordance with the goodness of fit criteria suggested by Engel et al.[14].

SPSS 23.0 software package was used to calculate the descriptive statistics and correlation coefficients and for the regression analysis while the path analysis was performed in AMOS 23.0 software. The significance level was accepted to be P<0.05.

RESULTS

Descriptive statistics regarding live weight (Y), back length (X_1), body length (X_2), shin girth (X_3), chest depth (X_4), rump width (X_5), chest girth (X_6) and withers height (X_7) measurements of Karacabey Merino sheep are given in *Table 1*.

Correlation coefficients predicted for examining the relationships between live weight (Y) and back length (X_1), body length (X_2), shin girth (X_3), chest depth (X_4), rump width (X_5), chest girth (X_6) and withers height (X_7) and among the body measurements are shown in *Table 2*. Accordingly, correlation coefficients among all the variables were found to be statistically significant (P<0.001). It was seen that there was a highly positive relationship between live weight and chest girth, a moderately positive relationship between live weight and back length, body length, rump width and withers height, and a lowly positive relationship between live weight and shin girth.

According to the preliminary assessment performed with the multiple linear regression analysis to establish the theoretical infrastructure of the path model, back length (t=5.638, P<0.001), body length (t=4.707, P<0.001), shin girth (t=3.924, P<0.001), chest depth (t=4.013, P<0.001), rump width (t=2.533, P=0.012) and chest girth (t=10.227, P<0.001) affected live weight on an important level and withers height (t=0.996, P=0.320) had no important effects on live weight. It was observed that chest girth is a more important measurement for live weight estimation

Table 1. *Descriptive statistics regarding live weights and body measurements of Karacabey Merino sheep*

Variables	n	Arithmetic Mean	Standard Deviation	Standard Error	Median	Minimum	Maximum
Live weight (Y)	249	51.36	5.99	0.38	52.00	35.00	68.00
Back length (X_1)	249	67.66	3.09	0.20	68.00	55.00	75.00
Body length (X_2)	249	56.19	3.15	0.20	56.00	33.00	68.00
Shin girth (X_3)	249	8.57	0.33	0.02	8.50	7.80	9.70
Chest depth (X_4)	249	31.49	1.84	0.12	32.00	26.00	36.00
Rump width (X_5)	249	19.40	1.49	0.09	19.00	16.00	29.00
Chest girth (X_6)	249	94.33	5.03	0.32	95.00	79.00	106.00
Withers height (X_7)	249	68.41	2.98	0.19	68.00	60.00	91.00

Table 2. *Correlation coefficients among dependent and independent variables*

Variables	Y	X_1	X_2	X_3	X_4	X_5	X_6	X_7
Live weight (Y)	1	0.657***	0.578***	0.378***	0.801***	0.617***	0.846***	0.555***
Back length (X_1)		1	0.437***	0.289***	0.564***	0.412***	0.509***	0.403***
Body length (X_2)			1	0.270***	0.460***	0.387***	0.452***	0.408***
Shin girth (X_3)				1	0.252***	0.200***	0.240***	0.260***
Chest depth (X_4)					1	0.550***	0.793***	0.533***
Rump width (X_5)						1	0.588***	0.466***
Chest girth (X_6)							1	0.501***
Withers height (X_7)								1

**** P<0.001*

Table 3. *Parameter estimations for the path model*

Dependent Variables	Independent Variables	Regression Coefficients			Z	P
		Unstandardized		Standardized		
		Beta	Standard Error	Beta		
Live weight (Y)	Back length (X_1)	0.357	0.062	0.186	5.765	<0.001
	Body length (X_2)	0.285	0.057	0.151	5.02	<0.001
	Shin girth (X_3)	1.997	0.487	0.111	4.099	<0.001
	Chest girth (X_6)	0.907	0.050	0.769	17.966	<0.001
Chest girth (X_6)	Chest depth (X_4)	1.822	0.116	0.665	15.775	<0.001
	Rump width (X_5)	0.779	0.137	0.230	5.676	<0.001

than other independent variables. It was also aimed at investigating the effects of these variables on chest girth for determining whether the effects of all other independent variables on live weight was through chest girth. Accordingly, chest depth (t=11.765, P<0.001) and rump width (t=4.097, P<0.001) measurements had important effects on chest girth, but the effects of back length (t=0.810, P=0.419), body length (t=1.319, P=0.189), shin girth (t=0.241, P=0.809) and withers height (t=0.914, P= 0.362) on chest girth were not important. When examining the Variance Inflation Factor (VIF) and the tolerance values, no multicollinearity problems were found in either of the models. Since withers height was found to be statistically insignificant in both models, it was not included in the path model. Since the effects of chest depth and rump width on chest girth were found to be important, the effects of these variables on live weight were considered being indirect through chest girth.

According to the path diagram in *Fig. 3*, the two-equation causal model was used in live weight (Y) estimation through the body measurements of back length (X_1), body length (X_2), shin girth (X_3), chest depth (X_4), rump width (X_5) and chest girth (X_6). The covariance among the error terms of these variables were also added to the model as a common variance is expected due to the high-level relationship between chest girth and live weight variables.

Parameter estimations and significance of path coefficients for both models are given in *Table 3*. As determined by the standardized regression coefficients, it is seen that the highest contribution to the explanation of live weight in Model 1 was by chest girth and the highest contribution to the explanation of chest girth in Model 2 was by chest depth.

As seen in the path diagram of *Fig. 3*, measurements of back length, body length, shin girth and chest girth had direct effects on live weight while chest depth and rump width affected live weight indirectly through chest girth. Size of the direct and indirect effects abovementioned are given in *Table 4*. Depending on the high correlation between live weight and chest girth, it can be concluded that the highest effect was the direct effect of chest girth on live weight.

Goodness of fit criteria, fit limits, and the goodness of fit values for the model achieved in the study are presented in *Table 5*. Accordingly, it was determined that all the goodness of fit criteria obtained in the model exhibited good fit; in other words, the model fits the data.

DISCUSSION

Path analysis helps reveal the relationships among variables in detail provided that variables have both individual effects and effects along with other variables. There

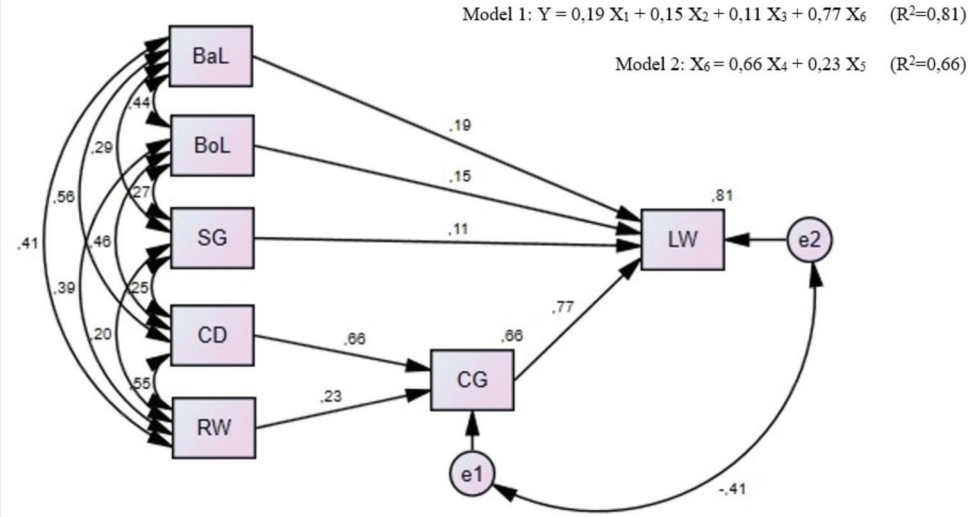

Fig 3. Path diagram of live weight estimation through body measurements

Table 4. *Causal (direct and indirect) effects for the path model*

Dependent Variables	Independent Variables	Back Length	Body Length	Shin Girth	Chest Depth	Rump Width	Chest Girth
Live weight (Y)	Total effect	0.186	0.151	0.111	0.511	0.177	0.769
	Direct effect	0.186	0.151	0.111	0.000	0.000	0.769
	Indirect effect	0.000	0.000	0.000	0.511	0.177	0.000
Chest girth (X_6)	Total effect	-	-	-	0.665	0.230	-
	Direct effect	-	-	-	0.665	0.230	-
	Indirect effect	-	-	-	0.000	0.000	-

Table 5. *Goodness of fit criteria and fit limits*

Goodness of Fit Criteria	Good Fit	Acceptable Fit	Goodness of Fit Values for the Model Achieved
χ^2	$0 \leq \chi^2 \leq 2df$	$2sd \leq \chi^2 \leq 3df$	4.682
P value	$0.05 < P \leq 1.00$	$0.01 \leq P \leq 0.05$	0.321
χ^2/df	$0 \leq \chi^2/df \leq 2$	$2 \leq \chi^2/df \leq 3$	1.171
RMSEA	$0 \leq RMSEA \leq 0.05$	$0.05 \leq RMSEA \leq 0.08$	0.026
NFI	$0.95 \leq NFI \leq 1.00$	$0.90 \leq NFI \leq 0.95$	0.995
CFI	$0.97 \leq CFI \leq 1.00$	$0.95 \leq CFI \leq 0.97$	0.999
GFI	$0.95 \leq GFI \leq 1.00$	$0.90 \leq GFI \leq 0.95$	0.995
AGFI	$0.90 \leq AGFI \leq 1.00$	$0.85 \leq AGFI \leq 0.90$	0.963

χ^2: *Chi-square value,* ***df:*** *Degrees of freedom,* ***RMSEA:*** *Root mean square error of approximation,* ***NFI:*** *Normed fit index,* ***CFI:*** *Comparative fit index,* ***GFI:*** *Goodness of fit index,* ***AGFI:*** *Adjusted goodness of fit index*

are many studies using path analysis in sheep and goat breeding [3,15-17], poultry farming [7,18-20] and cattle breeding [21-25] and other livestock breeding [26,27].

In this study, it was seen that chest girth (r=0.846) and chest depth (r=0.801) was highly related to live weight when examining the correlation coefficients that showed the relationships between live weights and body measurements of Karacabey Merino sheep. This finding shows similarity with findings of other studies that examined the relationships between live weight and body measurements [6,8,10,28-30].

In the model obtained with the multiple linear regression analysis that was applied in this study, sheep's live weight was explained by their back length, body length, rump width, chest depth, shin girth and chest girth whereas the body measurement which had the highest effect on live weight was found to be chest girth. It was also reported in other studies with similar purposes [10,29-31] that chest girth has the highest level of effect on live weight.

In the studies which estimated live weight with path analysis and using body measurements, it was observed that the path models that were established were one-equation causal models [6,8,10,30]. However, in this model, a two-equation causal model was established when estimating live weight with path analysis as the indirect effects of chest depth and rump width through chest girth were examined. The goodness of fit criteria for the model established differently from the studies abovementioned were examined and it was determined that the model met all the criteria. The body measurement with the highest direct effect on live weight was found to be chest girth (0.77); and parallel findings were observed in studies performed by Yakubu [8] with Yankasa sheep, Norris et al.[10] with native goat breeds in South Africa, Çankaya [30] with German Fawm x Hair Crossbreed goats, Dekhili and

Aggoun [32] with Ouled-Djellal breed sheep and Yunusa et al.[33] with West African Dwarf sheep. Yakubu and Mohammed [6] reported in their study with Red Sokoto goats and Tyasi et al.[34] indicated in their study with South African Indigenous sheep that body length had the highest direct effect on live weight. Ogah et al.[35] reported in their study with West African Dwarf goats that rump width had the highest direct effect on live weight. It is thought that the differences among the studies are caused by genetic factors such as types and breeds of the livestock and environmental factors such as feeding conditions, climate and area of breeding. In consideration of these differences, it was found that the most important body measurement to be considered when estimating live weight is chest girth.

Whereas multiple linear regression model is rather based on the explanation of dependent variable by independent variables, it is possible with path models to examine causal relationships among independent variables. In this study, it was shown that back length, body length, shin girth and chest girth directly affected live weight and chest depth and rump width not only had direct effects on chest girth but also indirect effects on live weight through chest girth. Beside its advantages, since path analysis allows for establishing different path models with the same dataset, there may be uncertainties about which of the models is superior; therefore, consulting expert opinion in the identification of the optimum model is of importance for the consistency and reliability of estimations. Another issue to be considered in path analysis is that causation and cause-effect relationship are not mentioned and only the concept of effect is used in a path model which is established without a theoretical basis.

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Research Article

Comparison of Wintering Ability and Colony Performances of Different Honeybee (*Apis mellifera* L.) Genotypes in Eastern Anatolian/Turkey Conditions

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Abstract

In this study, wintering ability and some physiological properties of Buckfast, Carniolan, Caucasian and Erzurum honeybee genotypes were investigated in Eastern Anatolian conditions. During the wintering season of 2014, a total of 48 colonies, 12 from each of Buckfast, Carniolan, Caucasian and Erzurum honey bee genotypes, were taken from the equilibrated colonies of Langstroth type wooden hives for wintering. In the 2015 production period, a total of 43 colonies,11 from each of Buckfast, Carniolan, Erzurum genotypes and 10 from Caucasian genotype were used in the study. In the production season, the average number of combs covered with bees in genotype groups were found as 11.72±0.63, 12.17±0.62, 9.52±0.51 and 10.72±0.55 per colony, and the average brood areas were found as 2713.7±237.9, 2797.6±238.3, 2036.5±166.4, 2364.3±197.2 cm2/colony (19.04.2015-4.10.2015). The difference between the groups was found statistically significant (P<0.01) in terms of number of combs covered with bees and brood areas. Averarage honey yields were determined as 28.08±2.37, 29.94±2.17, 19.28±2.13 and 23.36±2.15 kg/colony, respectively. The difference between groups in honey yield was found statistically significant (P<0.05).

Keywords: *Honeybee, Apis mellifera L., Wintering ability, Honey yield, Genotype*

Doğu Anadolu-Türkiye Koşullarında Farklı Bal Arısı (*Apis mellifera* L.) Genotiplerinin Kışlama Yeteneği ve Koloni Performanslarının Karşılaştırılması

Özet

Bu çalışmada, Doğu Anadolu koşullarında Buckfast, Karniyol, Kafkas ve Erzurum genotiplerinin kışlama yeteneği ve bazı fizyolojik özellikleri araştırılmıştır. Araştırmada Langstroth tipi ahşap kovanlardaki güçleri eşitlenmiş kolonilerden 2014 yılı kışlatma döneminde Buckfast, Karniol, Kafkas ve Erzurum bal arısı genotiplerinin her birinden 12'şer olmak üzere toplam 48 adet koloni kışlatmaya alınmıştır. 2015 yılı üretim döneminde ise 11'er adet Buckfast, Karniol, Erzurum ve 10 adet de Kafkas genotipine mensup toplam 43 adet koloni kullanılmıştır. Üretim sezonunda genotip grupların ortalama arılı çerçeve sayıları sırasıyla 11.72±0.63, 12.17±0.62, 9.52±0.51 ve 10.72±0.55 adet/koloni; ortalama kuluçka alanları 2713.7±237.9, 2797.6±238.3, 2036.5±166.4, 2364.3±197.2 cm2/koloni olarak bulunmuştur (19.04.2015-4.10.2015). Arılı çerçeve sayısı ve kuluçka alanı bakımından gruplar arasındaki fark istatistiksel açıdan önemli (P<0.01) bulunmuştur. Grupların ortalama bal verimleri; 28.08±2.37, 29.94±2.17, 19.28±2.13 ve 23.36±2.15 kg/koloni olarak belirlenmiştir. Bal verimleri bakımından gruplar arasındaki fark istatistiksel açıdan önemli (P<0.05) bulunmuştur.

Anahtar sözcükler: *Bal arısı, Apis mellifera L., Kışlama yeteneği, Bal verimi, Genotip*

INTRODUCTION

Turkey has several climatic and topographic regions and consequently it has many honeybee races and ecotypes adapted to the different climates and regions [1,2]. Caucasian bee *(Apis mellifera caucasia)* live at the northeast region, Persian bee *(Apis mellifera meda)* and Syrian bee *(Apis mellifera syriaca)* at the southeast region, Carniolan *(Apis mellifera carnica)* at Thracian region, subspecies of Anatolian bee *(Apis mellifera anatolica)* at the remaining areas in our country [3,4]. Local bees in Erzurum province are smaller yellow coloured and more aggressive than Caucasian and

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Anatolian bees [5]. This local bee ecotype is not commonly used by the beekeepers; it is only traditionally reared by the some local beekeepers. The Caucasian bees are native to north-eastern Anatolia and are the most popular honeybee genotypes in Turkey. They were adapted to the temperate climate and high elevation regions especially the north-eastern part of the country [6]. Carniolan bee is native to Slovenia, southern Austria, and parts of Croatia, Bosnia and Herzegovina, Serbia, Hungary, Romania, and Bulgaria. Carniolan honey bee is spread worldwide, today. Due to its soft temper, adaptation to extreme low temperatures, good honey production, Carniolan honey bee is in many countries well accepted and popular honey bee variety [7]. Buckfast bees are actually a hybrid. They were developed in 20th century by Brother Adam. Buckfast bees are resistant to Tracheal mites and do well in cool climates. They are very gentle and easy to work with and are excellent honey producers. They have a low tendency to swarm and are economical in the use of winter stores [8,9]. In order to increase productivity in beekeeping, the comparative analysis of the physiological characteristics of genotypes in different regions should be made, and the appropriate genotype should be determined for each region. While searching for the suitability of a genotype for a region, survival and wintering ability are the main features of a genotype that should be laid emphasis on [5]. Because the vast majority of colony losses occur in the winter months [10]. It was reported in a study performed in the United States that wintering losses were above the ratio of 30% [11]. The losses ranging from 30% to 80% have been reported in various regions of our country [12-14].

The dead colony ratio was used as the indicator of survival ability in a study conducted with Caucasian, Anatolian, Mugla and Thrace bee groups in Thrace, and this rate was found as 35.71%, 38.46%, 28.57% and 36.36%, respectively for the groups [15]. The wintering abilities of Carniol, Mugla, Tokat, Italian, Caucasian-Camili and Caucasian-TKV genotypes were identified as 64.86%, 63.91%, 61.59%, 57.85%, 56.93% and 51.98% respectively [16].

The colony population size at the beginning of the production period should be higher in order to increase honey yield. It was reported in the studies conducted that the brood development of the colonies support the increase in the number of adult bees, and there is a positive relationship between adult bee development and brood production (r = +0.76) [16]. The correlation between the average brood production efficiency of the colonies and the honey yield was found as r = +0.817 in another study [15].

The mean honey yield per colony was reported as 30.62±3.22, 32.63±5.17 and 35.41±5.36 kg colony, respectively in the study conducted to determine the performance of Caucasian, Anatolian and Erzurum local ecotype in Erzurum conditions [5]. The average honey yields of Buckfast and European Black bee (local bee) colonies were reported as 38.49 and 26.76 kg/colony, respectively in a study conducted in Poland [9].

The average honey yields of Buckfast, Italian, Carniolan and Middle European (local bee) colonies were reported as 37.79, 38.1, 42 and 33.2 kg/colony, respectively in a study conducted for three years in Finland [17]. The mean honey yields of the Mugla, Nigde local ecotype, Caucasian and Carniolan genotypes in Nigde conditions were determined as 28.60, 15.40, 23.40 and 31.60 kg/colony respectively [18]. In a study conducted in Slovenia with carniolan bees, the average honey production was reported as 9.5 kg and the area of capped brood was 7061 cm^2 [19].

Due to the high yield of honey, Buckfast and Carniolan genotypes, widely used in the world have been used in our country, especially by migratory beekeepers. In recent years, these two bee genotypes have been shown great interest by beekeepers in Northeast Anatolia and Eastern Anatolia. In this study, it was aimed to identify the genotype suitable for the region by investigating the various physiological characteristics of Buckfast, Carniolan *(A. m. carnica)*, Caucasian *(A. m. caucasica)* and Erzurum domestic bees in Erzurum conditions.

MATERIAL and METHODS

The study was carried out at the apiary of Narman Vocational School (40°21'3.70" E longitude, 37°56'28" N latitude and 1650 m high) in Narman District of Erzurum Province in Eastern Anatolia Turkey. During the wintering season of 2014, a total of 48 colonies, 12 from each of Buckfast, Carniolan, Caucasian and Erzurum honey bee genotypes, were taken from the equilibrated colonies of Langstroth type wooden hives for wintering. In the 2015 production period, a total of 43 colonies, 11 from each of Buckfast, Carniolan, Erzurum genotypes and 10 from Caucasian genotype were used in the study.

The Buckfast and Carniolan queens were supplied from Germany, The Caucasian bees were supplied from Artvin-Camili, the Erzurum bees were supplied from local beekeepers in Erzurum. All of the colonies were investigated with regard to the properties such as colony development, brood area and honey yield for one season, and those, superior than others in the same conditions, were seperated to be used as a breeder.

The grafted larvae raised with Doolittle method were introduced into starter colonies. Larvae <24 h old were grafted onto royal jelly that was diluted with water in the proportion of 1:1. For each genotype, 4 starter colonies were used and 30 larvae were grafted into of them. During the experiment, queen bee rearing colonies were fed with sugar syrup [20,21]. The queen cells, which were accepted and inclosed by raising colonies, were harvested and then

transferred to mating hives 10 days later than larva transfer operation. The queens, transferred to the mating hives, were daily monitored as of 6th day, and then test groups were created with mating queens. The colonies' food consumptions in wintering were calculated by subtracting the weights of the colonies before wintering from those after wintering and their wintering abilities were calculated by using the following formula [22];

Wintering Ability = (The number of combs covered with bees managing to survive until spring/The number of combs covered with bees entering to wintering) x 100

The values, received from the combs covered with bees, existing in the test colonies equilibrated in terms of the presence of bees and broods, at intervals of 21 days during the period up to the honey harvest, were used as the measure of adult bee development [15,18]. The brood area was measured by the PUCHTA method (S = 3.14xA/2xa/2) in cm^2, taking closed brood areas over all combs with brood into account [18,23]. In order to determine the honey yield of the colonies, the amount of honey they made apart from their own needs were based on [5,15].

For the test groups' struggle to Varroa infestation in spring, 8 g crystal thymol was pulverized by means of a grinder and then mixed with 22 g powdered sugar and placed on the top of combs in each colony by the help of newsprint cut in 4x4 size [24,25]. For the the test groups' struggle to Varroa infestation in autumn, 44.8 g of oxalic acid was prepared by being supplemented with sugar-water solution in the ratio of 1:1 to 1000 ml in the late autumn. The prepared 3.2% oxalic acid solution was instilled with a large-scale syringe so that 5 ml solution would exist on each honeycomb with bees [25,26].

"SPSS 20.0 for Windows" package program was used in the calculations, and multiple comparison test was performed for the properties, considered to have significant effect. In order to determine the wintering ability of the groups; while (arcsine √y/100) arcsine transformation in the case of percentages was performed to the population decrease rates prior to the analysis of variance, directly variance analysis was applied to the values as to food consumption, number of comb with bees, brood area, honey yield [27,28].

RESULTS

Wintering Ability

During the wintering period, while the highest consumption of food occured with 8.40±0.70 kg at Erzurum genotype, the lowest consumption of food occured with 6.63±0.51 kg at Caucasian genotype. The difference between the genotypes is statistically insignificant in terms of food consumption. While the difference between Buckfast, Carniolan and Erzurum local ecotype was found as insignificant in terms of population decrease, the population decrease of colonies belonging to Caucasian genotype was found higher than other groups (P<0.05) *(Table 1)*. The numbers of combs with bees of genotype groups entering into wintering and getting out of wintering are given in *Table 1*.

Development of Adult Bee

In respect to adult bee development, colonies constantly increased throughout the season, reaching the highest population in August. Carniolan bee ranked first with respect to the speed of comb with bees, which was followed by Buckfast, Erzurum and Caucasian genotypes respectively *(Fig. 1)*. The differences between the groups in respect to the number of comb with bees were found statistically significant (P<0.01).

Development of Brood Area

The average brood areas for Buckfast, Carniolan, Caucasian and Erzurum local ecotype were determined as 2713.7± 237.9, 2797.6±238.3, 2036.5±166.4, 2364.3±197.2 cm^2/colony. The differences in brood production, observed between groups, were also found statistically significant (P<0.01). As it can be seen from *Fig. 2*, the brood output of the groups steadily increased and reached the highest level during the nectar flow.

Honey Yield

The average honey yields of Buckfast, Carniolan, Caucasian and Erzurum genotypes were determined as 28.08±2.37, 29.94±2.17, 19.28±2.13 and 23.36±2.15 kg/colony. The differences between groups in respect to honey yields were found statistically significant (P<0.05).

Table 1. *Mean and percantage values of wintering ability of genotype groups*

Groups	n	Food Consumption (kg/koloni) X±Sx	Before Wintering Number of Combs with Bees X±Sx	After Wintering Number of Combs with Bees X±Sx	Population Decline (%) X±Sx	Wintering Ability (%)
Buckfast	11	7.97±0.55^{is}	7.90±0.06^{a}	5.31±0.16^{a}	32.68±2.10^{b}	67.21^{a}
Carniolan	12	6.92±0.49^{is}	7.79±0.11^{a}	5.12±0.15^{a}	34.11±2.32^{b}	65.72^{a}
Caucasian	10	6.63±0.51^{is}	7.05±0.26^{b}	3.95±0.13^{b}	43.95±2.13^{a}	56.02^{b}
Erzurum	12	8.40±0.70^{is}	7.87±0.09^{a}	5.45±0.18^{a}	30.87±1.84^{b}	69.25^{a}
Total	45	7.51±0.30	7.67±0.08	5.00±0.12	35.08±1.26	64.55

^{a,b} Different letters indicate significant differences among the means (P<0.05), Duncan, is insignificant

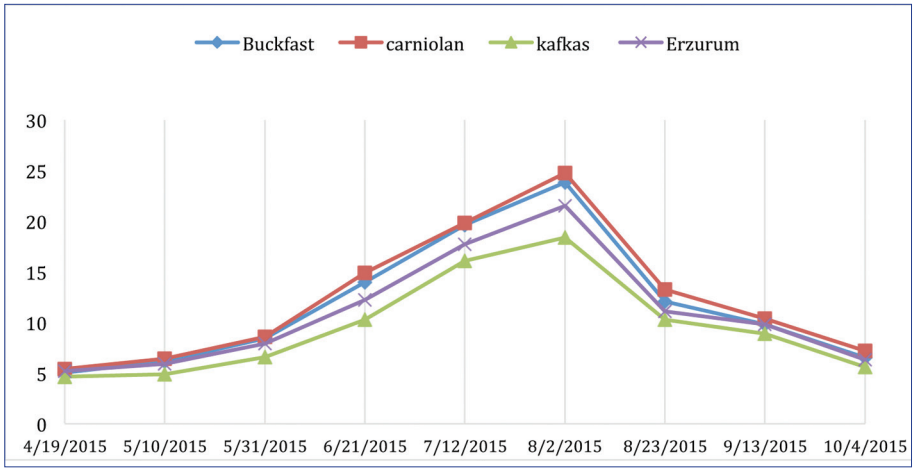
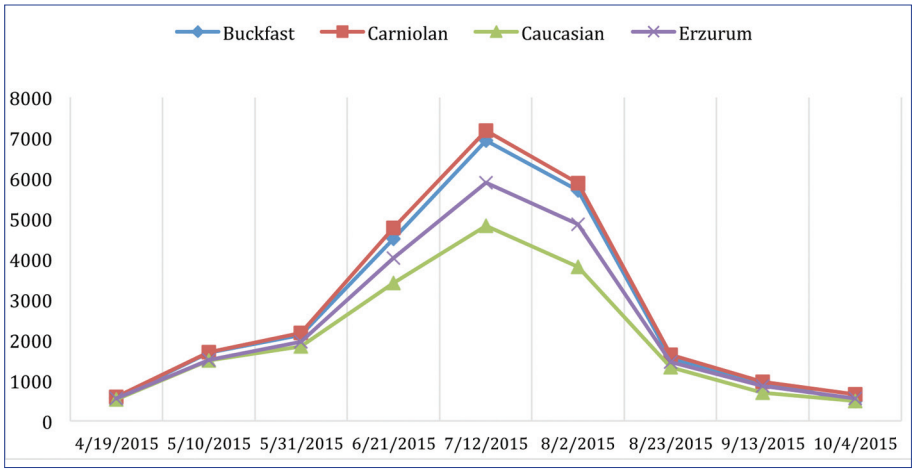


Fig 1. The average number of combs with bees of the genotypes

Fig 2. The average brood areas (cm²/colony) of the genotypes



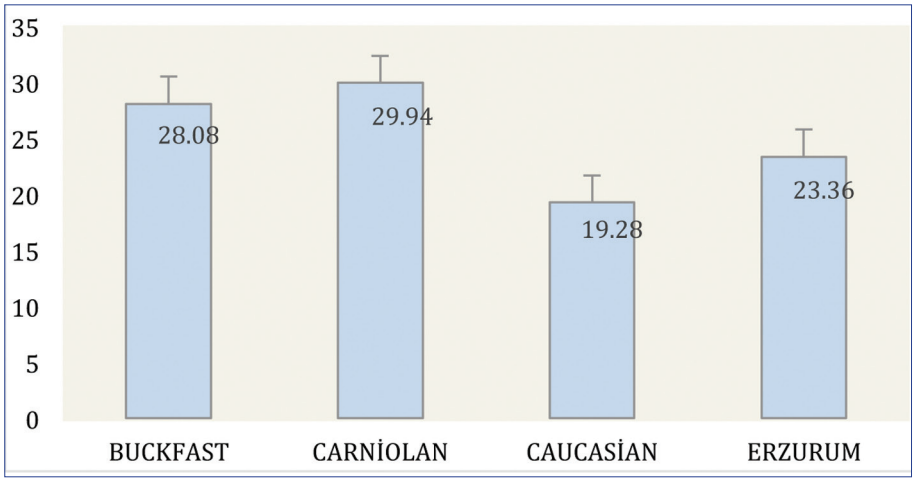


Fig 3. Average honey yield(kg/colony) of the genotypes

DISCUSSION

The results from *Table 1* show that wintering abilities of Buckfast and Carniolan bees are close to Erzurum bees, indigenous to the region. As a matter of fact, Buckfast and Carniolan bees consumed less food than Erzurum bees, but incurred more population losses than Erzurum bees. As well as the difference in population decrease between Buckfast, Carniolan and Erzurum bees was found statistically insignificant, the difference in population decrease between these three genotypes and Caucasian bee was found significant ($P<0.05$). When colony losses and population declines are taken into consideration, the lowest rate of wintering is obtained from Caucasian bees, consistent with the literature report [5]. The results of the wintering ability of the Buckfast, Carniolan and Erzurum groups are compatible with literature reports stating that these bees show high wintering ability [5,29,30]. Since an

increase in population losses of genotype groups means a decrease in existing food consumption, as a matter of course, less food was consumed in colonies where more bee losses occured. Having high wintering ability for a genotype means that colonies belonging to that genotype will manage to survive until spring with minimum bee loss and minimum food consumption. But, due to the fact that climate conditions differ every year, there is a need for more comprehensive studies to be made on this issue.

Buckfast and Carniolan adult bee developments are not different from each other, but the Erzurum group development is higher than Caucasus bee. It can be stated that in the research area, Erzurum group formed a larger population than Caucasian bees, and that Buckfast and Carniolan bees could gain more population development than region bees.

The average number of combs of Buckfast, Carniolan, Erzurum and Caucasian colonies in present study were found higher than result of Gençer [31] (informed as 7.64, 6.99, 7.90, 8.76, 8.23 number/colony). The results obtained from this study were found lower than result of by Genç et al.[5] (informed as 15.62, 17.08 and 18.49 number/colony), agree with the result of Akyol et al.[18] (informed as 11.24, 9.51, 8.11 and 12.38 number/colony).

The study's findings showed that Carniolan bees are the genotype, which produce the highest number of brood, which is followed by Buckfast bees, and Erzurum genotype produces more broods than Caucasian group. The Caucasian genotype ranked last in terms of this property evaluated.

The average brood areas for Buckfast, Carniolan, and Erzurum local genotypes and the Caucasian colonies in present study were found agree with the result of Akyol et al.[18] (informed as2825.0, 2160.6, 1701.9 ve 2883.0 cm^2/colony). The results obtained from this study were found lower than result of by Honko and Jasinski [17] (informed as 4002, 4091, 2750, 4035 and 3638 cm^2/colony), higher than result of Güler and Kaftanoğlu [32] (informed as 1112.6, 1184.8, 2387.5cm^2, 2030.2±188,6 cm^2, 1433.9 and 1501.5 cm^2/colony).

It was determined in a study that the brood and adult bee production of colonies support honey yield [33]; in another study, a positive and very significant (P<0.01) association r= +0.82 was found between colony population and honey yield [15].

Carniolan genotype produces more honey by 8.6%,36.27%, 55.29% than Buckfast, Erzurum local ecotype, Caucasian genotypes respectively under the same environmental and management conditions.

The average honey yield value, obtained for Buckfast bee, was found higher than the value reported by Olszewski [30], but lower than the value reported by Honko and Jasinski [17]. Carniolan group related average honey yield value was found consistent with the value reported by Akyol et al.[18] was found higher than the values reported by Arslan [16] and Gregorc and Locar [19]; was found lower than the value reported by Honko and Jasinski [17]. The average honey yield of the Caucasian group is lower than the values reported by Güler and Kaftanoğlu [32], higher than the values reported by Dodologlu and Genç [27], and consistent with the values reported by Gençer ve Karacaoğlu [34]. The average honey yield of Erzurum group was found lower than the value reported by Genç et al.[5], and consistent with the value reported by Cengiz [23].

The following results were obtained with the comparison of four honey bee genotypes in terms of food consumption, wintering ability and performance in Eastern Anatolian Turkey conditions.

1. Food consumption is not dependent on genotype in wintering, however, Caucasian and Carniolan group consumed less honey than Buckfast and Erzurum group.

2. While the differences between Buckfast, Carniolan and Erzurum genotypes were found insignificant in terms of population decrease, the population decrease at Caucasian genotype-related colonies was determined higher than other groups.

3. The highest wintering ability was found in Erzurum (69.25%) group, which was followed by Buckfast (67.21%), Carniolan (65.72%) and Caucasian (56.02%) groups respectively.

4. Development rate of the Caucasian bee is lower than other genotypes, and Buckfast and Carniolan genotypes have similar characteristics in this regard.

5. Carniolan and Buckfast genotypes, which produce the highest number of brood, have similar characteristics in respect to brood production, Erzurum genotype produces more broods than Caucasian group.

6. The differences between groups in terms of honey yield were found statistically important (P<0.01). The highest amount of honey was obtained from the Carniolan group while the lowest honey yield was obtained from the Caucasian group.

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Research Article

Feasibility Study of Inertial Sensor Technology on Ponies for Equine-Assisted Therapy (EAT)

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Abstract

Ponies used in equine-assisted therapy (EAT) (hippotherapy) often carry imbalanced riders, which is a cause for concern as regards the health of the ponies. A low degree of lameness or an abnormal gait is not always detectable by a veterinarian, subjectively, but this is enabled by using a motion analysis equipment. The aim of this study was to evaluate the feasibility of inertial sensor technology utilization to analyze ponies' kinematic motion at walking gait. Ten ponies were instrumented with the inertial sensors and made to walk 20 m in two trials (departure and return) for the forelimb data set (n=10), which was then repeated in the second round for the hindlimb (n=3). The ponies were assigned three interventions: walking with no rider, walking with a rider with typical development (normal rider), and walking with a rider with physical disability (disabled rider). The movement speed, stride length, and stride duration were measured by a video camera. The limb range of motion and the angular velocity were detected by inertial sensors. The results showed that there were no significant differences in the kinematic motion of the forelimb at walking gait for all interventions and no significant differences between the left and the right forelimbs except in the case of the anterior phase of the angular velocity of the arm when walking with a disabled rider ($P<0.05$). The hindlimb data set was not statistically compared due to insufficient "n" number. In conclusion, the inertial sensor technology is feasible to use on pony kinematic motion, especially when the sensor is attached to the forelimb. It seems that the ponies could modify the natural kinematic motion when walking with a load on them.

Keywords: *Pony, Walk, Kinematic, Equine-assisted therapy, Hippotherapy, Inertial sensor*

At-Destekli Terapi İçin Ponilerde Atalet Belirleme Teknolojisinin Fizibilite Çalışması

Özet

At-destekli (equine-destekli) terapide (hipoterapi) kullanılan ponilerin sıklıkla denge sorunlu binicileri taşımaları bu ponilerde sağlık sorununa neden olabilmektedir. Düşük dereceli topallık veya anormal yürüyüş daima veteriner hekim tarafından belirlenemeyebilir ve hareket analiz ekipmanı gerektirebilir. Bu çalışmanın amacı atalet belirleme teknolojisinin fizibilitesini ponilerin kinematik hareketlerinde test etmektir. On adet poni atalet sensorları ile donatıldı ve iki hat boyunca (kalkış ve dönüş) 20 m yürütüldü. Ön ayak veri seti (n=10) sonradan ikinci turda arka ayak (n=3) için tekrar edildi. Poniler 3 uygulamaya maruz bırakıldı: binicisiz yürüme, tipik gelişimli binicili yürüme (normal binici) ve fiziksel engelli binicili yürüme (engelli binici). Hareket hızı, adım uzunluğu ve adım süresi video kamera ile ölçüldü. Hareketin adım sırası ve açısal hızı atalet sensörleri ile belirlendi. Tüm denemelerde ön ayakların kinematik hareketlerinde yürüyüş bakımından gruplar arasında bir fark belirlenmedi. Engelli ile birlikte yürümede ön ayaklarda açısal hızın anterior fazı dışında ($P<0.05$) sol ve sağ ön ayaklar arasında da uygulamalar arasında bir fark bulunmadı. Arka ayak veri seti yetersiz n sayısı nedeniyle istatistiksel olarak karşılaştırılmadı. Sonuç olarak, atalet sensör teknolojisi ponilerin kinematik hareketlerini belirlemede özellikle de sensör ön ayaklarda takılı ise kullanılabilir. Poniler doğal kinematik hareketlerini yüklerine bağlı olarak adapte edebilmektedirler.

Anahtar sözcükler: *Poni, Yürüme, Kinematik, At-destekli terapi, Hipoterapi, Atalet sensörü*

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INTRODUCTION

Equine-assisted therapy (hippotherapy) is a physical, occupational, speech, and psychological treatment strategy through horseback riding that stimulates patients to have better emotional response, and to improve the balancing and weight transfer between themselves and horses. This activity is advantageous for children with musculoskeletal abnormalities and movement control failure [1]. Ponies are usually used in this activity as their size is suitable for young patient. For the most effective result, a pony with good physical and mental status is essential as is awareness regarding their welfare.

Good balance and body communication between a horse and its rider improves the horse's performance and welfare, including decreasing of stress, frustration, risks of injuries, and accidents. Thus, an experienced rider positively affects the cooperation of the horse with regard to the temperament, experience, and physical abilities of the horse [2]. On the other hand, riders with poor balance and body control often cause discomfort and stress to the ridden horses, leading to poor performance by the animal [3]. Therefore, it is possible that the horses or ponies used for equine-assisted therapy may (or may not) be at risk of physical discomfort, and this may be a cause for concern as regards the welfare of the animal. However, to the authors' knowledge, this matter has never been investigated and resolved.

Subjective lameness evaluation by a trained equine veterinarian is a routine diagnostic technique for lameness; however, it is not always reliable due to bias and it is less sensitive than motion sensors, especially for detection of mild lameness [4]. Kinematic analysis has eventually been developed to measure gait and motion in human patients with musculoskeletal and neuromuscular problems for the better treatment decision making and result evaluation with qualitative and quantitative assessment. This method has been incessantly developed and also applied in animals such as dogs and horses. Inertial sensor technology has been recently developed for human and horse kinematic analysis using WIFI signals which would replace wire connection or cameras. It enables objective evaluation by data collection and analysis as the horse walks on the ground [4]. Therefore, this technique is potentially beneficial for studying pony movement when it is being mounted by various types of riders including riders with typical development (normal rider) and those with physical disabilities (disabled rider). Therefore, the aim of the present study was to assess the feasibility of using inertial sensor technology to analyze ponies' kinematic motion at walking gait without riders, with normal riders, and with disabled riders.

MATERIAL and METHODS

The study received ethical approval from the ethics committee of the Faculty of Veterinary Medicine and the Faculty of Associated Medical Sciences, Chiang Mai University.

Animals

Ten (7 geldings and 3 mares) ponies (one from the Faculty of Veterinary Medicine, Chiang Mai University, 2 ponies from Laddaland Equestrian Club, and 7 ponies from the Pack Squadron, Chiang Mai) that had had previous equine-assisted therapy (EAT) experience were included in this study. All the ponies were healthy and had no history of illness in the previous 6 months, and were not lame, based on the results of subjective lameness evaluation before the study. The mean ± standard deviation (SD) age of these ponies was 13±3.43 years; the bodyweight (BW) was 295±30.66 kg; and the height was 136.2±8.95 cm. The ponies were led by their familiar handlers. Their temperament and performance were also observed during the course to be aware of any possible danger to both riders and ponies. Ponies with poor temperament status and unpleasant behaviors, including kicking, bucking, biting, reluctance to walk, and other aggressions, on the study day were temporarily excluded. Ponies that were temporarily excluded 3 times were permanently excluded from the study as were ponies that had any signs of illness or lameness during the course.

Riders

Two brothers of ages 9 and 14, with weight 27.5±0.71 kg and height 137.5±10.61 cm, were the riders. The 9-year-old rider was a child with typical development (normal rider) and the 14-year-old rider was one who had a physical disability from cerebral palsy with spastic diplegia (disabled rider) who suffered from balancing and body movement problems. Both riders wore equestrian helmets and safety vests every time they were on horseback.

Preparation of Horses

The ponies were tacked with their own set of riding equipment which consisted of a halter for the horse, both-side-restrained leashes, and a saddle. The adhesive tape was applied around the legs at positions that enabled the equipping of the inertial sensors. A pony equipped for forelimb analysis is shown in *Fig. 1*. The horse was leashed on both sides in this study because of the result obtained in the pilot study which revealed that handler being on one side had an effect on the kinematic results generated by the inertial sensor. The equipped ponies were warmed up by leading them to walk for 5 min and rest for 5 min before the study.

Each pony was instrumented with five inertial sensors (size 36 mm × 15 mm × 46.5 mm and weight 30 g; *Fig. 2)* which had a three-axial accelerometer, a gyroscope, and a magnetometer. For forelimb kinematic analysis, the sensors were placed, as shown in *Fig. 3*, at the following positions: (1) withers; (2-3) cranial aspect of mid-radius

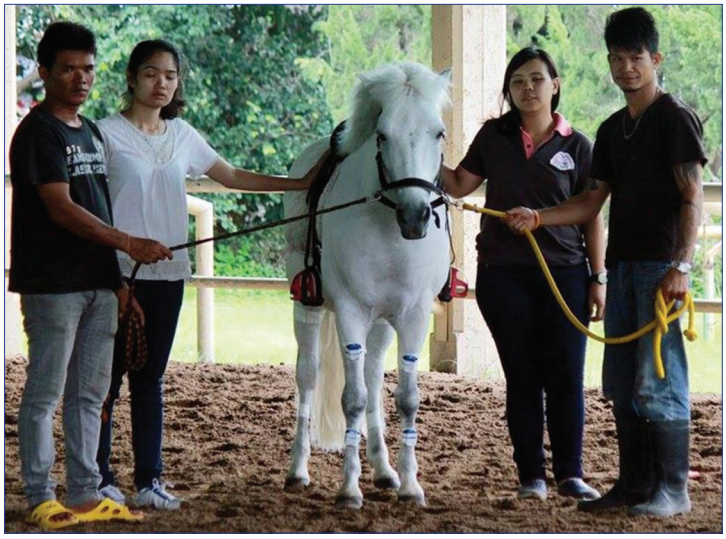

Fig 1. Equipped pony with a halter, both-side leashes, and a saddle. The five inertial sensors (STT-IBS, STT Systems, Spain) were attached at the forelimbs and the withers to prepare the pony for forelimb kinematic data collection

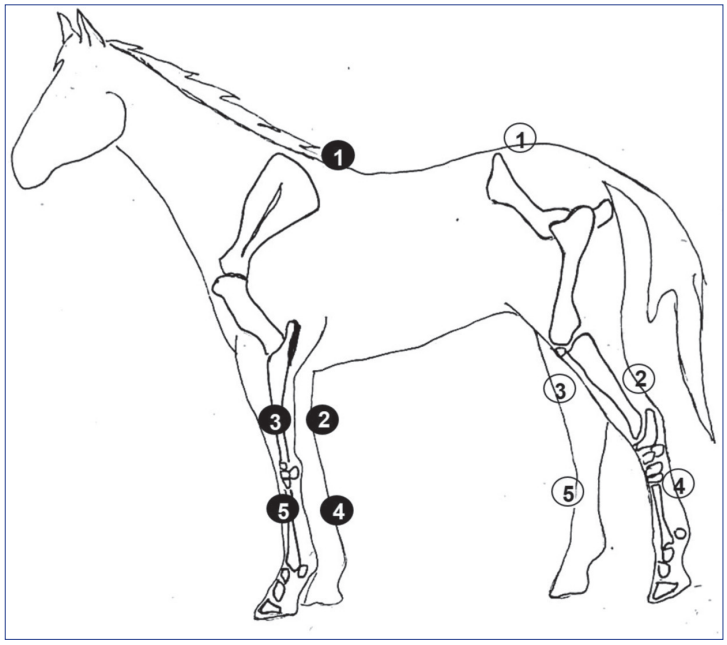

Fig 3. Schematic representation of the position of the inertial sensor in the forelimb and the hindlimb. Forelimb: withers (1), cranial aspect of mid-radius at both legs (2-3), and dorsal aspect of mid-metacarpus at both legs (4-5). Hindlimb: tuber sacrale (1), cranial aspect of mid-tibia at both legs (2-3), and dorsal aspect of mid-metatarsus at both legs (4-5)

at both legs; and (4-5) both the dorsal aspects of mid-metacarpus at both legs. For the hindlimb analysis, the sensors were placed at the following positions: (1) tuber sacrale; (2-3) cranial aspect of mid-tibia at both legs; and (4-5) both the dorsal aspects of mid-metatarsus at both legs. All the sensors were manipulated by the same person to ensure consistency throughout the study. The inertial sensors were placed initially for the forelimb analysis and then changed to the hindlimb afterward.

Fig 2. Examples of inertial sensors (STT-IBS, STT Systems, Spain)

Inertial Sensor Configuration

To set up the inertial sensors, a portable personal computer (laptop) with the suitable inertial software (STT Systems, Spain) was connected to the sensors via Bluetooth technology. The position of each sensor fixed on the pony's body was indicated and paired for their reference by the software. During walking, the movement of the sensors to their referred sensor was recorded and automatically quantified. A validity test of the sensors was performed to verify the accuracy of the data acquired *(Fig. 4)*. Upon using two inertial sensors placed at mid-radius and mid metacarpus with a 90-degree-flexed carpus, it was observed that the equipment measured the carpal angle at 90±2 degrees.

Walking Course Pattern

Five cone markers were placed every 5 m along the 20-meter-straight track *(Fig. 5)*. A walking course for the ponies for each intervention consisted of two round trips of this track. The first round consisted of two trials (departure and return) for the forelimb data set, and these were repeated in the second round for the hindlimb. The ponies were led straight from the first cone marker, made to pass the 20-meter-point, and then made to take a U-turn and walk straight back to the initial point *(Fig. 6)*. The data retrieved during the U-turn point were excluded.

Video Camera Setup

A digital video camera (EOS 70D Digital SLR Camera CANON®, 25 Hz frame rate, shutter speed 1:4000) was used for measuring the movement speed and the stride length. A camera monitor was set up to cover 10 m of the third and the fifth cone markers, with the height of the lens at 1.2 m and without magnification.

The movement speed was defined as the distance of the pony movement in one second. A stride consisted of a cycle of one hoof (this study observed the right forehoof

Fig 4. Validation test of the inertial sensor (Note that a book was applied as the reference for 90-degree flexion)

and the left forehoof during departure and return, respectively) movement completed when it regained the initial position. The stride length was the average distance of each pony stride calculated by the distance of the walking track (10 m) divided by the number of strides within the 10-m track. The stride duration was defined as the duration since one hoof lifted up from the ground till it regained its position on the ground.

Interventions

Each pony was assigned three interventions in random order and allowed at least a 5-min rest before starting the next intervention. The three interventions comprised walking with no rider, walking with a normal rider, and walking with a disabled rider.

Data Collection

Each data set was collected in duplicate. The data obtained by body-mounting the sensors were transmitted using wireless technology at 125-250 Hz in real time to a portable computer.

The parameters of each intervention from the sensors were the range of motion and the angular velocities (anterior and posterior) obtained from at least six complete strides.

The ranges of motion *(Fig. 7)* consisted of the following: (1) the arm swing range: the summation of the forward-moving and backward-moving angles from its referral line, which is the imaginary line drawn from the tip of the dorsal spinous process to the middle of the antebrachium when the pony is standing square; (2) the knee range: the summation of the forward-moving and backward-moving angles from its referral line, which is the imaginary line drawn from the middle of the antebrachium to the middle of metacarpus when the pony is standing square; (3) the thigh swing range: the summation of the forward-moving and backward moving angles from its referral line, which is the imaginary line drawn from the tuber sacrale to the middle of tibia when the pony is standing square; and (4) the hock range: the summation of the forward-moving and backward moving angles from its referral line, which is the imaginary line drawn from the middle of tibia to the middle of metatarsus.

Angular velocity is defined as the change in angular displacement per second. The angular velocity was also measured in the anterior and the posterior phases. In the anterior phase, the angular velocity was considered as the velocity of the angular changes when the limb moved forward from the vertical lines, and in the posterior phase, the angular velocity was considered as the velocity of the angular changes when the limb moved backward from the vertical lines.

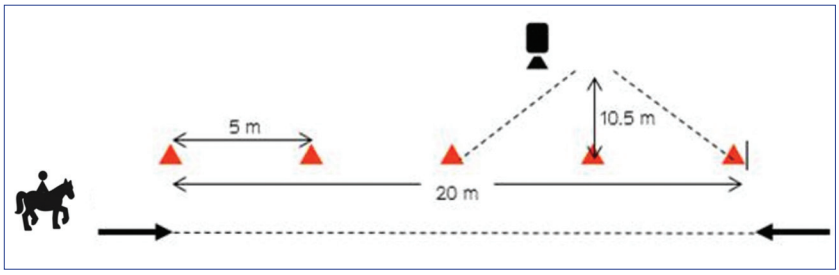

Fig 5. Walking track designed for the experiment

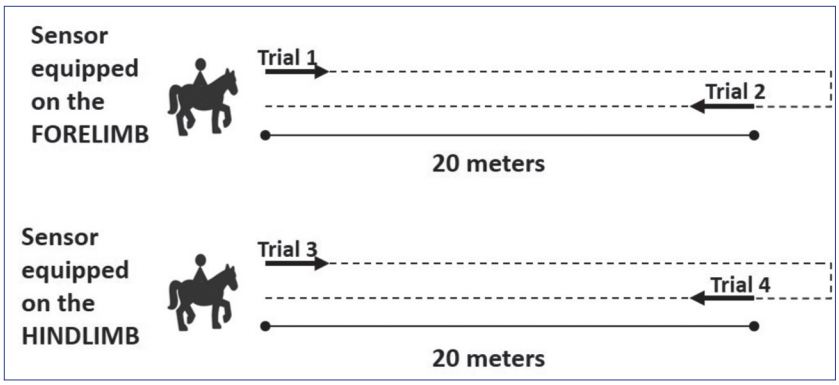

Fig 6. Horses' walking pattern for each intervention

Statistical Analysis

The speed, stride length, stride duration, and forelimb ranges of motion, as well as the angular velocities were

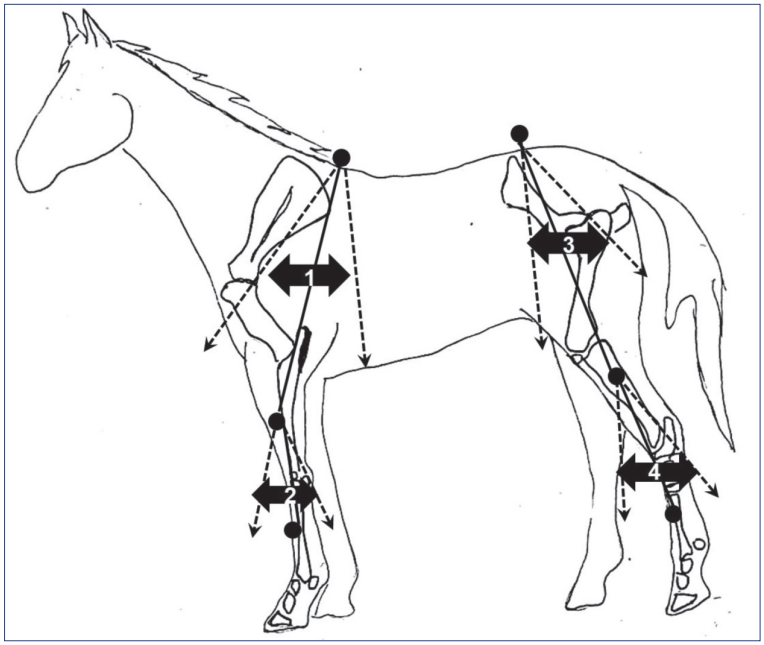

Fig 7. Schematic presentation of the range of motion of (1) arm swing range, (2) knee range, (3) thigh swing range, and (4) hock range. The front dashed line indicates the anterior phase and the back one denotes the posterior phase of each location

compared between the three interventions using repeated-measures analysis of variance (ANOVA) with the Tukey-Kramer's multiple comparison test. The ranges of motion and the angular velocities between the left and the right legs were compared using paired Student *t*-test. A P-value <0.05 was considered to be of statistical significance. The statistics were analyzed using the RStudio Version 0.98.501 software (RStudio, Boston).

RESULTS

The forelimb data were collected from all of the 10 ponies in this experiment. However, the hindlimb data could be obtained only from three ponies because seven ponies were very uncomfortable with attaching the devices to their hind legs and so, it was considered too dangerous for the riders. Therefore, the hindlimb ranges of motion and angular velocities were only descriptively analyzed. They could not be statistically compared between interventions and sides due to insufficient "n" number.

Table 1. *Mean ± Standard Deviation for Movement Speed, Stride Length, and Stride Duration Compared between Walking without Rider (no rider), with a Rider with Typical Development (normal rider), and with a Rider with CP Spastic Diplegia (disabled rider)*

Parameter	Intervention of Ponies		
	No Rider	Normal Rider	Disabled Rider
Speed (m/s)	1.20±0.15	1.17±0.15	1.12±0.15
Stride length (cm)	1.44±0.17	1.44±0.17	1.41±0.16
Stride duration (s)	1.21±0.13	1.25±0.12	1.24±0.12

n=10; P>0.05 for each parameter between interventions

Overall, the movement speed of each intervention, as well as the stride length and duration are shown in *Table 1*. There was no significant difference in the movement speed, stride length, and stride duration between interventions.

The results (mean ± standard deviation of the ranges of motion and angular velocities of the forelimbs are shown in *Fig. 8-13*. The summary of the kinematic motion values (mean ± standard deviation and range) in ponies walking with no rider is shown in *Table 2*.

Ponies walking with both types of riders had no statistically significant differences in the ranges and angular velocities

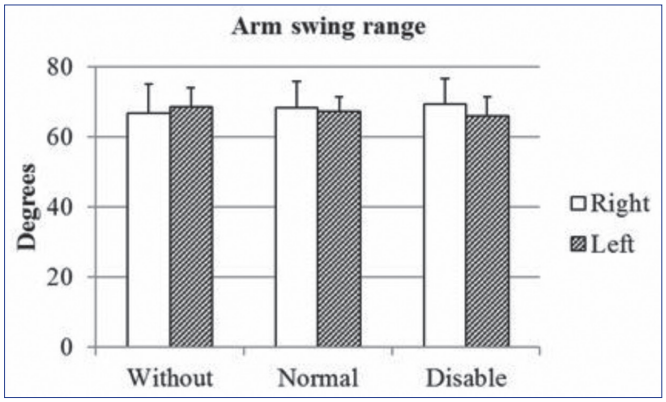

Fig 8. Means of the arm swing range compared between ponies walking with no rider (without), walking with a rider with typical development (normal rider), and walking with a rider with CP spastic diplegia (disabled rider) (n=10). The white bars represent the results from the right limb and the patterned bars represent the results from the left limb. Standard deviations are presented as T bars on the bar graphs. Significance is denoted as *P<0.05

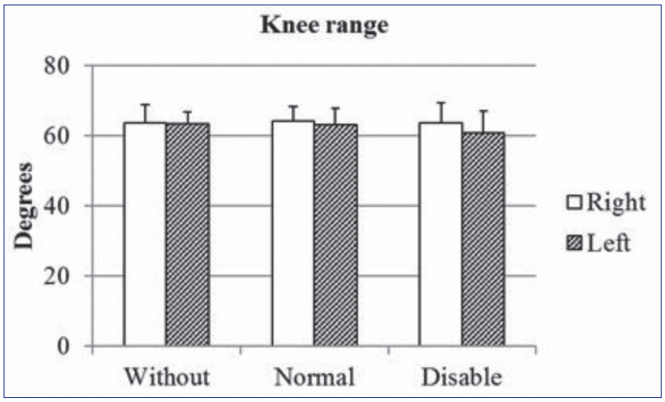

Fig 9. Means of the knee swing range compared between ponies walking with no rider (without), walking with a rider with typical development (normal rider), and walking with a rider with CP spastic diplegia (disabled rider) (n=10). The white bars represent the results from the right limb and the patterned bars represent the results from the left limb. Standard deviations are presented as T bars on the bar graphs. Significance is denoted as *P<0.05

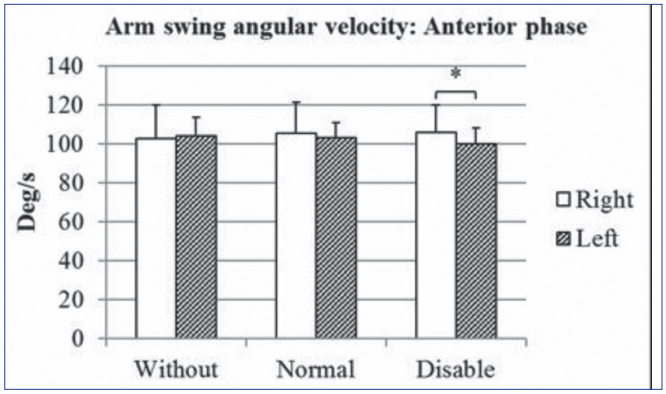

Fig 10. Means of the arm swing angular velocity (anterior phase) compared between ponies walking with no rider (without), walking with a rider with typical development (normal rider), and walking with a rider with CP spastic diplegia (disabled rider) (n=10). The white bars represent the results from the right limb and the patterned bars represent the results from the left limb. Standard deviations are presented as T bars on the bar graphs. Significance is denoted as *P<0.05

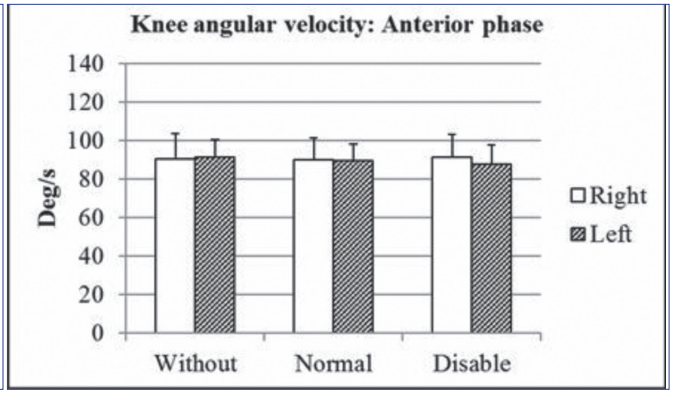

Fig 11. Means of the knee angular velocity (anterior phase) compared between ponies walking with no rider (without), walking with a rider with typical development (normal rider), and walking with a rider with CP spastic diplegia (disabled rider) (n=10). The white bars represent the results from the right limb and the patterned bars represent the results from the left limb. Standard deviations are presented as T bars on the bar graphs. Significance is denoted as *P<0.05

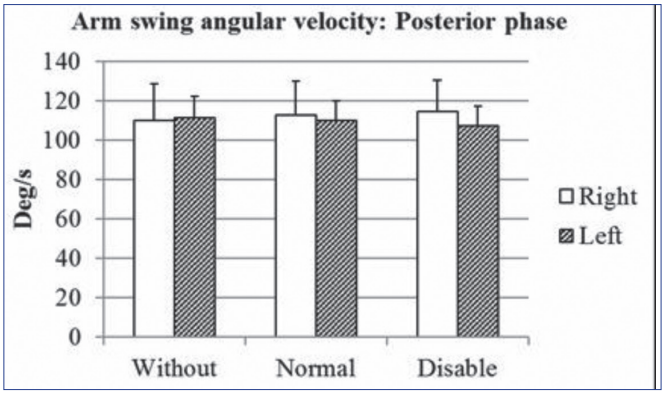

Fig 12. Means of the arm swing angular velocity (posterior phase) compared between ponies walking with no rider (without), walking with a rider with typical development (normal rider), and walking with a rider with CP spastic diplegia (disabled rider) (n=10). The white bars represent the results from the right limb and the patterned bars represent the results from the left limb. Standard deviations are presented as T bars on the bar graphs. Significance is denoted as *P<0.05

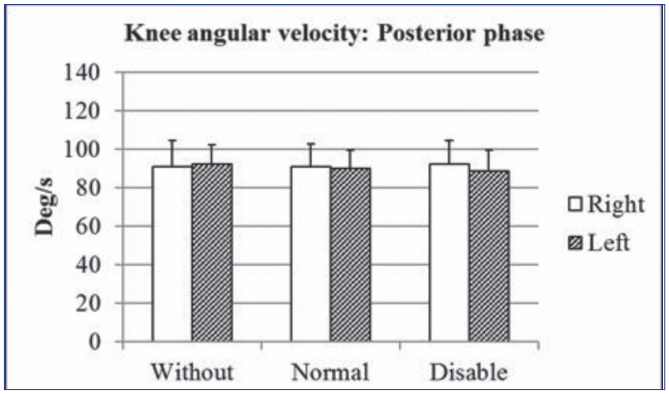

Fig 13. Means of the knee angular velocity (posterior phase) compared between ponies walking with no rider (without), walking with a rider with typical development (normal rider), and walking with a rider with CP spastic diplegia (disabled rider) (n=10). The white bars represent the results from the right limb and the patterned bars represent the results from the left limb. Standard deviations are presented as T bars on the bar graphs. Significance is denoted as *P<0.05

of the forelimbs compared to no-rider ponies. Similar results were obtained when a comparison was made between the left and the right forelimbs except in the case of the anterior phase of the angular velocity of the arm when walking with the disabled rider, in which the left side had a lower angular velocity than the right *(Fig. 10)*.

The results, including the mean and the standard deviation of the hindlimb ranges of motion and angular velocities, are shown in *Table 3* and *Table 4* for thigh and hock, respectively. It can be observed that the left thigh had a greater swing range than the right *(Table 3)*. On the other hand, the left hock range and the angular velocity were lower than the right hock range and the angular velocity *(Table 4)*.

DISCUSSION

This is the first study in which the kinematic motion of ponies for equine-assisted therapy (EAT) was evaluated with regard to the walking gait by applying the inertial sensor technology. The inertial sensor technique has been in use for equine motion analysis, especially in the analysis of the trotting gait with the aims of improving sports performance and fine detection of lameness [4] as well as studying the differences in motion between types of horses [5]. Inertial sensor technology is highly sensitive and accurate with quantitative data, and is suitable for detection of even the slightest asymmetry in movement [6].

The major components of gait speed are stride frequency and stride length. Stride length has a positive relation with the speed of gait [7]. In the present study, the ponies in all the interventions were allowed to walk in their comfortable movement speed. According to the results, loading the ponies with riders did not influence the movement speed, stride duration, or stride length. This is in accordance with the findings of a previous study, a study by Sloet et al.[8] who found that the stride duration in

nine Dutch Warmblood horses walking on a treadmill did not differ significantly between being mounted, or loaded, and unloaded. Gottlieb et al.[9] also found no differences in the stride length between horses whether or not they were pulling a load. This could imply that the horses are capable of maintaining their natural speed and stride during work.

Range of motion and angular velocity are important factors of normal limb movement. Horses or ponies that have a normal range of motion and angular velocity in all articular joints would have comfortable motion. This study, unfortunately, could not measure these parameters in every joint due to the limitation with regard to number of sensors. However, the experiment was designed for monitoring at positions that could be most representative of the limb movement. There were no statistically significant differences found in the ranges of motion and angular velocities of arm swing and knee between the three interventions in this present study. This might be taken to imply that loading and type of load do not effect forelimb movement. These results are in accordance with the findings of Miró et al.[10] who reported no significant differences between the ranges of motion of shoulder, elbow, and carpal joints when the horse was handled while walking or was cart-driven in the walking gait. However, based on the findings of this study, it is too early to state that the ponies are capable of maintaining their forelimb kinematic motion during EAT working because not every joint was monitored. It has also been reported by a previous study that there was more fetlock extension while the horse was being mounted to walk [8]. Moreover, significant differences in the anterior phase of arm swing angular velocities were detected in this study between the left and the right limbs when the ponies were ridden by a disabled rider.

Healthy horses usually have a symmetrical kinematic motion. Low angular velocity at the anterior phase of the left arm when walking with a disabled rider might indicate motion compensation to the imbalanced rider. Disabled riders cannot balance their weight transfer and usually shift their weight to the left side, so the ponies try to maintain the range of motion by increasing the angular velocity in the anterior phase of the opposite leg.

Asymmetrical range of motion between the left and the right hindlimbs was observed. Although the data came from only three ponies and could not be confirmed statistically, the explanation of this might be as follows: improper sensor position, the animal's preference of side, or subclinical lameness. In the author's opinion, as the sensors were placed in the same manner as in the case of

Table 2. *Summary of Kinematic Motion Values (mean ± standard deviation and range) in Ponies Walking with No Rider Using Inertial Sensor Technology*

Parameter		Limb	
		Right	Left
Forelimb (n=10)			
Arm swing range (degree)		66.85±8.35 (47.66-74.38)	68.49±5.67 (60.01-79.93)
Arm swing angular velocity (degree/s)	Anterior phase	102.94±16.99 (66.52-124.46)	104.40±9.40 (86.98-117.64)
	Posterior phase	110.30±18.47 (70.26-131.26)	111.67±10.91 (90.02-123.20)
Knee range (degree)		63.61±5.28 (56.60-71.45)	63.35±3.52 (54.98-68.17)
Knee angular velocity (degree/s)	Anterior phase	90.49±13.36 (71.17-106.22)	91.33±9.09 (76.68-101.60)
	Posterior phase	91.19±13.58 (70.16-107.34)	92.35±10.27 (76.88-103.90)
Hindlimb (n=3)			
Thigh swing range (degree)		36.40±2.63 (34.15-39.29)	47.01±5.48 (40.90-51.48)
Thigh swing angular velocity (degree/s)	Anterior phase	47.70±5.54 (43.14-53.87)	71.82±7.38 (65.29-79.84)
	Posterior phase	53.54±6.57 (48.49-60.96)	76.81±8.77 (68.55-86.02)
Hock range (degree)		43.38±3.81 (40.07-47.55)	36.77±4.97 (31.49-41.36)
Hock angular velocity (degree/s)	Anterior phase	57.49±4.14 (55.06-62.28)	39.41±9.32 (28.67-45.36)
	Posterior phase	50.21±3.13 (Range: 46.66-52.56)	35.31±8.00 (Range: 26.49-42.08)

Table 3. *Ranges of Motion and Angular Velocities of Thigh of Hindlimb*

Pony No.	Thigh Swing Range						Thigh Swing Angular Velocity											
							Posterior Phase						Anterior Phase					
	Right			Left			Right			Left			Right			Left		
	W	N	D	W	N	D	W	N	D	W	N	D	W	N	D	W	N	D
8	39.3	40.1	38.8	51.5	46.0	43.6	51.2	54.8	47.8	68.6	65.0	59.6	46.1	49.9	42.6	65.3	61.1	55.9
9	34.2	33.3	34.7	40.9	42.2	40.8	48.5	53.2	60.5	75.9	73.6	72.6	43.1	47.1	53.4	70.3	68.4	66.6
10	35.8	36.0	35.2	48.7	48.5	48.9	61.0	63.3	59.9	86.0	88.2	89.3	53.9	56.9	52.8	79.8	81.7	82.3
Mean	36.4	36.5	36.2	47.0	45.6	44.5	53.5	57.1	56.1	76.8	75.6	73.8	47.7	51.3	49.6	71.8	70.4	68.3
SD	2.6	3.4	2.2	5.5	3.1	4.1	6.6	5.5	7.1	8.8	11.7	14.9	5.5	5.1	6.1	7.4	10.4	13.3

n = 3; ***W*** *= walking with no rider,* ***N*** *= walking with a rider with typical development (normal rider), and* ***D*** *= walking with a rider with CP spastic diplegia (disabled rider)*

Table 4. *Ranges of motion and angular velocities of hock*

Pony No.	Hock Range						Hock Angular Velocity											
							Posterior Phase						Anterior Phase					
	Right			Left			Right			Left			Right			Left		
	W	N	D	W	N	D	W	N	D	W	N	D	W	N	D	W	N	D
8	47.6	49.5	48.5	41.4	45.1	45.3	51.4	53.6	44.7	42.1	49.7	46.8	55.1	55.2	49.2	45.4	53.2	51.8
9	42.5	45.4	42.9	31.5	35.4	37.5	52.6	53.7	49.9	26.5	30.6	35.9	62.3	63.5	57.6	28.7	38.8	42.4
10	40.1	40.6	41.0	37.5	36.0	36.0	46.7	48.0	40.3	37.4	35.4	20.8	55.1	55.2	42.8	44.2	37.9	19.3
Mean	43.4	45.2	44.1	36.8	38.8	39.6	50.2	51.7	45.0	35.3	38.6	34.5	57.5	58.0	49.9	39.4	43.3	37.9
SD	3.8	4.5	3.89	5.0	5.4	5.0	3.1	3.2	4.8	8.0	10.0	13.1	4.1	4.8	7.5	9.3	8.6	16.7

n = 3; ***W*** *= walking with no rider,* ***N*** *= walking with a rider with typical development (normal rider), and* ***D*** *= walking with a rider with CP spastic diplegia (disabled rider)*

the forelimbs, it is unlikely to cause this difference. Also, the validity test with the equipment had shown reliable results. Limb preference exists in horses. Siniscalchi et al.[11] found that limb preference in horses is task dependent. In addition, horses, including ones that are used in EAT, are usually trained to be leashed and mounted on the left side, which might explain the greater swing range at the left than the right thigh, but this does not explain the lower hock swing range at the left. Another possible reason could be that the ponies had unobservable mild lameness, such as a subclinical joint problem or chronic osteoarthritis, which results in an unequal swing range of the two limbs. McCracken et al.[4] reported that objective lameness evaluation using an inertial sensor system would be able to detect lower levels of lameness compared to subjective lameness evaluation.

The limitations of the present study are as follows: (1) insufficient number of sensors, causing inability to measure the kinematic motion of all the joints at the forelimbs and the hindlimbs at the same time; (2) insufficient number of ponies that cooperated in the hindlimb study. For further studies, the authors suggest careful selection of ponies and increased numbers of both ponies and sensors.

This study showed that inertial sensor technology is feasible for use on pony kinematic analysis, especially when the sensors are attached to the forelimbs. There were no significant differences in any of the kinematic motions of ponies at walking gait between the different interventions except in the case of the anterior phase of the angular velocity of the arm when compared between the left and the right when walking with a disabled rider. It was supposed that the ponies may modify the natural kinematic motion when walking with a load.

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Research Article

Uterine Immune Response After Single and Double Fresh Sperm Insemination in Mares [1]

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Abstract

The aim of the study was to evaluate the effects of single and double inseminations on the inflammation of the uterus in mares. Nine mix breed mares with endometrial biopsy scores grade I and IIA, aged 5-15, were used in the study. Two experiments were performed over two estrous cycles: (1) single insemination, (2) double insemination. All mares were used in both insemination group. In the single insemination cycles one insemination was performed 24 h after hCG injection, while in the double insemination cycles two inseminations were carried out 24 h and 48 h after the hCG injection. Endometrial biopsies were collected in both cycles immediately before (=0), and 6, 30 and 54 h after the first insemination. Gene expressions of the inflammatory markers IL-1β, IL-6, IL-8, iNOS, SAA, COX-2 and CASP-3 and histopathological alterations of the endometrium were determined. The 6 h expression levels of IL-6 were higher when compared with 30 h and 54 h ($P<0.05$). CASP-3 expression levels in single insemination cycles were higher at 0 h and 6 h when compared with 54 h ($P<0.05$). In conclusion expression levels of the IL-1β,-6,-8, iNOS, SAA and COX-2 were not different between single or double insemination cycles. This study provides preliminary evidence to further characterize the changes in the expression of relevant genes in response to fresh semen.

Keywords: *Mare, Postbreeding endometritis, Proinflammatory cytokines, Insemination*

Kısraklarda Tek ve Çift Taze Sperm İle Tohumlama Sonrası Uterin İmmun Yanıt

Özet

Çalışmada kısraklarda tek ve çift tohumlama sonrası immun yanıt değerlendirildi. Farklı ırktan, 5-15 yaşlarında ve endometriyal biyopsi skoru I ve IIA olan 9 adet kısrak kullanıldı. İki östrus siklusunda iki uygulama yapıldı: (1) tek tohumlama (2) çift tohumlama. Herbir kısrak tek ve çift tohumlama olmak üzere iki deney grubunda da yer aldı. Tekli uygulamalarda hCG uygulaması sonrası 24. saatte tohumlama yapılıyorken; çiftli uygulamalarda hCG uygulaması sonrası 24. ve 48. saatte tohumlama yapıldı. Tohumlamadan hemen önce (0.) ve tohumlamadan sonraki 6., 30. ve 54. saatte uterus biyopsi örnekleri alındı. Yangı parametrelerinden IL-1β, IL-6, IL-8, iNOS, SAA, COX-2 ve CASP-3 mRNA ekspresyonları ve endometriumdaki histopatolojik değişimler değerlendirildi. Tekli gruptaki kısraklarda 6. saatdeki IL-6 mRNA expresyonları 30. ve 54. saatlerdeki değerlere göre yüksek bulundu ($P<0.05$). 0. ve 6. saatlerdeki CASP-3 ekspresyonları ise 54. saatdeki değerlere göre yüksek bulundu ($P<0.05$). Sonuç olarak tekli ve çiftli gruplar arasında IL-1β,-6,-8, iNOS, SAA ve COX-2 expresyonları açısından fark bulunmadı. Taze semen ile yapılan tohumlamalardan sonra bahsi geçen gen expresyonlarına bakarak immun yanıt hakkında ön fikir elde edilebileceği anlaşıldı.

Anahtar sözcükler: *Kısrak, Tohumlama sonrası endometritis, Yangı öncesi sitokinler, Tohumlama*

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INTRODUCTION

Artificial insemination (AI) and natural breeding, may cause local inflammation of the uterus in mares [1]. Postbreeding endometritis is a physiologic event in mares after artificial insemination (AI) or natural mating and serves to eliminate excessive sperm and bacteria introduced into the uterus during breeding. Breeding fertile mares results in endometritis with a transient neutrophilic inflammation that typically is resolved within 48 hours following the breeding [2].

Shortly after breeding or introduction of pathogens to the uterine lumen, polymorphonucleated neutrophils (PMN) migrate into the uterine lumen and intrauterine fluid containing inflammatory mediators accumulates [3]. Activation of the innate immune system acts in combination with mechanical clearance to assist the uterine lumen to clear the local inflammatory response following breeding. Mares differ from each other in terms of their ability to eliminate inflammation and related harmful inflammatory products [4]. Although the causes for these differences in the ability to clear inflammation are unknown, a defective or poorly orchestrated innate immune response has been suggested [5].

The characteristics and grade of endometrial inflammation may vary depending on the sperm number and concentration, seminal plasma, semen extender and number of insemination [6,7]. If mares are inseminated twice with an interval of 24 h, sperm in the second inseminate induce directly an inflammatory response. Inflammatory environment impairs sperm motility and sperm binding to PMN, which results in formation of slow-moving big clusters. This causes the number of sperm transported to the oviduct to be reduced in the second insemination [7,8].

Cytokines are intercellular signaling proteins released by both immune and non-immune that play an important role in modulating local and systemic inflammatory responses, and the resolution of the inflammation. IL-1β, IL-6 and tumor necrosis factor alpha (TNF-α), also known as proinflammatory cytokines, modulate the acute phase response that involves potent systemic and local effects [9]. Infiltration of leukocytes and increased synthesis of cytokines in response to insemination are necessary to enhance reproductive success, however, prolonged inflammation after insemination may yield undesired outcomes [5]. Leukocyte infiltration and increased synthesis of cytokines in response to insemination is necessary to enhance the reproductive success [10], but a prolonged inflammation after breeding is considered to have an adverse consequence [1,4].

Serum amyloid A (SAA) is a rapidly responding acute phase protein and a very sensitive marker of inflammation. It is suggested that the expression of SAA in the epithelial surface of organs associated with external environment may play a role as a first line of defense against invasion by microorganisms and injuries [5].

Nitric oxide (NO) is a main mediator of smooth muscle relaxation in different organs including the uterus. The inducible NOS (iNOS) is typically expressed at sites of inflammation, and produces large amounts of NO for a prolonged time. Nitric oxide (NO) may play a role in determining susceptibility of mares to PBIE through an inhibitory effect on uterine contractility [11]. Cyclooxygenase (COX) is the enzyme required for the biosynthesis of prostaglandins from arachidonic acid [12] and it may produce prostaglandin during inflammation [13]. Furthermore, COX-2 is required for cell growth. Cyclooxygenase 2 gene expression is induced by stimuli or mediators [12]. Caspases are crucial mediators of programmed cell death (apoptosis). Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins [14].

Although the endometrial immune response has been investigated in mares after insemination, there are no reports relating the cytokine in mares after single and double insemination. The aim of the study was to investigate the inflammatory response in mares after single or double insemination by evaluating uterine mRNA expressions of IL-1β,-6,-8, iNOS, SAA, COX-2, CASP-3 and histopathological findings. Based on previous data, it was hypothesized that the inflammatory changes in response to insemination occur in a time related manner and inflammation-related gene expressions may vary according to the number of insemination.

MATERIAL and METHODS

Animals and Study Design

The animal application procedures were approved by the Local Ethics Committee for Experimental Animals (No: 2015-17). Nine mix breed mares with endometrial biopsy scores (Kenney and Doig [15]) of grades I and IIA, aged 5-15, were used in the study. All mares had normal clinical and gynecological characteristics before entering the study. Two experiments were performed over two estrous cycles: (1) single insemination, (2) double insemination *(Fig. 1)*. All mares were used in both insemination group.

As soon as a follicle was measured to be ≥35 mm, the mares were evaluated for the presence of intrauterine polymorphonuclear neutrophils (PMN) using a cytobrush (Minitube, Tiefenbach, Germany). Mares that had more than three PMN in five fields (400x) were excluded from the study. Human chorionic gonadotropin (hCG,1500 IU, I.V.Chorulon, MSD, Turkey) was administered to mares and insemination was performed 24 h after hCG injection. Before the insemination procedure, ultrasonography was performed and PMN were counted once again. Only mares without ovulation with negative cytology were included in the study and inseminated. Any mares that had a positive cytology and/or ovulation before insemination were

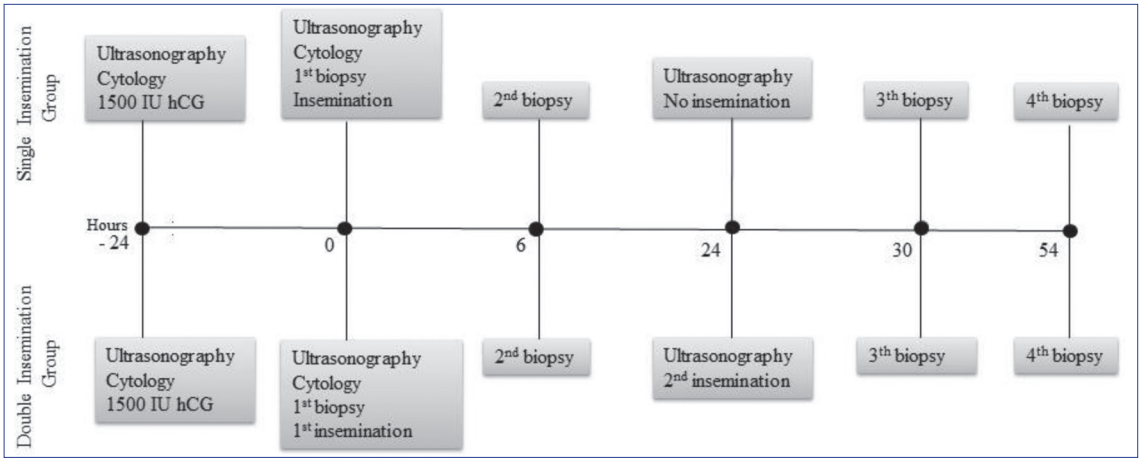

Fig 1. Experimental design of the study

Table 1. *Sequences of the gene-specific primers used in the analysis of gene expression levels*

Target (gene)	Forward Primer Sequence	Reverse Primer Sequence
IL-1β	5′- CGTTTCCCAGAGCCAATCCT -3′	5′- TGCTCATCAGAAGCTGGGTG -3′
IL-6	5′- ATGGCTACTGCTTTCCCCAC -3′	5′- GGCAGAGATTTTGCCGAGGA -3′
IL-8	5′- TGCTTTCTGCAGCTCTGTGT -3′	5′- TGTGGCCCACTCTCAATCAC -3′
iNOS	5′- CCCTTCAACGGCTGGTACAT -3′	5′- CAGCTTGTGTGTTTCCAGGC -3′
SAA	5′- GTTCACAGGCCTCGTCTTCT -3′	5′- TAGCATGTCCCAAGTCCCTC -3′
COX-2	5′- GATCCTAAGCGAGGTCCAGC -3′	5′- AGGCGCAGTTTATGCTGTCT -3′
CASP-3	5′- TCATCCAGTCGCTTTGTGCT -3′	5′- CCATGGCTACCTTGCGGTTA -3′
B2M	5′- CTACTCTCCCTGACTGGCCT -3′	5′- ATTCTCTGCTGGGTGACGTG -3′
GAPDH	5′- CATCAAATGGGGCGATGCTG -3′	5′- ACATTGGGGCATCAGCAGAA -3′
ACTB	5′- GGGCCAGAAGGACTCATACG -3′	5′- TCGATGGGGTACTTGAGGGT -3′

excluded for that cycle, treated, and re-entered the study during a subsequent cycle. In the single insemination group (n=9) mares received one insemination at 0 hour (= 24 h after hCG) while in the double insemination group (n=9) the mares received two inseminations at 0 and 24 h. Ultrasonography was performed again at 24 h and only ovulated mares were included in the study. In this way all mares ovulated between 24-48 h after hCG administration.

Endometrial biopsies were collected at 0, 6, 30 and 54 hours after first insemination. Biopsy samples were obtained at the same time during both cyles. Biopsies were taken from the endometrium using a uterine biopsy forceps (Equivet®, Kruuse, Denmark). Immediately after biopsy, the samples were cut in two pieces with nearly the same size using a sterile scalpel. One portion of the sample was used for histopathological examination and the other portion was used for gene expression analysis. Samples collected for gene expression were snap frozen in liquid nitrogen and kept at -80°C until further processing.

Preparation of Sperm for Insemination

Semen was collected from an Arabian stallion with known fertility. E-Z Mixin BF (ARS, USA) was used as extender. Sperm were counted immediately after collection with Makler Counting Chamber (Sefi-Medical Instruments, USA). The insemination dose contained 500 million progressively motile sperm in 30 mL of extended semen.

RNA Isolation and cDNA Synthesis

RNA isolation was performed by using the RNAzol®RT RNA Isolation Kit (MRC, Canada) according to the manufacturer's recommendations. Briefly, 100 mg of frozen tissue was disrupted and homogenized in 1 mL of RNAzol®RT solution using TissueRuptor (Qiagen, Germany). After the isolation, total RNA was eluted in 50 µL RNase free water. In order to detect the quantity and quality of the total RNA, samples were evaluated using Nano-Drop 2000 (Thermo Scientific, USA). cDNA synthesis was performed using 200 ng of total RNA and SCRIPT cDNA Synthesis Kit (Jena Bioscience, Germany).

Quantitative Real-Time PCR Analysis

Gene-specific primers were designed manually and the sequence homologies were confirmed using the BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer sequences are given in *Table 1*.

qRT-PCR was carried out using gene-specific primers, qPCR Green Master with UNG (Jena Bioscience, Germany) and CFX 96 Real Time Instrument (Bio-Rad, Germany) with EVA Green detection module. All samples were studied in duplicate and their average cycle threshold (Ct) values were taken for further analysis. Mean of Ct values of β-actin (ACTB), glydecaldehyde-3-phosphate-dehydrogenase (GAPDH) and β-2-microglobulin (B2M) were used to normalize the values. Analysis of relative expression data was performed by comparative Ct method (2-DDCt method) and the results were expressed as relative quantification (RQ) values. Melting curve analysis was used to confirm the specificity of the amplified products.

For expression analysis in endometrial biopsy samples at different time points and between the two insemination cycles, expression levels of the studied genes (IL-1β,-6,-8, iNOS, SAA, COX-2 and CASP-3) were compared.

Histopathological Examination

Uterine biopsy samples of the mares were fixed in neutral buffered formalin, routinely processed, embedded in paraffin, cut at 4-5 µm thickness and then stained with Hematoxylin and Eosin (H&E) for evaluation by light microscopy.

Biopsy specimens were assessed separately by two pathologists who were blinded to the identification of the samples. Histological inflammatory parameters modified from similar studies such as hyperemia, edema, hemorrhage, surface epithelial disruption (SED), polymorphonuclear leukocyte (PMN) infiltration, lymphoplasmacytic (LP) infiltration and fibrosis and cystic dilation (F+CD) of endometrial glands were evaluated to determine inflammatory status in the uterine tissue. Specimens were scored using a 0-3 scale for each parameter. Score 0 = none; 1 = mild; 2 = moderate; 3 = severe changes [16,17]. While estimating the intensity of PMN, the number of total neutrophilic leukocytes was determined in 3 separate fields at a magnification of 400x. And the scores were given on the basis of the severity of neutrophilia. No neutrophilia (<10 neutrophils in total of three fields; score=0), moderate neutrophilia (10-99 neutrophils in total; score=1), severe neutrophilia (100–149 neutrophils in total; score=2) and very severe neutrophilia (>150 neutrophils in total; score=3) [18].

Statistical Analyses

Repeated measurement of ANOVA in SPSS 10.0 statistical package (SPSS, 1999) was used to analyze data for gene expression. The model included biopsy time as a within-subject effect and insemination as a between-subject effect, and also biopsy time × insemination interaction. Significance control was assessed by using the least significant difference procedure. Comparison between gene expression levels were conducted by using RQ values and results were expressed as mean and standard error (S.E.). Independent samples t-test was applied to compare single versus double uterine insemination at a specific time point for gene expressions.

The Friedman test was used to compare different biopsy time points with respect to different histological parameters in each insemination group and the Wilcoxon test was applied to compare single versus double uterine insemination at a specific time point for histological parameters.

RESULTS

Gene expression of IL-1β,-6,-8, iNOS, SAA, COX-2 and CASP-3 in single and double insemination groups were compared at different time points (0, 6, 30 and 54 h).

Effects of One and Two Inseminations

Expression levels of IL-1β, IL-6, IL-8, SAA, COX-2 and iNOS genes were not different between single or double insemination cycles *(Table 2)*. Additionally, IL-1β, IL-8, SAA and iNOS were similar between different each time point for those gene expressions within the same groups.

IL-6 gene expression in single insemination cycles were higher at 6 h when compared with 30 h and 54 h ($P<0.05$). Although there was a similar trend in double insemination group, it was not significant ($P>0.05$). No differences ($P>0.05$) were detected between single and double insemination group at any time point.

COX-2 mRNA expression in single insemination cycles was increased at 6 h after insemination ($P<0.05$). A significant decrease was obtained at 54 h when compared with 6 h ($P<0.05$). COX-2 mRNA expression in double insemination group did not differ at any time point. No differences ($P>0.05$) were detected between single and double insemination group at any time point.

CASP-3 expression levels in single insemination cycles were higher at 0 h and 6 h when compared with 54 h ($P<0.05$). Double insemination group showed an increased gene expression at 0 h when compared with 30 h ($P<0.05$). Single insemination group had a higher level of expression at 6 h when compared with double insemination group ($P<0.05$).

Comparison of Histopathological Parameters

Hyperemia and edema were observed in all samples immediately after the first insemination application. When the surface epithelial status was assessed, it was detected that the epithelial lining was disrupted *(Fig. 2A)* or infiltrated by PMN at varying levels. PMN was seen right below the surface epithelium, among the uterine glands and in perivascular areas *(Fig. 2B,2C)*. In both insemination groups, PMN increased apparently at the second biopsy time points. Hemorrhage was not a specific finding in either of the samples *(Fig 2D)*.

Table 2. *Gene expressions of inflammatory markers immediately before (hour 0) and 6, 30 and 54 hh after first insemination with fresh sperm during single and double insemination groups. Gene expression values given as mean Relative Quantitation and Standard Error*

Genes	Biopsy Time (hours)	Insemination Procedures		Sig.##
		Single Insemination (n=9)	Double Insemination (n=9)	
IL-1β	0	6.66±2.24	2.24± 0.55	ns
	6	11.29±2.12	6.60±3.12	ns
	30	10.18±3.69	5.98 ±1.42	ns
	54	2.72±0.55	3.21± 1.08	ns
Sig.#		ns	ns	
IL-6	0	5.49±2.37^{ab}	1.22±0.52	ns
	6	8.35±1.95^{a}	4.21±1.42	ns
	30	0.92±0.51^{b}	2.77±1.22	ns
	54	0.71±0.15^{b}	0.77±0.36	ns
Sig.#		*	ns	
IL-8	0	6.95±3.37	1.62±0.83	ns
	6	7.47±2.47	2.72±1.34	ns
	30	5.69±2.81	1.90±0.81	ns
	54	0.81±0.20	0.53±0.26	ns
Sig.#		ns	ns	
CASP-3	0	2.72±0.52^{a}	1.55±0.42^{a}	ns
	6	1.93±0.40^{a}	0.66±0.13^{ab}	*
	30	1.13±0.32^{ab}	0.45±0.86^{b}	ns
	54	0.73±0.12^{b}	0.62±0.10^{ab}	ns
Sig.#		*	*	
SAA	0	33.28±29.05	15.66±7.50	ns
	6	48.97±14.67	154.21±124.26	ns
	30	89.03±64.67	41.50±18.48	ns
	54	81.17±29.69	18.80±11.92	ns
Sig.#		ns	ns	
COX-2	0	1.42±0.46^{a}	1.55±0.53	ns
	6	3.25±0.72^{b}	2.35±1.37	ns
	30	4.25±1.48^{ab}	1.08±0.45	ns
	54	0.75±0.26^{a}	0.85±0.57	ns
Sig.#		*	ns	
iNOS	0	14.31±5.54	30.82±9.09	ns
	6	22.12±7.20	11.00±2.71	ns
	30	25.00±13.78	13.49±3.64	ns
	54	16.59±3.99	25.88±15.27	ns
Sig.#		ns	ns	

Different letters in the same column indicate significant differences. ***Sig.#*** *-values for differences within the same insemination cycle. Sig ## -values for differences between single and double insemination cycles. * P<0.05* ***ns:*** *P>0.05*

Statistically significant differences were noted between single and double insemination at 30th h biopsy with respect to SED (P<0.05), LP infiltration (P<0.05) and F+CD (P<0.05), all of which were found to have increased in the double insemination group.

When biopsy time points in each insemination group were compared among each other, statistically significant differences were detected in terms of edema, SED and PMN. Edema peaked at the 6th h biopsy in the single insemination group and decreased at the 30th h and 54th h biopsy (P<0.05). On the other hand, edema was at the highest level in the first biopsy samples of the double insemination group and decreased at the 6th h biopsy and 30th h biopsy and then increased again at the 54th h biopsy (P<0.05).

SED differed significantly among different time points only in the double insemination group (P<0.05). SED was less severe in the first and last time points when compared with its severity at 6th h biopsy and at 30th h biopsy.

There was a marked significant difference with respect to PMNs both in the single (P<0.05)and double (P<0.001) insemination groups. PMN infiltration was quite low in the first biopsy samples of the single and double insemination groups and increased significantly at 6th h biopsy and at 30th h biopsy. The number of PMN in last biopsy double insemination group was still at a higher level than the first biopsy. All statistical results are given in *Table 3*.

DISCUSSION

Based on this study, we investigated the immune response of endometritis in mares after single or double insemination. The results of the present study demonstrated that mRNA expressions of IL-1β,-6,-8, iNOS, SAA and COX-2 were not different in these two groups of mares.

mRNA expressions of IL-1β, IL-6 and IL-8 tend to be higher at 6 h after insemination in the present study. A gradual increase was found only for IL-6 at 6 h after insemination in the single insemination group. Fumoso et al.[9] reported that mRNA expression of IL-1β, IL-6 and TNF-α for susceptible or resistant showed no variation in 24 h after stimulus of artificial insemination with killed sperm. Resistant mares had lower endometrial mRNA expression for IL-1β and TNF-α in dioestrus than susceptible mares. Twenty-four hours after AI, gene expression for the three cytokines was significantly increased in resistant mares compared to baseline expression in the same mares during estrus of the previous cycle (without treatment). The difference between the results of these two studies might be due to the usage of killed or motile spermatozoa. In additionally, the mares were not classified as susceptible or resistant to persistent breeding induced endometritis (PBIE) but endometrial biopsy scores of all mares were grade I and IIA and they were considered as potential resistant to PBIE.

Gene expressions by equine endometrial cells in response to intrauterine infusion of *E. coli* were investigated in a study. The researchers detected an up regulation of IL-1β, IL-6, IL-8 and TNF-α at 3 h after *E. coli* inoculation in resistant mares [19]. Even though the only IL-6 had significant upregulation in our results for interleukin gene expressions, all these parameters for IL-1β, IL-6, and IL-8 had the highest values at 6 h after insemination with a

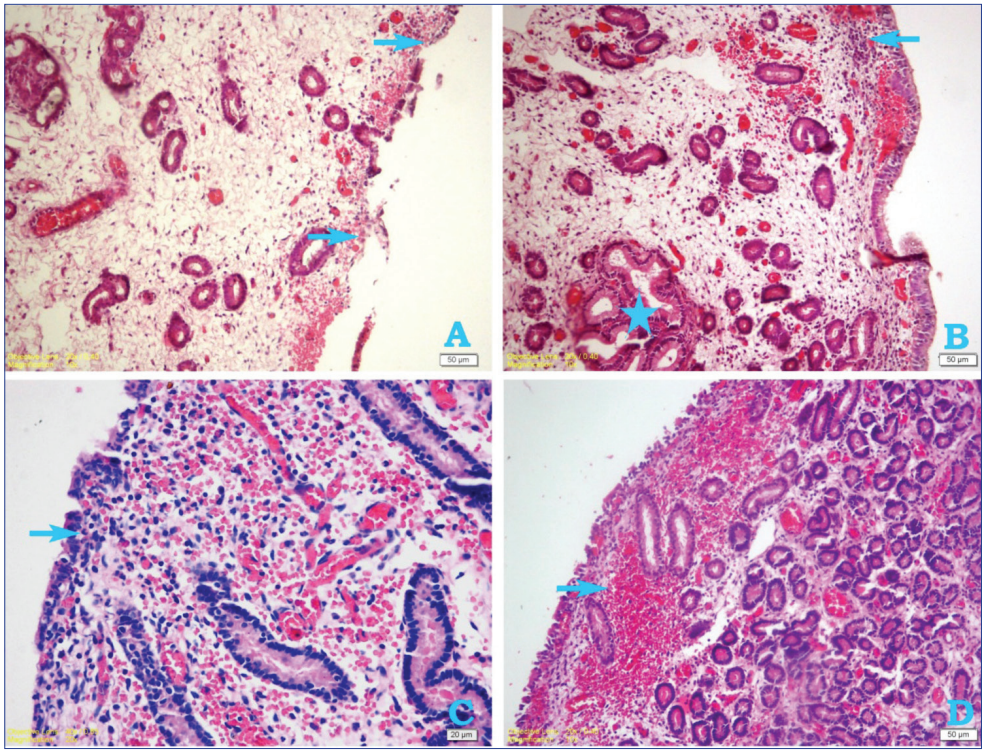

Fig 2. A. Surface epithelial disruption *(arrows),* edema in the mucosa, H&E staining, Bar=50 µm, B. PMNs infiltration in the subepithelial area *(arrow)* and mucosa, nesting uterine glands *(star),* H&E staining, Bar=50 µm, C. Severe PMNs infiltration in the subepithelial area *(arrow)* and mucosa, H&E staining, Bar=20 µm, D. Hemorrhage in the subepithelial area *(arrow),* H&E staining, Bar=50 µm

similar pattern of an up regulation in the first few hours of uterine immune system activation by inflammatory substances as bacterial invasion or sperma.

IL-6, originating from inflammed tissues, is one of the main acute phase proteins. It is suggested that IL-6 initially promotes acute inflammation and PMN recruitment, and subsequently induces PMN apoptosis and phagocytosis which leads to termination of inflammation [9] while IL-8, a crucial pro-inflammatory mediator for chemotaxis [20], is responsible for the continuing migration of PMN's into the uterine lumen [19]. IL-1β is responsible for initiating and down-regulating the pro-inflammatory response [19]. PMN plays role in clearance of inflammatory by-products from uterus by inducing myometrial contractions via stimulating synthesize and secretion of $PGF_{2\alpha}$ from endometrium [5]. Although the absence of statistical significance in double insemination group, mRNA expressions of IL-1β, IL-6, IL-8 were consistent with immunohistochemical findings. As double insemination was performed at 24 h of the study, gene expressions of interleukins which were peaked at 6th h remains higher at 30 h in the present study. There was significance at SED, LP infiltration and F+CD between the groups at 30th h. The higher scores of those parameters in double insemination group represent the inflammatory changes which were moderated by acute phase proteins.

Increased nitric oxide (NO) produced by iNOS serves as an aid in the removal of pathogens but can also be cytotoxic [21-23]. In addition, mares susceptible to PBIE had increased iNOS activity and NO production com pared to resistant mares at 13 h after insemination [11]. Troedsson et al.[24] suggested that contractility deficiencies in susceptible mares between 6 and 19 h after insemination may be associated with increased NO production. However, expression levels of iNOS were not comparable within the single or double insemination groups in our study. According to the endometrial biopsy scores which were determined at beginning of this study, the mares were not susceptible to PBIE. The unchanging NO production after insemination might be explain with the resistance of the mares. The results should be compared with further studies which includes PBIE susceptible mares.

CASP-3 mRNA expression has been detected in placenta and in the uterus of cycling and pregnant cows [25]. The present study is the first report of uterine mRNA expression of CASP-3 in mares after insemination. It is an important enzyme for the first step of apoptosis that can be activated by intrinsic or extrinsic pathways [25]. The rate of apoptosis in the tissue was not taken account in the present study. However, a gradual decrease was observed in CASP-3 expression at each biopsy sampling time after the first insemination in both groups. This decrement might be associated with a potential down-regulation of this protein throughout consecutive sampling times.

In the present study no difference in endometrial gene expression of SAA was found between the two groups. Previous studies have shown an endometrial SAA expression were significantly and rapidly up-regulated in response to inoculation, and *E. coli* endometritis thus provoked a marked and transient inflammatory response [5] but same researcher's another study mRNA expression of SAA was not influenced after *E. coli* infusion. The different expression of SAA between two studies reflected the course of inflammation within the uterus [19].

Neutrophils are the potent producers of cytokines. PMN migration into the uterus peaks about 6 h after experimental introduction of bacteria to the uterine

Table 3. *Histological characteristics of the endometrium immediately before (0 h) and 6, 30 and 54 h after first insemination in single and double single and double insemination cycles*

Histological Inflammatory Parameters	Biopsy Time (hours)	Single Insemination (n=9) Median (Min-Max)	Double Insemination (n=9) Median (Min-Max)	Sig.##
Hyperemia	0	1 (1-2)	2 (1-2)	ns
	6	1 (1-2)	2 (1-2)	ns
	30	1 (1-2)	1 (1-2)	ns
	54	1 (0-2)	1 (1-3)	ns
Sig.#		ns	ns	
Hemorrhage	0	1 (0-3)	1 (0-3)	ns
	6	1 (0-3)	2 (0-3)	ns
	30	1 (0-2)	1 (0-3)	ns
	54	2 (0-3)	2 (0-3)	ns
Sig.#		ns	ns	
Edema	0	2^{ab} (0-3)	2^{a} (1-3)	ns
	6	2^{a} (1-3)	1^{b} (1-3)	ns
	30	1^{b} (0-2)	1^{c} (0-2)	ns
	54	1^{b} (0-2)	2^{ab} (0-3)	ns
Sig.#		*	*	
Surface epithelial disruption	0	1 (0-3)	1^{ac} (0-2)	ns
	6	1 (1-2)	2^{b} (1-2)	ns
	30	1 (0-2)	2^{ab} (0-3)	*
	54	1 (0-2)	1^{c} (0-2)	ns
Sig.#	P	ns	*	
PMN infiltration	0	0^{a} (0-1)	0^{a} (0-1)	ns
	6	2^{b} (0-3)	2^{b} (0-3)	ns
	30	2^{bc} (1-2)	2^{bc} (1-2)	ns
	54	1^{ab} (0-2)	1^{d} (0-1)	ns
Sig.#		*	**	
LP infiltration	0	1 (1-3)	1 (1-2)	ns
	6	1 (0-2)	2 (1-2)	ns
	30	1 (0-2)	2 (1-2)	*
	54	1 (0-2)	1 (1-2)	ns
Sig.#		ns	ns	
Fibrosis and cystic dilation of endometrial glands	0	0 (0-1)	0 (0-3)	ns
	6	0 (0-2)	1 (0-2)	ns
	30	0 (0-3)	1 (0-3)	*
	54	0 (0-3)	2 (0-2)	ns
Sig.#		ns	ns	

Different letters in the column indicate significant differences. ***Sig.#*** *-values for differences within the same insemination cycle.* ***Sig ##*** *-values for differences between single and double insemination cycles. * P<0.05 ** P<0.001* ***ns:*** *P>0.05*

lumen, and normally the response will remain elevated for at least 72 h [26]. The biopsy sampling times (0, 6, 30 and 54 h) of this study was specified with the light of this information. Neutrophil activation was also same between single and double insemination groups. Neutrophils increased after 6 h insemination in both groups and gradually decreased after 54 h insemination with parallel to proinflammatory gene expressions. Other researchers obtained similar results 5-12 h after uterine infusion of bacteria [26,27] and 6 h after natural mating or insemination with chilled or frozen/thawed semen [1]. The increased number of neutrophils after the first insemination was an expected result of this study. However, the number of neutrophils and chemokines like IL-6 and IL-8 which provide chemotaxis for neutrophils did not change after the second insemination. The reason of this result might be the binding between spermatozoa and inflammatory cells after the second insemination in 24 h, as explained by Troedsson [8]. The increase of neutrophils after second

insemination might be detected in uterine lumen rather than uterine tissue.

In conclusion, according to our result, uterine mRNA expressions of IL-1B,-6,-8, iNOS, SAA, COX-2 were not affected by the number of inseminations. In order to establish whether the mares are resistant to PBIE, it is necessary to expose both resistant and susceptible mares to this study and to determine whether gene expression changes are dependent upon the inflammatory status of the uterus. This study provides preliminary evidence to characterize further the changes in the expression of relevant genes in response to live semen. The effect upon gene expression by treatment with other preparations of fresh, chilled or frozen/thawed semen also needs to be investigated in both resistant and susceptible mares.

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Research Article

Ultrasound Evaluation of Negative Energy Balance-Induced Fatty Liver in Sheep [1]

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Abstract

Fatty liver disease is a common liver disease in humans and animals alike, and macrofauna are considered an ideal model of fatty liver disease for ultrasonography (US)-based diagnostic research. Here, a model of fatty liver disease in lactating thin-tail sheep induced by negative energy balance was developed by restricted feeding. Ultrasonographic images were divided into the following fat classes according to liver triglyceride concentrations (ratio of triglyceride weight to wet liver weight): healthy liver, <2%; mild hepatic steatosis (HS), ≥2% to 5%; moderate HS, ≥5% to 10%; and severe HS, ≥10%. Characteristics of Ultrasonographic images according to HS severity were evaluated. As the results, mild to severe HS was detected by restricted feeding. The sensitivity of 5 MHz for diagnostic differentiation of healthy liver, and mild, moderate, and severe HS were 66.7%, 60.0%, 66.7%, and 82.5%, respectively, with a moderate κ statistic ranging from 0.667 to 0.735 among observers. The sensitivity of 3.5 MHz for diagnosis of healthy liver, and mild, moderate, and severe HS were 69.7%, 60.5%, 81.5%, and 75.4%, respectively, with moderate κ statistics ranging from 0.575 to 0.684 among observers. The sensitivities of 3.5 and 5 MHz for moderate to severe HS reached 97.4% and 98.2%, respectively. In summary, visualization at 3.5 and 5 MHz was ideal for diagnosis of HS except mild cases with established US diagnostic standards. Furthermore, with the established model, newer methods, such as image digitizing analysis, can be used for US-based diagnosis of fatty liver in further investigations.

Keywords: *Ultrasound, Fatty liver, Sheep, Negative energy balance*

Negatif Enerji Dengesiyle Oluşturulmuş Koyun Yağlı Karaciğerinin Ultrasonografik Değerlendirmesi

Özet

Yağlı karaciğer hastalığı insanlarda ve benzer olarak hayvanlarda yaygın bir karaciğer hastalığı olup, makrofauna yağlı karaciğer hastalığının ultrasonografi (US)-temelli diagnostik araştırmaları için ideal bir modeldir. Bu çalışmada, laktasyondaki ince kuyruklu koyun ırkında negatif enerji dengesi ile yağlı karaciğer hastalığı modeli beslemenin kısıtlaması yoluyla oluşturulmuştur. Ultrasonografik görüntüler karaciğer trigliserid konsantrasyonu (yaş karaciğer ağırlığının trigliserid ağırlığına oranı) baz alınarak yağ sınıflarına ayrılmıştır; sağlıklı karaciğer, <2%; hafif hepatik steatozis (HS), ≥2% ile 5% arası; orta derecede HS, ≥5% ile 10% arası ve şiddetli HS, ≥10%. HS şiddetine göre ultrasonografik görüntülerin özellikleri değerlendirildi. Sonuç olarak kısıtlı besleme ile hafif ve orta derecelerde HS tespit edildi. Sağlıklı, hafif, orta ve şiddetli HS'nin diagnostik ayırımı için 5 MHz'nin hassasiyeti sırasıyla %66.7, %60.0, %66.7 ve %82.5 olarak belirlendi. Gözlemciler arasında ortalama κ istatistik 0.667 ile 0.735 arasında değişim gösterdi. Sağlıklı, hafif, orta ve şiddetli HS'nin diagnostik ayırımı için 3.5 MHz'nin hassasiyeti sırasıyla %69.7, %60.5, %81.5 ve %75.4 olarak belirlendi. Gözlemciler arasında ortalama κ istatistik 0.575 ile 0.684 arasında değişim gösterdi. Orta ve şiddetli HS için 3.5 ve 5 MHz'lerin hassasiyetlikleri sırasıyla %97.4 ve %98.2'ye ulaştı. Özet olarak, 3.5 ve 5 MHz'lerde görüntüleme hafif vakalar haricinde HS'nin tanısı için tespit edilmiş olan US tanı standartlarında idealdir. Ayrıca, bu model görüntü dijital analiz gibi yeni metotlar ile birlikte yağlı karaciğerin US temelli tanısında ileriki araştırmalarda kullanılabilir.

Anahtar sözcükler: *Ultrasonografi, Yağlı karaciğer, Koyun, Negatif enerji dengesi*

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INTRODUCTION

Fatty liver disease is a common in both humans and a variety of mammals, including dairy cows, sheep, dogs, and cats [1,2]. The degree of fatty liver disease ranges from simple hepatic steatosis (HS) to nonalcoholic steatohepatitis (NASH) with or without fibrosis or cirrhosis [3]. Although the correlation between excessive fat intake and non-alcoholic fatty liver disease in humans remains controversial [4], its prevalence has continued to increase concomitant with the epidemic of obesity. In addition, a severe negative energy balance (NEB) during the early lactation period, as a consequence of insufficient energy intake to sustain the high energy requirements for milk production, can also cause fatty liver, especially in dairy cows [5-7], thereby resulting in major economic losses to the dairy industry [8,9].

Although liver biopsy is the gold standard for assessment of fatty liver disease, the procedure is invasive and only evaluates approximately 0.002% of the liver parenchyma [10]. In contrast, ultrasonography (US) is a non-invasive and convenient testing modality with high sensitivity and specificity that is widely used for the diagnosis of fatty liver. US can reflect both metabolic derangements and histological changes caused by HS. However, intra- and inter-observer agreements of US findings are inconsistent across various studies and the diagnostic accuracy of liver US is higher for severe HS than mild HS and NASH [3]. Thus, further research is needed to establish criteria for highly sensitive diagnostic US of fatty liver. In this study, US findings of NEB-induced fatty liver in sheep were evaluated for establishing a practical US diagnostic standard of estimating the degree of fatty liver.

MATERIAL and METHODS

Ethical Approval

The study protocol was approved by the Ethics Committee on the Use and Care of Animals of Heilongjiang Bayi Agricultural University (Daqing, China)

Fatty Liver Model in Lactating Sheep

A total of 14 pregnant thin-tail ewes with parities of 2-3 were acquired. After lambing, the sheep were fasted for 12 h and allowed to nurse for lamb freedom and then allocated to two experimental groups: a control group (CG, n = 4), comprised of sheep that gave birth to a single lamb that remained with the ewe in individual pens and received a normal diet of 2 kg of peanut hay and 300 g of a concentrate composed of 2.1 kg of dry matter/day, 12.8 MJ of net energy for lactation (NEL)/day, and 173.7 g of absorbable protein/day twice per day (09:00 and 16:00 h), and a fatty liver model group (MG, n = 10), which included six single birth sheep (SMG) and four double birth sheep (DMG) that received a restricted diet of 200 g/day of peanut hay and 60 g of concentrate composed of 0.4 kg of dry matter/day, 2.6 MJ of NEL/day, and 34.7 g of absorbable protein/twice per day (09:00 and 16:00 h) to supply the ewes with sufficient energy for lactation. Ewes in both groups were fed with the designated diets for 16 days.

Abdominal US

Abdominal US was performed prior to the morning feeding on postpartum days 4, 7, 10, 13, and 16 by a single expert physician. Images were obtained using the SIUI CTS-7700V US system (Shantou Institute of Ultrasonic Instruments Co., Ltd., Shantou, China) with a 2.5-5-MHz curved transducer operated at 3.5 MHz and a 4-8-MHz linear array transducer operated at 5.0 MHz. The following settings of the equipment were fixed and used throughout the study period: depth range of image, 8.7 cm; dynamic range, 80 dB; time gain compensation, neutral (sliders in the center position); overall gain, 66 dB for 3.5 MHz and 80 dB for 5.0 MHz; and transmit focus, 3 cm.

The sheep were examined in the left lateral decubitus position without sedation. After carefully shaving and degreasing the skin with 70% alcohol, transcutaneous US of the liver region was conducted at the 8th-13th intercostal spaces of the right flank of the animal. For diagnosis and grading of the severity of HS, standardized transverse and longitudinal views of the right hepatic lobe, including the right kidney, diaphragm, and intestines, as well as the portal vasculature [11], were obtained.

Liver Biopsy and Histological Analysis

Liver biopsies were performed after liver US on postpartum days 4, 7, 10, 13. Briefly, the incision site was shaved, disinfected, and injected with 1 mL of 2% xylocaine. After 10 min, liver biopsy specimens (approximately 20 mg each) were collected through a 0.5 cm right-side incision between the 11th and 12th rib from the mid-scapula to the tuber coxae using a biopsy paracentesis needle (Cone TZ14-16; Cone Instruments, LLC, Cleveland, OH, USA). The liver biopsy specimens were immediately stored at –80°C for triglyceride analysis using an enzymatic kit (Applygen Technologies, Inc., Beijing, China).

On the final day of the experimental period (postpartum day 16), after liver US, each sheep was anesthetized by intramuscular injection of ketamine hydrochloride (50 mg/kg BW) and xylazine hydrochloride (50 mg/kg BW), and euthanized by exsanguination. After exsanguination, the liver was removed and cleaned with saline, and part of the liver was frozen in liquid nitrogen for Oil Red O staining and triglyceride analysis. The remaining tissue was fixed in 4% formaldehyde for hematoxylin/eosin (H&E) and picrosirius red staining.

Imaging Analysis

In this study, 70 US images were obtained from the 14

sheep on postpartum days 4, 7, 10, 13, and 16. Images were subdivided into the following fat classes according to liver triglyceride concentrations (expressed as the ratio of triglyceride weight to wet liver weight.): healthy liver, <2%; mild HS, ≥2% to 5%; moderate HS, ≥5% to 10%; and severe HS, ≥10% [12]. According to the fat classes, the following four widely accepted items were used for image analysis to establish standards for fatty liver grade of sheep [13,14]: echogenicity of liver parenchyma (bright liver) compared with the echogenicity of the renal cortex (L-K contrast), visualization of intrahepatic vessels (vessels blurring), and visualization of the posterior hepatic lobe, including the diaphragm, postcaval vein, rumen, and intestines. Visualization of these image characteristics were classified as absent, slight, intermediate, and marked. Three radiologists evaluated the images according the developed standards for sensitivity, specificity, and kappa analysis.

Statistics Analysis

All data were analyzed using SPSS statistical software (version 19.0; IBM-SPSS, Inc., Chicago, IL, USA). Liver triglyceride concentrations are presented as means ± SD and differences were identified by repeated measures analysis of variance. A probability (P) value of <0.05 was considered statistically significant. Interobserver agreement was analyzed using κ statistics, where κ <0.4 indicated poor agreement, 0.4-0.6, indicated moderate agreement; >0.6 0.8 indicated good agreement, and >0.8-1 indicated excellent agreement.

RESULTS

Triglyceride Analysis and Pathological Changes in the Liver

In this study, 70 liver biopsy samples were obtained for tri-glyceride analysis. As shown in *Fig. 1A*, the liver triglyceride concentrations of the treated sheep were significantly increased after post-partum day 4 and severe HS was detected in DMG sheep after postpartum day 7. Based on the estimated triglyceride concentrations, the samples were divided into the following hepatic fat classes: healthy liver (n = 22), mild HS (n = 10), moderate HS (n = 19), and severe HS (n = 19) *(Fig. 1B)*.

On postpartum day 16, the sheep were anesthetized and then euthanized by bloodletting from the carotid arteries, and liver were collected for biopsy and histological analysis. As shown in *Fig. 2*, liver histopathological analysis showed various degrees of hepatic fatty infiltration in MG sheep. When the liver triglyceride concentration was increased, lipid droplets tended to accumulate in the portal area. Similarly, Oil red O staining clearly revealed the tendency of hepatic fatty infiltration to spread from the liver portal area to the hepatic lobule and, importantly, there was a relative correlation between triglyceride concentration and fatty infiltration. In this study, picrosirius red was employed to assess the degree of liver fibrosis and steatohepatitis, which revealed no significant difference in the degree of fibrosis between the two groups [15].

Abdominal US

US was used to image the liver at the 8th-13th intercostal spaces. As show in *Fig. 3*, the hepatic caudate lobe process and cranial pole of the kidney were imaged at the 12th-13th intercostal spaces, while the distal caudal vein of the right hepatic lobe and the interface between the liver and diaphragm, rumen, and postcaval vein were imaged at the 11th-12th intercostal spaces. Most of the right hepatic lobe, copious veins, portal vein, and the interface between the liver and rumen, intestine, and omasum were imaged in the 10th-11th intercostal spaces, while the liver appeared very narrow at the 8th-10th intercostal spaces. In this study, US was used to observe the liver at the 10th-13th intercostal spaces.

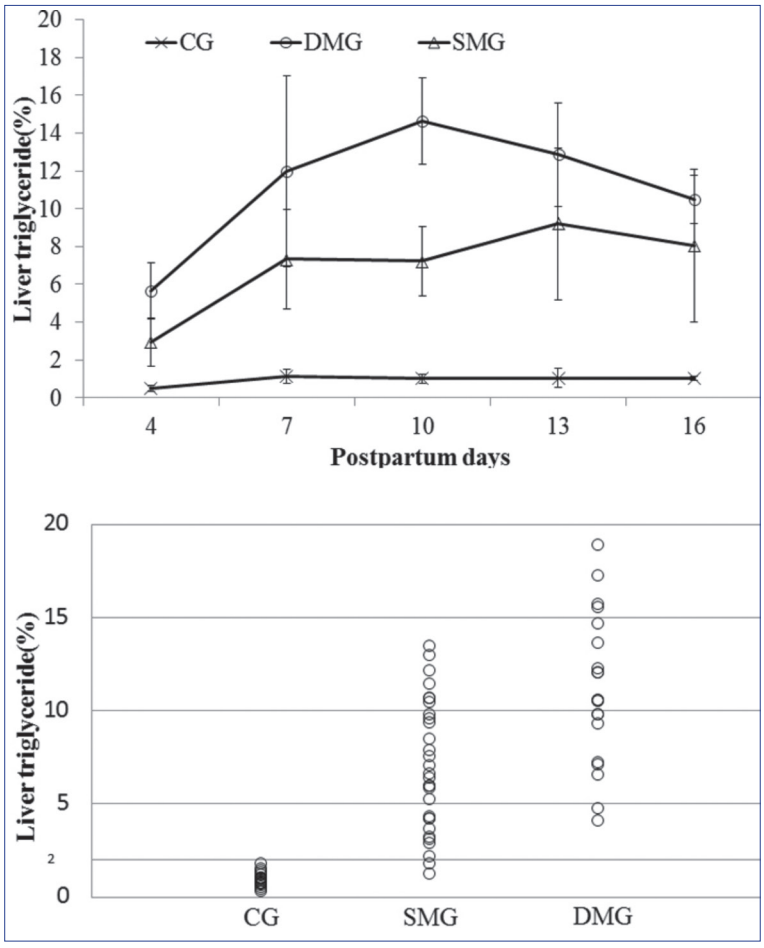

Fig 1. Polygram (A) and scatterplot (B) of sheep liver triglyceride concentration

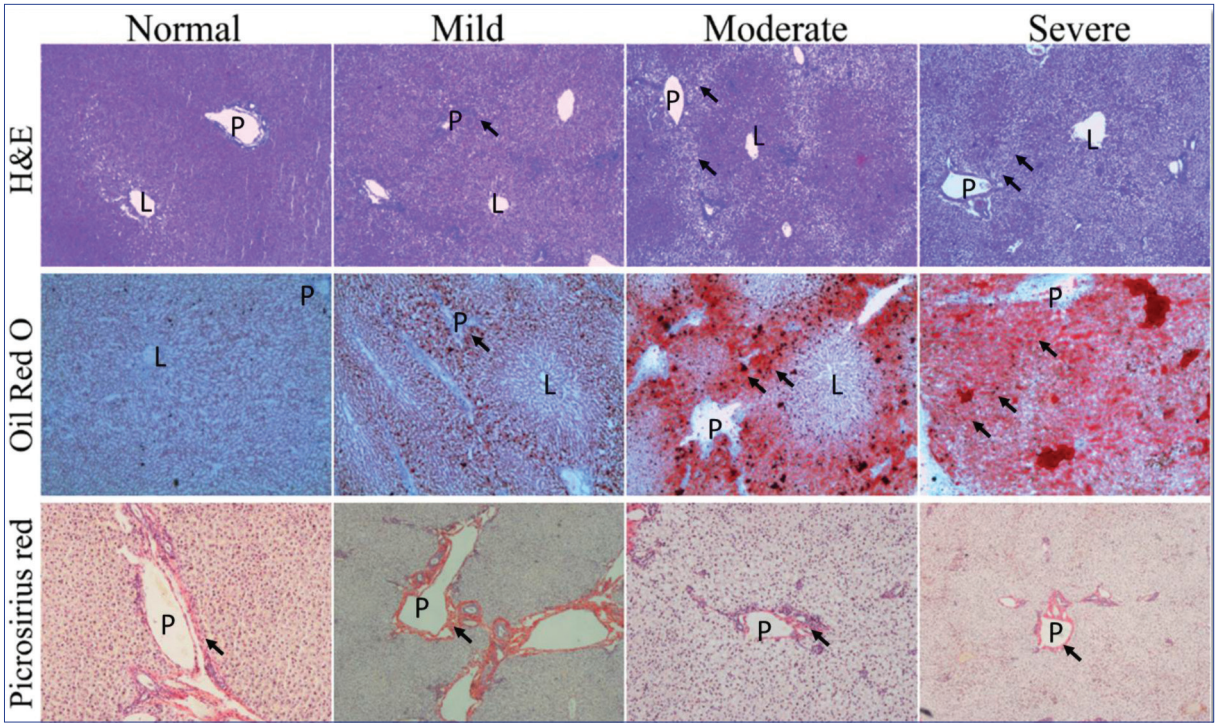

Fig 2. Representative liver pathological images of a healthy liver and those with mild, moderate, and severe HS staining by H & E, oil red O, and picrosirius red. P, hepatic portal area; L, hepatic lobules. H&E and oil red O staining (magnification 40×) show steatosis hepatocytes radially arranged around the portal area *(arrows)*. Picrosirius red staining (magnification 40×) shows only vessels stained in red *(arrows)*

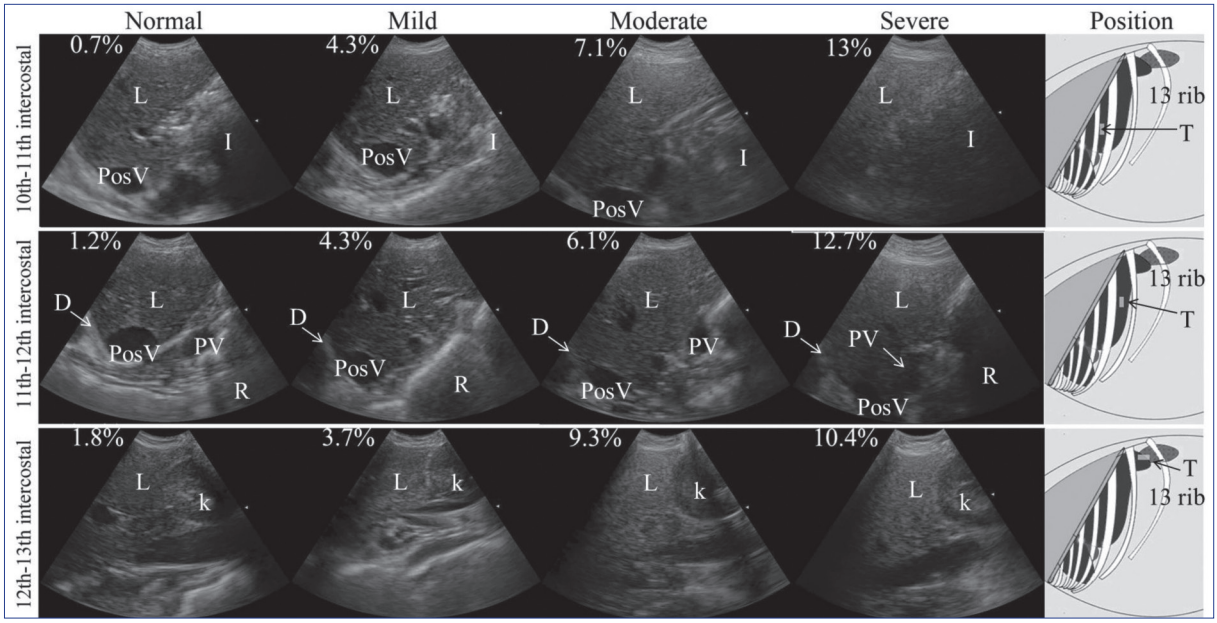

Fig 3. Schematic of image evaluation of fatty liver in sheep, as detected with a 3.5-MHz transducer. The corresponding triglyceride level (%) is shown for each image. L, liver; PosV, postcaval vein; I, intestines; D, diaphragm; PV, portal vein; R, rumen; K, kidney; T, detecting position of the transducer

Imaging Analysis

In this study, an image of a SMG sheep on postpartum day 4, revealed a liver fat concentration of 4.5%, which was eliminated because of unsatisfactory imaging details due to the thickness of the sebum layer, thus 69 images were included for analysis. In addition, images of L-K contrast could not be obtained with a 5-MHz transducer in this study. As shown in *Fig. 3* and *Fig. 4*, images of a healthy liver showed homogenous hepatic parenchyma, smooth vascular margins, clear hepatic margins, and legible outlines of the organs and tissues around the liver. With mild

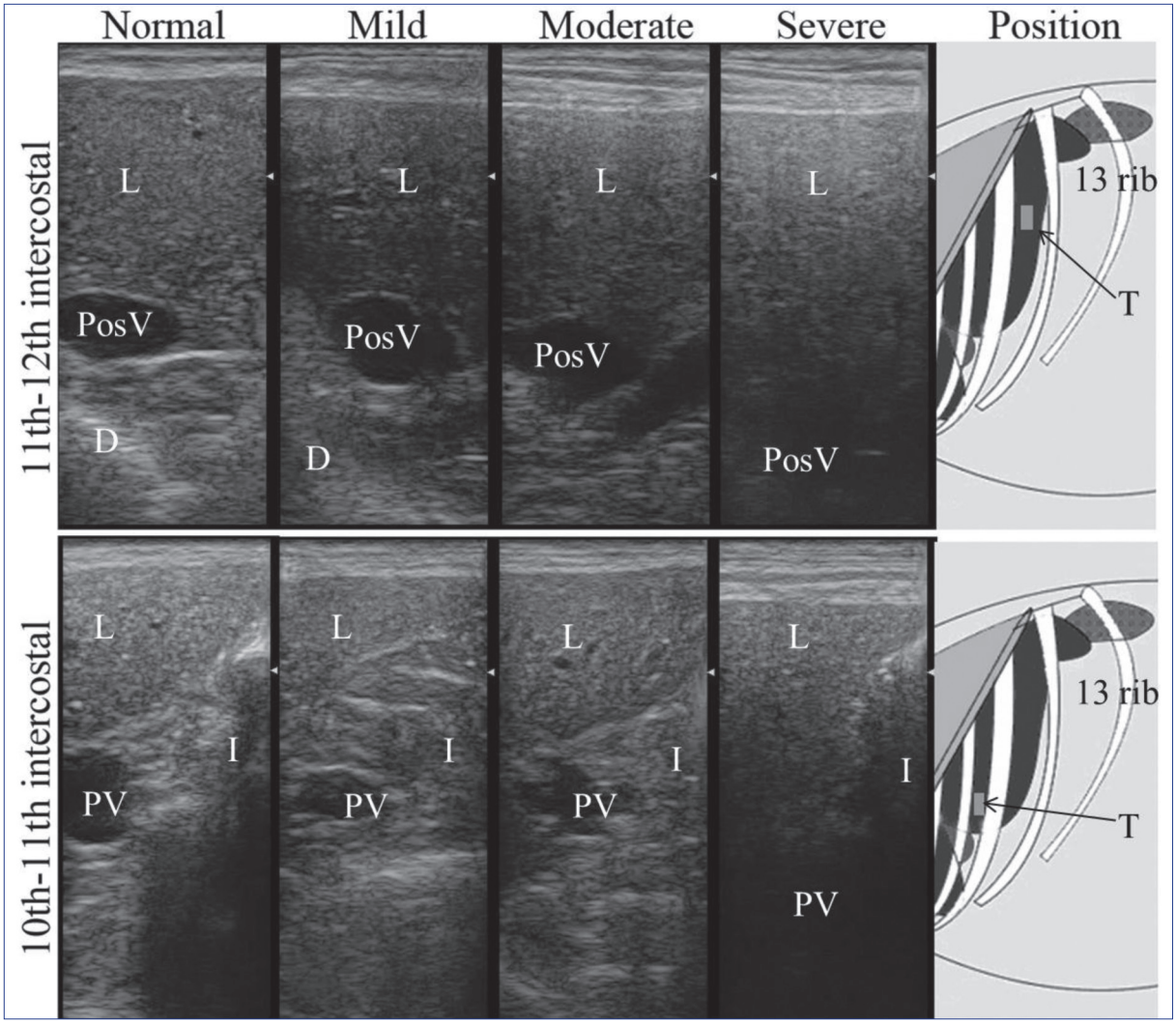

Fig 4. Schematic of image evaluation of fatty liver in sheep, as detected with a 5-MHz transducer. L, liver; PosV, postcaval vein; I, intestines; D, diaphragm; PV, portal vein; T, detecting position of the transducer

Table 1. *Sensitivity, specificity, and kappa coefficient values of observers for established US diagnosis standards*

Hepatic Steatosis Grade	3.5 MHz		5M Hz	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Healthy	69.7	94.3	66.7	93.8
Mild	60.5	92.9	60.0	93.4
Moderate	81.5	86.3	66.7	89.3
Severe	75.4	93.3	82.5	84.0
Interobservers agreement (κ)				
Radiologist 1 vs. 2	0.684		0.735	
Radiologist 1 vs. 3	0.575		0.702	
Radiologist 2 vs. 3	0.639		0.667	

HS, US showed a slight increase in L-K contrast and slight attenuation of echogenicity from the distal veins, vena cava, diaphragm, and rumen wall. Imaging of moderate HS was showed intermediate attenuation of the posterior beam, an increase in the echo ratio of the liver and kidney, decreased echo of the mid-field of the liver and intestines, significant attenuation of the echo of the diaphragm in the far end, loss of the vena cava margin, and obvious attenuation of the echo of the rumen. Images of severe HS showed a marked reduction in beam penetration, loss of echoes from most of the portal vein wall, while the ratio of the liver and kidney was marked and unclear to nearly non-visual of the mid-field of the liver and intestine.

Sensitivity, Specificity and Kappa Analysis

A total of 69 US images were evaluated by three observers according to the established diagnostic criteria. As show in *Table 1*, the sensitivity of 5-MHz diagnostic imaging of a healthy liver and those with mild, moderate, and severe HS were 66.7%, 60.0%, 66.7%, and 82.5%, respectively, with moderate κ statistics ranging from 0.667 to 0.735 among observers. The sensitivity of 3.5-MHz diagnostic imaging of a healthy liver and those with mild, moderate, and severe

HS were 69.7%, 60.5%, 81.5%, and 75.4%, respectively, with moderate κ statistics ranging from 0.575 to 0.684 among observers.

DISCUSSION

As a common liver disease in human and animals, fatty liver disease has been widely researched. Several animal models are widely used, such as high-fat diet or gene deletion mice, for studies of the mechanisms of fatty liver disease [16-18]. While macrofauna are considered ideal fatty liver models for US-based diagnosis research, in this study, a model of fatty liver using lactating thin-tail sheep was developed by restricted feeding. After 3 days of restricted feeding, mild to moderate HS was detected. Sheep with multiple births had greater milk yields and developed severe HS after day 7. While there were some limitations for NEB-induced fatty liver, severe NEB caused by insufficient energy intake failed to sustain the high energy demand of colostrum, especially in the early postnatal period. With the long period of restricted feeding management, the milk yield continued to decrease and body fat diminished, while the severity of HS lessened, indicating that hepatic injury caused by long-term HS was limited. In this study, pathological changes to the liver on post-partum day 16 were sufficient to develop a simple HS ovine model.

At present, liver US is widely used for diagnosis of HS in humans. According to widely accepted imaging parameters, US is deemed to be accurate for diagnosis only if the histological extent exceeds 33% [19]. A recent study reported that the pooled sensitivity of US was 84.8% for detection of HS with a histological extent of 20%-30% [20]. In this study, the imaging characteristics of a healthy liver and mild to severe HS were defined, which showed that the diagnostic standard of 3.5 MHz had poor sensitivity for mild HS. As indicated by the results of intra-observer evaluation, due to the severity of HS, part of the image with mild HS was considered to reflect healthy tissue. The image parameters of L-K contrast, far gain attenuation, and vessel blurring for mild HS were unremarkable, while visualization of mild HS at 5 MHz was better than at 3.5 MHz, but still not ideal. The sensitivities of US for moderate and severe HS were lower than previous reports [20], possibly because diseased tissue captured in the image was difficult to distinguish when HS was near 10%. With the established image diagnostic standard in this study, the sensitivity of 3.5 and 5 MHz for moderate to severe HS can reach 97.4% and 98.2%, respectively. Furthermore, with the established model, newer methods, such as image digitizing analysis [21-23], can be used for US-based diagnosis of fatty liver disease.

There were some limitations to US in this study. First, the images were graded according to liver triglyceride content, but some images showing accumulation of liver triglycerides near the train spacing point were usually difficult to diagnose. The images of severe HS were usually accompanied by a thick sebum layer because obese sheep showed a high degree of fat mobilization in response to restricted feeding that resulted in severe HS, which usually induce attenuation of the echo affecting image evaluation. In addition, different from fatty liver disease caused by a high-fat diet, triglycerides first accumulate around the hepatic lobules, while liver triglycerides of NEB sheep were first detected around the liver portal area [17,24]. Hence, further studies are needed to determine whether the area of triglyceride accumulation impacts the vessel blurring score.

In conclusion, the fatty liver model in lactating sheep induced by restricted feeding was an ideal model for ultrasound and radiology research. Visualization at 3.5 and 5 MHz was an ideal for diagnosis of moderate to severe HS with established US diagnostic standards.

Conflict of Interest

The authors declare that they have no competing interests.

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Research Article

Evaluation of Immunotherapeutic Effects of *Aloe vera* Polysaccharides Against Coccidiosis in Chicken

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Abstract

This study reports the immunotherapeutic effects of *Aloe (A.) vera* polysaccharides against coccidiosis in broiler chicken. For the purpose, polysaccharides were recovered from *A. vera* and analyzed by using HPLC. Three different hexose sugars including maltose, glucose and mannose were detected in hydrolyzed solution of *A. vera*. The extracted polysaccharides (graded doses) were evaluated for immunotherapeutic activities against coccidiosis in chicken. Results revealed that percent protection and daily weight gains were significantly higher (P<0.05) in chicken administered with *A. vera* polysaccharides as compared to control group. On the other hand, oocyst counts and lesion scores were lower (P<0.05) in polysaccharides administered chickens as compared to control. Moreover, anti-coccidial indices were also higher in chickens administered with polysaccharides (159.75-239.63) as compared to control (36.57). Except spleen, the organ-body weight ratios of all lymphoid organs of experimental and control groups were statistically similar (P>0.05). Based upon findings of this study, it was concluded that *A. vera* derived polysaccharides had immunotherapeutic activity against coccidiosis in chickens and might be further explored for its commercial feasibility for effective use in poultry industry to control avian coccidiosis.

Keywords: *Immunotherapeutic, Aloe vera , Polysaccharides, Coccidiosis, Chicken*

Tavuklarda Koksidiyoza Karşı *Aloe vera* Polisakkaritlerinin İmmunoterapötik Etkinliğinin Saptanması

Özet

Bu çalışma ile tavuklarda koksidiyoza karşı *Aloe (A.) vera* polisakkaritlerinin immunoterapötik etkisi rapor edilmektedir. Bu amaçla, *A. vera*'dan polisakkaritler elde edildi ve HPLC ile analizleri yapıldı. Maltoz, glikoz ve mannoz içeren üç farklı heksoz şekeri *A. vera*'nın hidrolize edilmiş solüsyonunda belirlendi. Ekstrakte edilen polisakkaritlerin (dereceli dozlarda) tavuklarda koksidiyoza karşı immunoterapötik etkisi araştırıldı. Sonuçlar; yüzde koruma ve günlük ağırlık kazanımının *A. vera* polisakkaritleri verilen tavuklarda kontrol grubuna oranla istatistiksel olarak daha yüksek olduğunu gösterdi (P<0.05). Oosit sayısı ve lezyon skoru ise polisakkarit verilen tavuklarda kontrol grubuna oranla istatistiksel olarak daha düşüktü (P<0.05). Anti koksidial belirtiler de polisakkarit verilen tavuklarda (159.75-239.63) kontrol grubuna oranla (36.57) daha yüksekti. Dalak dışındaki tüm lenfoid organların organ-vücut ağırlığı oranları deney ve kontrol grubundaki hayvanlarda benzerlik göstermekteydi (P>0.05). Çalışmanın bulgularına dayanılarak, *A. vera* polisakkaritlerinin tavuklarda koksidiyoza karşı immunoterapötik eksinin olduğu ve bu nedenle tavuklarda koksidiyozu kontrol altında tutabilmek amacıyla ticari kullanımının araştırılması gerektiği sonucuna varılmıştır.

Anahtar sözcükler: *İmmunoterapötik, Aloe vera, Polisakkaritler, Koksidiyozis, Tavuk*

INTRODUCTION

Coccidiosis is one of the most important protozoal infections of poultry industry, inflicting heavy economic losses in the form of high morbidity and mortality in affected flocks [1]. It is caused by different species of genus *Eimeria*, belonging to family *Eimeriidae*. Poor management such as damp litter, contaminated drinkers and feeders, high stock density and poor ventilation are the most important predisposing factors of this disease in intensive poultry production [2]. It has a negative impact on the production performance of affected birds in terms

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of retarded growth and poor feed conversion ratios in addition to high morbidity and mortality [3]. According to an estimate, it causes economic losses up to three billion US dollars annually worldwide [4,5].

In current era, the poultry industry largely relies upon the use of chemoprophylactic drugs and live vaccines to control coccidiosis [6,7]. At the same time, there is also increasing concern towards the use of alternative control measures due to some associated pitfalls of exiting control strategies including the emergence of drug resistant strains, high cost of vaccines and drug residues in meat and eggs [5,8]. At present, many alternative strategies are under investigation for effective, economical and environment friendly control of coccidiosis, including the use of medicinal plants [9,10]. In this context, *A. vera* has been reported for promising immunomodulatory effects in different animal models, highlighting it as a potential candidate for immunotherapy in different ailments [11]. *A. vera* is one of the most commonly used medicinal plants throughout the world with pronounced historical importance [12]. Its gel contains more than 75 active components including polysaccharides, minerals, phenolic compounds, proteins, sugars, vitamins, amino acids and saponins each with some pharmacological effects in different ailments [13,14]. Literature revealed that most of medicinal effects are due to polysaccharides present in inner leaf gel [15]. These polysaccharides had been extensively reported as a wound healing agent in different wound conditions by proliferation of fibroblasts and hydroxyproline and hyaluronic acid production in fibroblasts and thus extracellular remodeling in wound healing process [16]. It could inhibit inflammatory process by reduction of leukocytes adhesions and pro-inflammatory cytokines [17]. Its administration increases phagocytic and proliferative activity of reticuloendothelial system [18]. The *A. vera* polysaccharides are well documented for pharmacological activities in different animal disease models but only a few studies are available in the chicken model. Keeping in view, in continuation to our previous studies [12,19], this study aimed to investigate the immunotherapeutic efficacy of *A. vera* polysaccharides against coccidial infection in chickens.

MATERIAL and METHODS

Procurement and Processing of A. vera Leaves

Fresh leaves of *A. vera* were obtained from Botanical Garden, University of Agriculture, Faisalabad (UAF), Pakistan and their authenticity was confirmed from the concerned botanist of Department of Botany, UAF, Pakistan. The plant specimens were kept in the Ethnoveterinary Research and Development Centre, Department of Parasitology, UAF, Pakistan (Specimen Voucher No. 072). Fresh leaves after harvesting were subjected to surface sterilization by washing with chlorinated H_2O followed by formalin (0.005 ppm solution) and finally with distilled H_2O [19].

Separation of Leaf Gel

The mucilaginous leaf gel was separated from *A. vera* leaves within 3-4 h post collection to avoid aerodeterioration of gel contents. Briefly, the prewashed *A. vera* leaves were incised longitudinally with the help of a sharp sterilized knife followed by gentle scrapping of gel using a spatula. The gel was homogenized, filtered through cheese cloth and stored in screw capped jars at 4°C till further use.

Extraction and Hydrolysis of A. vera Polysaccharides

Polysaccharides were extracted from *A. vera* gel by following the methodology described by Chang et al.[20] with minor modifications. In brief, the *A. vera* gel was mixed with 95% ethanol (1:4) by vigorous shaking and incubated for 12 h at 4°C. The supernatant was discarded, and precipitate was subjected to centrifugation (6500×g for 10 min). The precipitate was mixed with dd-H_2O and incubated for 12 h and again precipitated with 95% ethanol (1:4). The procedure was repeated several times until all the colored material was removed. The final precipitate was mixed with dd-H_2O and treated with Sevag reagent [butanol:chloroform (1:4 v/v)]. The protein contents were removed by repeated oscillation and centrifugation procedures [21]. The deproteinated solution was mixed with 95% ethanol (1:3) to precipitate the polysaccharides. The precipitated polysaccharides were separated and subjected to further purification by washing 2-3 times with ethanol (absolute) followed by acetone and ethyl ether, respectively. Polysaccharides thus obtained were dried at 40°C for 24-48 h.

HPLC Analysis of Polysaccharides

The extracted polysaccharides were hydrolyzed to get the monomer units (monosaccharides) as described previously [22] with minor modifications. In brief, the polysaccharides were refluxed in trifloroacetic acid (2M; Sigma-Aldrich®, USA) at 100°C for 2 h in a round-bottom flask equipped with a reflux condenser. The TFA and water contents were removed by evaporation (75°C) and freeze drying (-65°C), respectively. The hydrolysed monosaccharides were analysed by using Shimadzu-10A HPLC workstation (Japan) equipped with a quaternary gradient pump unit and a refractive index detector (RID). The Rezex RCM-Monosaccharide Ca^{+2} column (Phenomenex, USA) was used to get absorption spectra at a wavelength of 235 nm at 80°C. Isocratic DD H_2O was used as mobile phase. Injection volume for each of monosaccharide standards and sample was taken as 20 µL.

The Infective Material

Sporulated oocysts of mixed *Eimeria (E.)* species including *E. tenella*, *E. acervulina*, *E. maxima* and *E. necatrix* maintained at Immunoparasitology Laboratory, UAF, Pakistan were used for this study to induce *Eimeria* infection in the birds. The infective dose was adjusted at the rate of 7×10^4

sporulated oocysts of per 2 mL of phosphate buffered saline (PBS).

Experimental Design

A total of 160 (one-day-old) broiler chicks were obtained from local market and reared under standard management conditions at Experimental Poultry Shed, Department of Parasitology, UAF. All the chicks were offered withdrawal feed and water *ad libitum* throughout the study and vaccinated according to the routine schedule against ND, IBD and HPS [5]. During the experimental trial, all procedures were performed in accordance to the guidelines of the Institutional Animal Care and Use Committee of UAF.

After 5 days of acclimatization, chicks were randomly divided into four equal groups A_1-A_4, each containing 40 chicks and were administered orally with graded doses of Aloe polysaccharides for three consecutive days i.e. 5^{th}-7^{th} days of age. Groups A_1, A_2 and A_3 were administered *A. vera* polysaccharides at the dose rates of 100, 200 and 300 $mg.kg^{-1}$ body weight, respectively while group A_4 was kept on PBS as a control.

Immunotherapeutic Evaluation

On day 14^{th} post-administration of *A. vera* polysaccharides, chickens of all the groups were challenged with infective dose of mixed species of genus *Eimeria* (local isolates; 7×10^4 sporulated oocysts per bird) with the help of an oral gavage [23]. In each group, chickens were monitored for oocysts per gram of faeces (OPG), daily weight gains, lesion scoring and mortality from day 3^{rd} to 12^{th} post challenge. For lesion scoring, dead and survived chickens in all the groups were killed humanely and scored for intensity of lesions as described by Johnson and Reid [24].

Further, the percent protection against lesions was calculated by using the formula described by Singh and Gill [25] as follows:

Per cent protection against lesions = (Average lesion score (IUG)-Average lesion score (IMG))/(Average lesion score (IUG)) × 100

Where,

IUG = Infected Untreated Group; IMG = Infected Medicated Group

Anti-coccidial Index (ACI)

Anti-coccidial index (ACI) was calculated to demonstrate the therapeutic efficacy of *A. vera* polysaccharides by following the formula described by Shah et al.[26] as follows:

Relative rate of weight gain was calculated by subtracting the body weight at the time of challenge from the body weight at the end of experiment. Survival rate was estimated by the number of survived chickens divided by the initial number of chickens. Lesion scores of the chickens from all groups were calculated by the method of Johnson and Reid [24] and oocyst value was calculated by using the formula described previously [27] as follows:

Development of Lymphoid Organs

The organ-body weight ratio of lymphoid organs including spleen, thymus, caecal tonsils and bursa of Fabricius were calculated on day 12^{th} post challenge with *Eimeria* species. Briefly, chickens of all the groups were weighed individually. Thereafter, birds were killed humanely and their lymphoid organs were incised out and weighed. The results were expressed as percent organ-body weight ratios as described earlier [5].

Statistical Analysis

Data thus collected were analyzed by using statistical analysis software (SAS® 2004) through one-way ANOVA and Duncan's Multiple Range (DMR) test. The differences were considered significant at $P<0.05$.

RESULTS

HPLC Profile of A. vera Polysaccharides

HPLC analysis of the hydrolysed solution of *A. vera* polysaccharides revealed the presence of three different monosaccharide units including mannose, glucose and maltose at peak retention times (min) of 12.55, 11.08 and 9.423, respectively. Molar concentrations (%) of detected monosaccharides are presented in *Table 1*.

Immunotherapeutic Evaluation of A. vera Polysaccharides

- Oocyst Counts and Daily Weight Gains Post-Challenge:

All the groups administered with graded doses of *A. vera* polysaccharides showed significantly lower ($P<0.05$) oocyst counts as compared to control from days 4^{th} to 12^{th} post challenge. Maximum OPG was recorded on day 9^{th} post infection in all groups. OPG count was lower in group A_2 and A_3 as compared to A_1 and control. However, the

Table1. *Quantitative analysis of monosaccharides detected in the hydrolyzed solution of A. vera polysaccharides*

Monosaccharides	Retention Time (min)	Area (mV.s)	Height (mV)	Quantity (molar %)
Maltose	9.423	11.837	0.54	0.04
Glucose	11.08	35.252	0.605	0.11
Mannose	12.55	36.885	0.612	0.02

difference between groups A_2 and A_3 was statistically non-significant (P>0.05; *Fig. 1)*. On the other hand, daily weight gains were significantly higher (P<0.05) in chickens administered with different doses of *A. vera* polysaccharides as compared to those of control group; although no graded dose response was detected *(Fig. 2)*.

- Percent Protection, Lesion Scoring and Percent Protection Against Lesions: The highest protection (70%) was observed in group A_2 administered with *A. vera* polysaccharides (200 mg.kg^{-1} BW) followed by group A_3 (60%) and group A_1 (55%) and control group (30%). Chickens of all the groups (both survived and dead chickens) were examined for lesion scoring on a scale from 0 to 4. Chickens of experimental groups administered with *A. vera* polysaccharides showed lesser lesions and thus higher percent protection against lesions as compared to those of control group. Among experimental groups, chickens of group A_2 showed the lowest score of severe lesions followed by A_1 and A_3 as compared to chickens in control group, which showed severe lesion scores *(Table 2)*.

- Estimation of Anti-coccidial Index: The group A_2 administered with *A. vera* polysaccharides at the rate of 200 mg.kg^{-1} showed the highest anti-coccidial index (239.63) followed by those of groups A_3 (195.31) and A_1 (159.75). A value of 36.57 was also recorded for control group that could be due to the self-limiting nature of the *Eimeria* infection in poultry *(Table 3)*.

Organ-body Weight Ratios in A. vera Polysaccharides Administered and Control Groups: All with mixed *Emeria* groups showed statistically similar organ-body weight ratios (P>0.05) except spleen, which showed significantly higher (P<0.05) spleen-body ratio in birds of control group as compared to those administered with *A. vera* polysaccharides *(Table 4)*.

Table 2. *Lesion scoring and percent protection against lesions*

Group	Lesion Scoring of Birds					Protection Against Lesions (%)
	0	1	2	3	4	
Caeca						
A_1	0	5	6	11	18	23.75ab
A_2	0	8	14	6	12	36.25a
A_3	0	0	8	16	16	20b
A_4	0	0	5	11	28	3.25c
Intestine						
A_1	0	11	6	8	15	33.125b
A_2	0	12	9	5	9	49.375a
A_3	0	16	7	5	12	41.875a
A_4	0	0	2	16	22	12.5c

Values sharing similar letters in a column are statistically non-significant (P>0.05); ***A_1*** *= A. vera polysaccharides at dose rate of 100 mg.kg^{-1} BW;* ***A_2*** *= A. vera polysaccharides at dose rate of 200 mg.kg^{-1} BW;* ***A_3*** *= A. vera polysaccharides at dose rate of 300 mg.kg^{-1} BW;* ***A_4*** *= Control group*

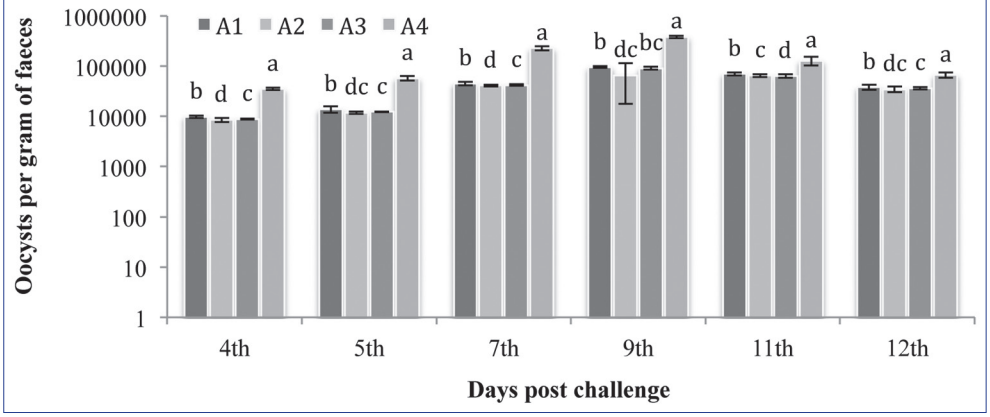

Fig 1. Oocysts per gram of faeces from day 4th to 12th post-challenge with *Eimeria* species. Bars sharing different letters on a particular day present a significant difference (P<0.05). A_1 = *A. vera* polysaccharides given at rate of 100 mg.kg^{-1} BW; A_2 = *A. vera* polysaccharides given at rate of 200 mg.kg^{-1} BW; A_3 = *A. vera* polysaccharides given at rate of 300 mg.kg^{-1} BW; A_4 = Control group

Fig 2. Daily weight gains of experimental and control chickens from day 3rd to 12th post challenge. Bars sharing different letters on a particular day present a significant difference (P<0.05). A_1 = *A. vera* polysaccharides given at rate of 100 mg.kg^{-1} BW; A_2 = *A. vera* polysaccharides given at rate of 200 mg.kg^{-1} BW; A_3 = *A. vera* polysaccharides given at rate of 300 mg.kg^{-1} BW; A_4 = Control group

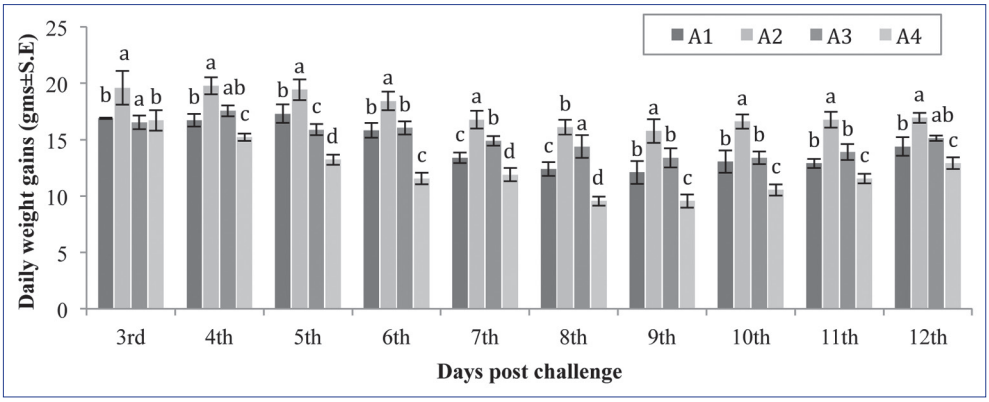

Table 3. *Anti-coccidial indices in experimental and control groups*

Groups	Relative Rate of Weight Gains	Survival Rate	Lesion Value	Oocyst Value	Anticoccidial Index
A_1	162.567	0.55	2.675	0.696	159.75
A_2	241.696	0.7	2.025	0.746	239.63
A_3	197.756	0.6	2.325	0.717	195.31
A_4	40.14	0.3	3.5	0	36.57

A_1 = A. vera polysaccharides given at rate of 100 mg.kg^{-1} BW; A_2 = A. vera polysaccharides given at rate of 200 mg.kg^{-1} BW; A_3 = A. vera polysaccharides given at rate of 300 mg.kg^{-1} BW; A_4 = Control group

Table 4. *Organ-body weight ratio post challenge in experimental and control chickens*

Group	Thymus (Mean ± SE)	Spleen (Mean ± SE)	Bursa (Mean ± SE)	Caecal Tonsils (Mean ± SE)
A_1	0.37±0.01	0.27±0.02^c	0.26±0.01	0.09±0.01
A_2	0.38±0.01	0.28±0.01^b	0.25±0.01	0.08±0.01
A_3	0.37±0.01	0.28±0.01^b	0.26±0.01	0.08±0.01
A_4	0.36±0.01	0.29±0.01^a	0.25±0.01	0.07±0.01

Means sharing similar letters in a column are statistically non-significant (P>0.05); A_1 = A. vera polysaccharides given at rate of 100 mg.kg^{-1} BW; A_2 = A. vera polysaccharides given at rate of 200 mg.kg^{-1} BW; A_3 = A. vera polysaccharides given at rate of 300 mg.kg^{-1} BW; A_4 = Control group

DISCUSSION

Coccidiosis is an important protozoal infection of poultry of high economic importance having a negative impact on the production performance and thus farm profitability [4,28,29]. Conventionally, disease is controlled through medication and vaccination strategies but each with certain limitations. As an alternative approach, modern trends are molding towards the use of native biomolecules from different medicinal plants for the treatment of various ailments in both animals and human beings [10]. In this regard, *A. vera* reported to have significant immunoregulatory and immunostimulatory activities, mainly antioxidant effects; stimulation of phagocytes and humoral immunity in different animal models [12,30,31]. Keeping in view, this study was conducted to evaluate the immunotherapeutic efficacy of *A. vera* polysaccharides against coccidiosis in chicken.

Polysaccharides isolated from *A. vera* were analyzed by using HPLC. Results indicated the presence of three different monosaccharides including maltose, glucose and mannose. Previously, several polysaccharides including acemannan, arabinoxylan, arabinorhamnogalactan, galactan, galactogalacturan, galactoglucoarabinomannan, gluco-galactomannan, glucomannan and glucuronic acid had been isolated from different parts of *A. vera* plant [15,32]. Further, presence of saccharides including aldopentose, glactose, glucose, L-rhamnose and mannose in *A. vera* polysaccharides were also reported [33,34]. Tan et al.[13] analyzed *A. vera* polysaccharides by HPLC and reported presence of mannose as a monomeric unit of isolated polysaccharides. In other studies, different polysaccharides of variable molecular sizes have been isolated from *A. vera* [35,36] and this variability might be associated with isolation methodology along with seasonal and cultivational variations [34].

For immunotherapeutic evaluation of *A. vera* polysaccharides, chickens of all the groups (experimental and control) were challenged with mixed *Eimeria* species in this study. Significantly lower oocyst counts in *A. vera* treated groups might be correlated to the development of resistance induced by *A. vera* polysaccharides against *Eimeria* species [12,37]. Yim et al.[38] also reported that *A. vera* extract can be used as a safe dietary supplement against coccidiosis. Some other studies had also reported the similar findings in broilers and rabbits [39-41]. Lesion score is the most common method for assessing intestinal condition during coccidiosis [42]. Chickens administered with polysaccharides, showed higher daily weight gains and lesser lesions on the caeca and intestine as compared to control. These lesser intestinal lesions might be due to the effects of *A. vera* on intestinal tract microflora, reduced bowel putrefaction that subsided/decreased inflammation [36] or lining of intestine layer with *Aloe* biomolecules [43]. Improved intestinal health in *A. vera* polysaccharides administered chickens might be responsible for better higher weight gains and thus better production performance [44].

Maximum protection (70%) in polysaccharides administered chickens might be correlated immunostimulatory activity of Aloe polysaccharides like acemannan which had been reported to reduce the opportunistic infections and stimulate wound healing [45]. Further, previous studies reported that carbohydrate polymers (glucomannans) present in *A. vera* played role in healing process [46] and inhibited cyclooxygenase pathway resulting in decreased prostaglandin production from arachidonic acids [47]. Vahedi et al.[48] reported that *A. vera* polysaccharides led to stimulate cellular and humoral immune responses by increased synthesis and release of T-lymphocytes and cytokines, which might be speculated to neutralize the pathogenic organisms like *Eimeria* species. Earlier, *A. vera* extracts administered at different dose rates had also revealed significantly elevated macrophages and white blood cell counts in mice [49]. Further, Cheesbrough [50] also reported *A. vera* polysaccharides to boost the activity of intestinal macrophages and T-lymphocytes up to 50 percent to prevent the penetration of pathogenic viruses,

bacteria and tumor cells. Results of present study also showed a similar response against coccidiosis in terms of higher survival percentage and reduced oocyst counts. Some previous studies on herbal biomolecules also reported the similar findings [5,10,28,45].

Anticoccidial index (ACI) reflects a comprehensive ability of any compound against coccidial infection. ACI values lower than 120 depict that compound/drug has no anti-coccidial activity; whereas values between 120 and 160 are considered partially effective but very effective at value > 160 [51]. In present study, *A. vera* polysaccharides administered at dose rates 200 and 300 mg.kg^{-1} of body weight showed ACI values higher than 160, so can be considered as very effective immunotherapeutic regimes against coccidiosis. *A. vera* polysaccharides did not show any significant effect on the development of different immune organs including thymus, caecal tonsils and bursa of Fabricius as compared to control. Only spleen-body weight ratios of chickens of control group showed significant difference from those administered with *A. vera* polysaccharides. Contrary to this, Darabighane et al.[45] reported a significantly higher relative weight of spleen in *A. vera* gel administered chickens; whereas, some previous similar studies reported a non-significant impact of herbal biomolecules on the development of lymphoid organs [5,12]. In this study, higher spleen-body weight ratio might be speculated due to cellular infiltration and spleen hypertrophy due to severity of coccidial infection in control group as compared to Aloe polysaccharide administered groups [52,53]. In conclusion, *A. vera* polysaccharids demonstrated the immunotherapeutic efficacy against coccidiosis in chickens and can be used successfully as a trustworthy alternative to anti-coccidial drugs, against which resistance has been emerged, to combat the avian coccidiosis.

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Research Article

Effects of Dietary L-glutamine Supplement on Performance, Egg Quality, Fertility and Some Blood Biochemical Parameters in Guinea Fowls *(Numida meleagris)*

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Abstract

The present study was conducted to investigate the effect of dietary supplementation of L-glutamine and synergistic effects between glutamic acid in wheat and glutamine on productive performance, egg quality characteristics, blood biochemical parameters and fertility traits of guinea fowls fed with corn-soybean meal-wheat based diets. 120 guinea fowls (*Numida meleagris*) were allocated to a completely randomized design with six treatments consisted of four replicates and 5 birds per replicate. Treatments were included: 1) corn-soybean meal based diet (control1), 2) corn-soybean meal-wheat based diet- (control2), 3) control1 containing 0.5% L-glutamine, 4) control1 containing 1% L-glutamine, 5) control2 containing 0.5% L-glutamine, 6) control2 containing 1% L-glutamine. Results showed that diet supplementing with L-glutamine significantly increased egg mass, egg production, egg weight, shell thickness, haugh unit, levels of follicle-stimulating hormone and luteinizing hormone, hatchability and one-day chick weight and also improved feed conversion ratio compared with control1 and 2 diets ($P<0.01$). The best response for fertility traits was achieved in birds fed with control2 containing 1% glutamine. It can be concluded that 1% glutamine has positive effects on performance, some egg quality traits and fertility parameters. Positive synergistic effect between wheat and glutamine on fertility traits can be valuable in guinea fowls.

Keywords: *Guinea fowl, Glutamine, Wheat, Performance, Egg traits, Fertility*

Gine Tavuklarında *(Numida meleagris)* Diyete L-glutamin İlavesinin Yumurta Kalitesi, Fertilite ve Bazı Kan Biyokimyasal Değerleri Üzerine Etkisi

Özet

Bu çalışma mısır-soya fasulyesi-buğday ile beslenen Gine tavuklarında diyete L-glutamin ilavesinin ve buğdaydaki glutamik asit ile glutamin arasındaki sinerjistik etkinin verim performansına, yumurta kalitesi parametrelerine, kan biyokimyasal değerlerine ve fertilite üzerine etkilerini araştırmak amacıyla yapılmıştır. Çalışmada 120 Gine tavuğu (*Numida meleagris*) tamamen rastgele dizaynda olmak üzere dört tekrar ve her tekrarda 5 tavuk olacak şekilde altı çalışma grubuna ayrıldı. Uygulamalar şu şekilde gerçekleştirildi: 1) mısır-soya fasulyesi temelli diyet (kontrol 1), 2) mısır-soya fasulyesi-buğday temelli diyet (kontrol 2), 3) %0.5 L-glutamin içeren kontrol 1, 4) %1 L-glutamin içeren kontrol 1, 5) %0.5 L-glutamin içeren kontrol 2, 6) %1 L-glutamin içeren kontrol 2. Elde edilen sonuçlar, L-glutamin ilave edilen diyet ile beslenen tavuklarda yumurta kütlesinde, yumurta üretiminde, yumurta ağırlığında, kabuk kalınlığında, Haugh biriminde, folikül stimüle edeci hormon ve luteinize edici hormon seviyelerinde, yumurtadan çıkma oranı ve bir günlük civciv ağırlıklarında anlamlı derecelerde artış olduğunu gösterdi ($P<0.01$). Ayrıca yem konversiyon oranı kontrol 1 ve 2 ile karşılaştırıldığında iyileşme göstermekteydi. Fertilite için en iyi değerler %1 glutamin içeren kontrol 2 ile beslenen tavuklarda gözlemlendi. %1 glutaminin performans, bazı yumurta kalitesi özelliklerine ve fertilite parametrelerine pozitif etkisi olduğu kanısına varıldı. Buğday ve glutamin arasındaki pozitif sinerjistik etki Gine tavuklarında fertilite bakımından değerli olabilir.

Anahtar sözcükler: *Gine tavuğu, Glutamin, Bığday, Performans, Yumurta özellikleri, Fertilite*

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INTRODUCTION

Guinea fowl are originating from Africa. Asia and Latin America raise semi-domesticated species while in Europe, North America and Australia breed large-scale production of dominated guinea fowl [1]. This topic well shows adaptability guinea fowls to any condition. Guinea fowls have been shown to have resistance against common diseases and also lower requirement to labor and management [2]. Guinea fowl meat has a higher protein content of approximately 28% compared to 20% for domestic fowl [2]. It has been reported that guinea fowl eggs, due to more thickness, have better storage time than chick eggs [3]. These birds have much advantage but those have problems. Guinea fowls only lay in warm season [4]. It has been shown that guinea fowl lays by 100 eggs during 9 months [3] and has low hatchability [4]. It seems feeding strategies can help the birds for improvement in fertility and performance traits.

Wheat is usually applied in some countries as the major energy source in poultry diets. The composition of wheat is commonly more variable than other cereals. Protein level can vary from 10-18%, depending on cultivars and growing conditions. Wheat is contained higher amounts of protein compared to corn, and it provides only slightly less energy. However, there are some potential problems from feeding much more than 30% in a diet. Wheat contains by 5-8% of pentosans, which can create problems such as viscosity which can subsequently lower digestibility [5]. Wheat protein is known to be low in some amino acids which are known as essential for the human diet, especially lysine and threonine, but they are rich in glutamic acid [6].

Glutamine, a semi-essential amino acid or in some condition essential, has mobilizable nitrogenous groups in its structure [7] and also modulates in intestine health of animals [8]. Glutamine can be synthesized form combination glutamic acid and ammoniac by glutamine synthetase, especially in muscle [7]. Glutamine and glutamic acid totally form by 14% of egg proteins [9]. It has been accepted glutamine role as energy source for intestine cells [10], increasing the mucin synthesis [11] and modulating in gene expression [12]. It has been shown glutamine modulation in digestive system can improve absorption and subsequently increase performance and other traits [9]. It has been shown glutamine role in cellular immunity [7] and fertility [13]. Glutamine improved hatchability by decrease in blood urea nitrogen and oxidation activity [13].

As mentioned guinea fowls have lower fertility and performance and on the other hand glutamine has positive role in improvement of performance, fertility and egg traits. In addition, wheat contains much amount glutamic acid which maybe subsequently converted to glutamine. Thus, we hypothesized replacement of part corn by wheat can help to increasing the glutamine and finally improvement in mentioned traits. Thus, this study was conducted to investigate the effect of dietary supplementation of L-glutamine on performance, egg quality characteristics, some blood biochemical parameters and fertility traits of guinea fowls fed with corn-soybean meal-wheat based diets.

MATERIAL and METHODS

Birds

The current study was conducted in East Azarbaijan Research Center for Agriculture and Natural Resources and all the used procedures were approved by standard committee of Research's Science University (Approval date: 04/02/2016; No: 10030). A total number of 150 Guinea fowl, 38 weeks of age with weight mean 1800±50 g, were selected for pre-trial period (two weeks). In this period, birds were fed with corn-soybean meal diets and finally 120 hens were selected. 120 guinea fowls *(Numida meleagris)* were allocated to a completely randomized design with six treatments consisted of four replicates and 5 birds per replicate (4 females and 1 male) for 40 days. Experimental conditions were similar for all birds including; light cycling 16 h light: 8 h dark, similar temperature and free access to feed and water. Birds were fed with corn-soybean meal-wheat based diets containing 0.5 and 1% L-glutamine. Experimental treatments were as follows; 1) corn-soybean meal based diets (control 1), 2) corn-soybean meal-wheat based diets (control 2), 3) control 1 containing 0.5% L-glutamine, 4) control 1 containing 1% L-glutamine, 5) control 2 containing 0.5% L-glutamine, 6) control 2 containing 1% L-glutamine. Glutamine was purchased from Wellife Korean Company. Diets were formulated according to the Guinea-Fowls [1]. The diets composition is presented in *Table 1*. The proximate analyses of diets were performed according to Association of official Analytical Chemists AOAC [14].

Analysis of Amino Acids

In the present study, corn, wheat and soybean meal ingredients are diets. Thus analyses of amino acid corn, wheat and soybean meal were important. Analysis of amino acids was performed using high performance liquid chromatography (HPLC) as explained by Moral et al.[15] and the data are presented in *Table 2*. The amino acid composition was reported as percentage of protein content (i.e., in g/100 g of protein).

Performance

The number of produced eggs and their weight mean were daily recorded. Feed intake (FI), egg production (EP), egg weight (EW) and egg mass (EM) were calculated each 10 days/once and feed conversion ratio (FCR) was also calculated. EM was calculated as was previously explained by Hou [16], as follows;

$$EM = EP\ (\%) \times EW\ mean$$

Table 1. *Ingredient and nutrient composition of experimental diets*

Ingredients (%)	Control1	Control2	Control1+0.5% Gln	Control1+1% Gln	Control2+0.5% Gln	Control2+1% Gln
Corn	59.35	39.1	59.35	59.35	39.1	39.1
Soybean meal	29.3	27	29.3	29.3	27	27
Wheat	-	22	-	-	22	22
Vegetable oil	2	2.5	2	2	2.5	2.5
Sand	1	1	0.5	-	0.5	-
L-glutamine	-	-	0.5	1	0.5	1
Oyster shell	5	5.1	5	5	5.1	5.1
DCP	2	1.95	2	2	1.95	1.95
Salt	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin premix[1]	0.25	0.25	0.25	0.25	0.25	0.25
Mineral Premix[2]	0.25	0.25	0.25	0.25	0.25	0.25
Met+ Cys	0.7	0.7	0.7	0.7	0.7	0.7
Analysis						
Dry matter (%)	91	90.8	91	91	90.8	90.8
ME (kcal/kg)	2839	2808	2839	2839	2808	2808
Crude protein (%)	18	18	18	18	18	18
Ca (%)	2.5	2.5	2.5	2.5	2.5	2.5
Available P (%)	0.5	0.5	0.5	0.5	0.5	0.5
Met+ Cys (%)	0.8	0.8	0.8	0.8	0.8	0.8
Ether extract (%)	2.80	2.40	2.80	2.80	2.40	2.40
Crude fiber (%)	4.00	4.30	4.00	4.00	4.30	4.30
Ash (%)	6.50	6.80	6.50	6.50	6.80	6.80

DCP: *Di-calcium phosphate;* ***ME:*** *metabolizable energy;* ***CP:*** *crude protein; Available* ***P:*** *Available phosphorous;* ***Lys:*** *Lysine.* [a] *Vitamin premix provided the following per kilogram of supplement: vitamin A, 9.000 IU; vitamin D_3, 2.000 IU; vitamin E, 1.800 IU; nicotinic acid, 30 mg; vitamin B_{12}, 0.015 mg; vitamin K_3, 4 mg; biotin, 0.15 mg; ; folic acid, 1.0 mg; niacin, 30.0 mg; panthotenic acid, 25.0 mg; pyridoxine, 2.9 mg; ; riboflavin, 6.6 mg; thiamin, 1.18 mg.* [b] *Mineral premix supplied the following per kilogram of supplement: manganese oxide, 100 mg; $FeSO_4$. $7H_2O$, 50 mg; zinc oxide, 100 mg; copper, 10 mg; I, 1.0 mg; Se, 0.2 mg*

Table 2. *Amino acid composition (g/100 g of protein) for used wheat, corn and soybean meal*

Ingredients	Ala	Arg	Asp	Cys	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val
Wheat	3.1	5.1	5.6	3.5	32	4.4	2.8	3.4	5.3	3.3	1.7	4.5	4.3	5.3	6.1	4.1	4.1
Corn	3.5	7.7	12.5	1.5	11.5	5.1	4.2	5.1	5.1	9.2	2.9	8.1	2.4	5.5	5.1	4.2	3.5
Soybean meal	3.9	7.1	11.4	1.6	17.1	4.1	2.6	4.7	7.8	6.5	1.3	5.5	4.5	5	4.4	4	5.1

Egg Quality Traits

Three eggs, close to mean, from each replicate were selected per 10 days/once and then egg weights, albumen percentage, yolk percentage and shell percentage were measured by a digital scale. Shell thickness was measured using micrometers (OSK 13469- Japan). Yolk color was also measured by color indexes (Iran), with numbers from 1 to 15. Haugh unit was calculated as was previously explained by Hou [17], as follows;

$$\text{Haugh unit} = 100\log [\text{albumen height} + 7.57 - 1.7\,(\text{EW})^{0.37}]$$

Blood Biochemical and Hematological Parameters

Three birds from per replicate were selected (each 10 days/once) and two blood samples were taken form wing vein (2 mL /bird). One blood sample was considered for measurement of glucose, triglycerides and cholesterol by using specified-kits (Bionik-Iran). Other part of same blood samples was used for assessment of total protein and albumin using specified-kits (Pars Azmoon-Iran). Blood biochemical parameters were analyzed by mentioned kits and auto analyzer (Hitachi 911-Japan). The levels of thyroid-stimulating hormone (TSH), triiodothyronine (T3), tetraiodothyronine (T4), luteinizing hormone (LH), follicle-stimulating hormone (FSH) were measured using kits (Liaison-Italy). Other sample was transferred to EDTA-containing tubes. Blood smear was prepared and white blood cells were evaluated with Gisma staining and light microscope as described by Thrall et al.[18].

Fertility Parameters

In the end of trial, a number of collected eggs during experiment (600 eggs) were firstly candled and suitable eggs were transferred to hatchery machine. Incubation period was lasted 27 days. Hatchability percentage and one-day chick weight was calculated

Statistical Analyses

The all data of bird's were subjected to statistical analysis (SAS) [19] using analysis of variance (ANOVA) appropriatefor a completely randomized design. When significant effects were detected by ANOVA, treatment means were compared using Duncan's multiple range test. Differences were considered significant at P<0.01. All of parameters were examined as follows:

$Yij = \mu + Ti + eij$

Where Yij is the individual observation, μ is the overall mean, Ti is the effect of treatment, and eij shows the random error.

RESULTS

Table 3 shows the effect of experimental diets on productive performance. Replacement of corn with wheat had not significant effect on productive performance (P>0.05; control 1 *vs* control 2). FI was significantly reduced in birds fed with control 1 containing 0.5% glutamine compared with other birds (*P*<0.05). The highest FCR and lowest EM, EP and EW were seen in birds receiving control 1 and 2 diets than those fed with diets containing glutamine (P<0.05); showing positive effects of glutamine on productive performance. In the corn-soybean meal based diets, EM, EP and EW increase and FCR decreases by increasing glutamine levels (P<0.05), while better response for FCR was achieved at level of 0.5% glutamine in corn-soybean meal-wheat based diets compared with level of 1% (P<0.05). Comparing the level of 0.5% in control 1 and 2 has showed a better response for level of 0.5% in diets containing wheat; showing that lower levels of glutamine have better interaction with wheat. However, diet supplementing with wheat had not negative effects on productive performance compared with corn-soybean meal diet (P>0.05).

Egg quality characteristics are presented in *Table 4*. Albumen percentage, shell percentage and albumen: yolk ratio were not influenced by nutritional modulations (P>0.05). Comparing control 1 and 2 did not show significant differences for egg quality traits (except yolk color); showing that adding wheat to diet had not positive or negative effects on egg quality traits. Yolk color was significantly higher in birds fed with control 2 compared with those fed with control 1 (P<0.05). Dietary inclusion

Table 3. *Effect of experimental diets on productive performance*

Treatments	FI (g)	EM (g/hen/d)	FCR (g/g)	EW (g)	EP(%)
Control 1	89.10^{a}	15.24^{c}	5.85^{a}	36.50^{b}	41.75c
Control 1 + 0.5% Gln	81.01^{b}	17.60^{b}	4.65^{b}	39.09^{a}	45.02^{b}
Control 1 + 1% Gln	89.60^{a}	22.03^{a}	4.10^{c}	39.09^{a}	56.35^{a}
Control	86.77^{a}	14.65^{c}	5.95^{a}	36.21^{b}	40.45^{c}
Control 2 + 0.5% Gln	88.02^{a}	21.45^{a}	4.15^{c}	39.04^{a}	54.94^{a}
Control 2 + 1% Gln	86.70^{a}	19.42^{ab}	4.50^{b}	39.22^{a}	49.51^{b}
P-value	0.006	0.004	0.002	0.004	0.005
SEM	1.44	1.29	0.45	0.63	1.12

SEM: *standard error of means. Footnotes (a-c) show significant differences each column (P<0.01)*

Table 4. *Effect of experimental diets on egg quality traits*

Treatments	Albumen (%)	Yolk (%)	Shell (%)	Albumen/Yolk	Shell Thickness (mm)	Haugh Unit	Yolk Index	Yolk Color
Control 1	54.47	30.79^{b}	14.74	1.77	0.47^{c}	85.97^{c}	37.74^{b}	11.62^{c}
Control 1 + 0.5% Gln	53.75	31.42^{ab}	14.83	1.73	0.51^{a}	87.59^{bc}	38.46^{ab}	12.31^{a}
Control 1 + 1% Gln	54.00	31.70^{ab}	14.30	1.70	0.50^{ab}	92.31^{a}	39.42^{ab}	12.31^{a}
Control 2	54.12	31.03^{b}	14.85	1.66	0.47^{c}	87.14^{bc}	39.07^{ab}	11.75^{b}
Control 2 + 0.5% Gln	54.37	31.37^{ab}	14.26	1.74	0.49^{b}	89.21^{b}	40.53^{ab}	12.06^{ab}
Control 2 + 1% Gln	53.80	32.00^{a}	14.20	1.71	0.50^{ab}	91.15^{a}	41.85^{a}	12.00^{ab}
P-value	0.61	0.003	0.55	0.56	0.0005	0.0001	0.004	0.0001
SEM	0.78	0.42	0.19	0.04	0.004	0.84	0.56	0.08

SEM: *standard error of means. Footnotes (a-c) show significant differences each column (P<0.01)*

of glutamine significantly increased haugh unit and shell thickness (P<0.05).

Effects of wheat and glutamine addition to diet on blood biochemical parameters are presented in *Table 5*. The serum concentrations of triglycerides, albumin and T3 were not influenced by dietary modulation (P>0.05). Comparing the control 1 and 2 did not indicate significant differences between both groups for glucose and total protein (P>0.05). The serum levels of cholesterol, FSH, LH and T4 were significantly increased in control 2 (P<0.05); showing that wheat addition to diet increased cholesterol, FSH, LH and T4 (P<0.05). Addition of glutamine to diet caused conflict results, so that the serum concentration of glucose was reduced by increasing glutamine levels, while the serum concentration of glucose was increased in level of 1% compared to 0.5%. The serum concentration of protein was significantly increased in control 2 diet containing 1% glutamine. Dietary inclusion of glutamine increased FSH and LH (P<0.05). Comparing the level of 0.5% in control diets showed better positive in control 2 compared with control 1; showing positive interaction between wheat and glutamine.

There were not significant differences between control 1 and 2 for hatchability (P>0.05), but 1% glutamine in both control groups, significantly increased hatchability (P<0.05). The weight of 1 day old chicks was increased significantly in control 2 compared with control 1 *(Table 6*; P<0.05). Also glutamine showed synergistic interaction effect with wheat for 1-d chick weight; so that 1-d chick weight was significantly higher in diets containing wheat and 1% glutamine compared to corn-soybean meal based diets containing glutamine.

Hematological parameters of guinea fowls are presented in *Table 7*. As it has been shown in *Table 7*, hematocrit percentage, basophile percentage and level of hemoglobin were not influenced by dietary treatments (P>0.05). The addition of wheat to diet was decreased lymphocyte count (control 2 vs control 1; P<0.05). The heterophil: lymphocyte ratio is reduced in control 2 compared with control 1 (P<0.05) and this is a suitable index for confortable in birds nourished with control 2. Dietary inclusion of wheat also increased white blood cells count (control 2 vs control 1; P<0.05); showing improvement in immunity by wheat. Birds receiving the glutamine showed the increased heterophil: lymphocyte ratio and heterophil count and also the reduced lymphocyte count compared with controls (P<0.05). Glutamine linearly increased white blood cells in corn-soybean meal based diets (P<0.05), while there were conflicting results for diets containing wheat, so that higher levels (1%) did not show significant differences with control 2 (P>0.05). However, glutamine increased white blood cells in corn-soybean meal based diets (P<0.05).

DISCUSSION

In this study, diet supplementing with wheat had not negative effects on productive performance compared with corn-soybean meal diet. According to our findings, Shakeri et al.[20] did not observe significant differences in growth performance of broiler chicks supplemented with corn based diets than those fed with wheat based diets. As *Table 2* shows, wheat is containing higher levels of glutamic acid which would be laterconverted to glutamine in the body, by glutamine synthetase, and finally it can improve productive performance. Thus, the presence of glutamic acid in wheat can be the major reason for indifference

Table 6. *Effect of experimental diets on fertility traits*

Treatments	Hatchability (%)	1-d Chick Weight (g)
Control 1	49.00^{d}	24.99^{c}
Control 1 + 0.5% Gln	52.00^{bc}	25.02^{c}
Control 1 + 1% Gln	71.00^{a}	25.85^{b}
Control 2	51.00^{c}	25.03^{c}
Control 2 + 0.5% Gln	54.00^{b}	25.83^{b}
Control 2 + 1% Gln	70.00^{a}	26.20^{a}
P-value	0.001	0.0001
SEM	0.86	0.08

SEM: *standard error of means. Footnotes (a-c) show significant differences each column (P<0.01)*

Table 5. *Effect of experimental diets on blood biochemical parameters*

Treatments	Glucose (mg/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	Albumin (g/dL)	Total protein (g/dL)	TSH (IU/L)	T3 (ng/dL)	T4 (µg/dL)	FSH (IU/L)	LH (IU/mL)
Control1	187.93^{a}	257.25^{b}	359.69	4.21	5.21^{b}	1.00^{a}	101.89	1.85^{d}	0.88^{d}	0.61^{d}
Control 1 + 0.5% Gln	168.31^{bc}	254.55^{b}	361.06	4.40	5.52^{ab}	0.74^{b}	102.95	2.02^{cd}	1.17^{b}	1.00^{b}
Control 1 + 1% Gln	174.62^{b}	280.25^{a}	371.88	4.05	5.28^{b}	0.61^{bc}	105.23	2.31^{b}	1.39^{ab}	1.13^{ab}
Control 2	182.50^{ab}	296.25^{a}	357.94	4.15	5.28^{b}	1.01^{a}	104.31	2.40^{b}	1.03^{c}	0.78^{c}
Control 2 + 0.5% Gln	162.75^{c}	294.75^{a}	411.69	4.47	5.52^{ab}	0.94^{a}	107.07	2.87^{a}	1.56^{a}	1.30^{a}
Control 2 + 1% Gln	188.00^{a}	281.68^{a}	389.56	4.21	5.78^{a}	0.58^{c}	102.04	2.09^{c}	1.31^{ab}	1.19^{ab}
P-value	0.007	0.001	0.13	0.20	0.006	0.0001	0.81	0.0001	0.0001	0.0001
SEM	4.91	6.88	0.485	0.12	0.09	0.04	3.01	0.06	0.003	0.04

SEM: *standard error of means. Footnotes (a-d) show significant differences each column (P<0.01)*

Table 7. *Effect of experimental diets on blood hematological parameters*

Treatments	Hematocrit (%)	Hemoglobin (g/dL)	Heterophil (%)	Lymphocyte (%)	Heterophil/ Lymphocyte	Monocyte (%)	Eosinophil (%)	Basophile (%)	White Blood cells$X10^3$ cells/µL
Control1	42.16	14.19	61.88^{d}	32.38^{a}	1.95^{d}	3.56^{a}	1.78^{bc}	0.40	203.73^{d}
Control 1 + 0.5% Gln	40.99	14.21	68.18^{b}	25.88^{cd}	2.70^{a}	3.60^{a}	1.92^{b}	0.42	212.75^{c}
Control 1 + 1% Gln	42.85	14.23	70.11^{a}	25.08^{d}	2.85^{a}	2.91^{b}	1.50^{c}	0.40	225.23^{a}
Control2	40.74	13.83	63.96^{d}	29.12^{b}	2.25^{c}	4.04^{a}	2.45^{a}	0.46	210.19^{c}
Control 2 + 0.5% Gln	41.74	13.88	68.97^{ab}	25.01^{d}	2.80^{a}	3.52^{a}	2.10^{a}	0.40	216.90^{b}
Control 2 + 1% Gln	40.11	13.26	66.55^{c}	27.46^{c}	2.48^{b}	3.54^{a}	2.03^{ab}	0.42	209.28^{c}
P-value	0.36	0.25	0.001	0.0001	0.0001	0.002	0.008	0.50	0.0001
SEM	0.93	0.31	0.73	0.70	0.09	0.18	0.12	0.02	1.24

SEM: *standard error of means. Footnotes (a-d) show significant differences each column (P<0.01)*

between control 1 and control 2. The role of anti-nutrient substances in wheat is well accepted, although their effects are well demonstrated in higher levels (more than 30%) [5]. Thus, lower levels (20% wheat) might be also a reason for indifference between control 1 and 2.

Glutamine improved productive performance, except FI, in guinea fowls. Similar to our observations, the other studies showed that diet supplementing with glutamine increased growth performance of birds [21,22]. Positive effects of glutamine on performance may be explained by several reasons. Firstly, glutamine improved performance by growth and development of digestive system [21]. An increase in gene expression and secreted enzymes activity [12] and increase in mucin synthesis [11] by glutamine can be another reasons for improvement of productive performance of guinea fowls. It is essential to mention that sexuality significantly affects FI and male birds usually consume more FI because of higher requirement [23]. Sexual ratio in all replicates was similar and it could not affect FI. The beneficial effects of glutamine on intestine microflorabeen reported by Francis and Griffiths [7] which can indirectly improve productive performance. A study has been shown the positive correlation between levels of FSH and LH and EP, EW and finally productive performance in laying hens [24]. They believed that these hormones improve productive performance by growth of oviduct and other productive system. On the basis of Ying et al.[24] findings, it can be strongly claimed that FSH and LH are responsible for improvement in productive performance, since their levels were significantly increased in groups containing glutamine *(Table 5)*. It can be concluded that glutamine improves productive performance by mentioned mechanisms but the expected synergistic effect between wheat and glutamine on performance was not seen.

In relation to egg quality, yolk color difference in wheat group compared with corn is not known. Oxycarotenoids are known as responsible for the pigmentation of egg yolk. Corn, wheat and barley are major feed components which can create significant variations in egg yolk pigmentation [25]. Corn and its by-products are known as the most important oxycarotenoid source for layers. A study showed that corn containing feed caused to a fan value of 10, while wheat and barley showed yolk color with a fan value of 4. Differences were explained by oxycarotenoid contents in the raw materials [25]. It seems to be more oxycarotenoid contents in the used wheat cultivar which is causedto increase the yolk color in comparison with corn. Rafuse et al.[26] did not observe significant differences among treatments for albumen height and eggshell in laying hens consuming wheat-based diets. Studies have been tried to increase egg quality and improve in wheat-based diets by adding enzymes. Mirzaie et al.[27] reported that dietary inclusion of xylanase had not any significant effect on egg quality traits. Çiftci et al.[28] reported that substitution 30% corn by wheat did not affect eggshell thickness of laying hens from 27 to 43 wk of age. Dietary inclusion of glutamine increased haugh unit and shell thickness. In contrast to our observations, Ying et al.[24] showed that glutamine addition to diet had not significant effect on haugh unit and shell thickness in laying hens. This difference between our findings and others may be explained by bird type, bird age, hygiene level, etc. Exact mechanism of glutamine in improvement of shell thickness and haugh unit would be needed more investigations.

Results showing that wheat addition to diet increased cholesterol, FSH, LH and T4. Smits et al.[29] showed that the increase in non-starch polysaccharide content, enough in wheat, lowered cholesterol absorption and plasma cholesterol concentration in broiler chicks. Other study also showed that triglyceride and total cholesterol concentrations were numerically decreased in wheat-based diet, although they were not significantly different [30]. It is believed that NSP can bind to bile salts, lipids and cholesterol which finally reduce cholesterol concentration [30]. It was expected to reduction the serum concentration of cholesterol by wheat, due to viscosity substances, but such result was not found and it may be explained because of using low levels of wheat in diet. Similarly Luo et al.[31] showed that wheat-based diets did not have significant effects on level of T4. They suggested more investigations for the relationship between wheat and hormone levels, because

of complexity. Mirzaie et al.[32] did not found significant effects of xylanase on thyroid hormones in wheat-based diets. The serum concentration of glucose was increased in level of 1% compared with 0.5%. This result is confusing and the mechanism of action is not known. Iwashita et al.[33] recommended glucose during exercise and post-exercise, due to lowering the glucose level. Another study has been shown glutamine as improving the serum concentration of cholesterol, albumin and total protein [34]. Several studies have indicated that glutamine supplementation could stimulate protein synthesis [35,36]. Studies have been shown that glutamine supplementing increased fat-free mass in athletes [37]. Dietary inclusion of glutamine increased FSH and LH. Ying et al.[24] showed that dietary inclusion of glutamine (0.4-0.8%) increases the level of FSH and LH in laying hens. They showed that glutamine improves gonadal hormone levels in animal body for a better genital system growth. Human studies have been shown to increase testosterone hormone after supplementing the glutamine [37]. It has been reported that glutamate biosynthesis from glutamine by binding to N-methyl- D-aspartate receptors was an event contributing to the pubertal activation of luteinizing hormone-releasing hormone [38] and pulsatile gonadotropin-releasing hormone secretion [39]. The relation between glutamine and T4 is not known.

An increase in 1-d chick weight can be explained by synergistic interaction effects between glutamic acid in wheat and glutamine supplement. Our claim was confirmed by Oliaei et al.[40] who indicated that *in ovo* administration of glutamine increased 1-d chick weight by 3.6%. Glutamine increased the hatchability in guinea fowls. Suchner et al.[13] suggested that glutamine improved hatchability by decreasing urea nitrogen and oxidation activity in blood. However, improved fertility traits in guinea fowls are valuable and this study suggests glutamine asa factor for improving the fertility traits in guinea fowls.

Heterophils are phagocytizing cells and their counts will increase against bacterial, microbial and chemical infections. Lymphocytes are the most leucocytes in normal condition and heterophil: lymphocyte ratio is an important index for evaluating stress conditions. Dietary inclusion of wheat increased white blood cells count (control 2). Also El-Katcha et al.[41] reported that dietary inclusion of wheat to diet increased humoral immunity in broiler chickens. The role of wheat for improving the immune system is not known, but it may be related to wheat components. Wheat is containing high amount of glutamic acid which can then converts to glutamine. The role of glutamine for improving the immune system function would be discussed; in this study Glutamine increased white blood cells. In agreement with our findings some researchers has been reported the glutamine role for improving the immune system under normal and stress conditions [20,22]. Glutamine is used at high levels in immune cells such as, lymphocytes, macrophages and neutrophils [42]; suggesting that glutamine involves in immune system. Also its role has been accepted in other immune cells, i.e. T-cell proliferation, B-lymphocyte differentiation, macrophage phagocytosis, antigen presentation and cytokine production [43] and also in anti-oxidant system against ischemia-reperfusion injury in rats [44].

The present study was conducted to investigate the synergistic effects between glutamine supplementation and wheat on performance, egg traits, blood and fertility parameters in guinea fowls, since wheat contains high amount of glutamic acid which can be subsequently converted to glutamine for the first time. The results showed that diet supplementing with L-glutamine significantly increased egg mass, egg weight, shell thickness, haugh unit, levels of follicle-stimulating hormone and luteinizing hormone, hatchability percentage and one-day chick weight and also lowered feed conversion ratio compared with control 1 and 2 diets. The synergistic effect between wheat and glutamine was observed in 1-d chick weight. Guinea fowls have low fertility so that the combination of glutamine and wheat can help to improve their fertility. Therefore glutamine can be suggested as an effective factor for improving the performance, some blood and hematological parameters and also fertility parameters in birds.

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Research Article

Histoanatomical Studies on the Fibrous Tunic of Eye in Dromedary Camel [1]

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Abstract

This study was conducted to understand the macroscopic and microscopic structures of the fibrous tunic of 20 healthy adult dromedary camel eyes. First, the fibrous tunic of the eye was anatomically examined in terms of appearance, dimensions, location and structure. Then prepared histological slides were studied microscopically after staining by H&E, Verhoeff, Trichrome masson's and P.A.S. The result showed that in the oval-shaped camel cornea, the internal angle is rounder than the external one. The average cornea length (lateral-medial), width (dorsal-ventral), and thickness were respectively measured as 2.91±0.05, 2.07±0.04, and 0.12±0.05 cm. The thickest part of sclera is in the posterior pole of the eyeball. There was not any Bowman's membrane in camels. Descemet's membrane includes a rather even, pink, thick and shapeless membrane. Descemet's membrane is characterized with a very high positive P.A.S. reaction. Caudal endothelium of cornea did not show positive P.A.S. reaction and is observed in the form of a thin band attached to descemet's membrane. In the structure of sclera, the collagen fibers are remarkably thick, and the longitudinal and transverse cross sections are observed in the form of alternating layers. There were small elastic fibers in camel sclera. In anatomy, except for some differences, the cornea and sclera of the camel are similar to those of the other ungulates. Except for a little differences in Bowman's layer and descemet's membrane, it is similar to the fibrous tunic of the other animals.

Keywords: *Dromedary camel, Eye, Fibrous tunic, Histoanatomy*

Tek Hörgüçlü Devede Gözün Fibröz Tabakasında Histoanatomik Çalışmalar

Özet

Bu çalışma tek hörgüçlü 20 adet sağlıklı erişkin devede gözün fibröz tabakasının makroskobik ve mikroskobik yapılarını araştırmak amacıyla yapılmıştır. İlk olarak gözün fibröz tabakası anatomik olarak görünüş, boyut, yerleşim ve yapı bakımından incelendi. Sonrasında, hazırlanan histolojik kesitler H&E, Verhoeff, Masson'un üçlü boyası ve PAS ile boyanarak mikroskopik olarak araştırıldı. Oval şekilli deve korneasında iç açının dış açıdan daha yuvarlak olduğu gözlemlendi. Ortalama kornea uzunluğu (lateral-medial), genişliği (dorsal-ventral) ve kalınlığı sırasıyla 2.91±0.05, 2.07±0.04 ve 0.12±0.05 cm olarak ölçüldü. Skleranın en kalın yeri göz yuvarlağının posterior kutbuydu. Develerde Bowman membranı bulunmamaktaydı. Descement membranı daha düz, pembe, kalın ve şekilsiz membrandan oluşmaktaydı. Descement membranı çok fazla PAS pozitif reaksiyon ile karakterize idi. Korneanın kaudal endoteliumu PAS pozitif reaksiyon göstermezken, descement membrana bağlı ince bir bant şeklinde gözlemlendi. Skleranın yapısında kollajen fibriller oldukça kalın olup longitudinal ve transversal kesitlerde yer değiştiren formlarda gözlemlendi. Deve sklerasında küçük elastik fibriller bulunmaktaydı. Anatomik olarak bazı farklar dışında devenin kornea ve sklerası diğer toynaklı hayvanlara benzerlik göstermekteydi. Bowman katı ve descement membranındaki küçük farklar dışında devenin fibröz tabakası diğer hayvanlarınkine benzerlik göstermekteydi.

Anahtar sözcükler: *Tek hörgüçlü deve, Göz, Fibröz tabaka, Histoanatomi*

INTRODUCTION

Eye is one of the important sensory organs of the body which plays a very important role in communication between the living creatures and their environment [1,2]. Because of its importance and critical nature, a lot of researchers have focused their studies on this issue [3,4]. Anatomically finding, the eye is made up of three important layers, namely nervous, vascular and fibrous layers [2,5,6]. Eye is among the sensitive parts of the body that, its anterior

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epithelium exposed to different types of threats, and it is examined in most illnesses for any probable changes in appearance [7,8]. Before studying the unnatural states of the eye, definitely we need to study its natural state and the different parts of the eye.

The eye of a one-humped camel looks like the eye of ruminants, especially cow. Most of the studies on the camel's anatomy between 1940 and 1970 have been conducted in some European and Arabian countries as well as India. A few studies have also been conducted on the anatomy of the camel's eye between 1980's and 1990's [9,10]. Some of the researchers have also studied the characteristics of the microscopic power of the camel's eye. For example, they have explicated that the adjacent parts of the camel's cornea is colored, the eyelid outgrowths are long, the eyelid have fissure, and the pupil of a camel's eye is oval in shape. Some researchers have studied the sensory receptors in the capsule of skeletal muscles in the camel's eye and have concluded that there are receptors in the muscles of a camel's eye, mostly located in the adjacency of the origin of the muscle; the elevator of upper eyelid has the most receptors in the sensory capsule [10,11]. In some animals, tapetum lucidum exists in the posterior wall of the eye hole. This layer is fibrous in some animals like equines and ruminants, but in some other animals like carnivores it is cellular; it also has the ability to reflect light; its color also varies across different animals [12,13]. Therefore, the present study is conducted with the purpose of understanding the macroscopic and microscopic structure of fibrous tunic of eye in one-humped camel.

MATERIAL and METHODS

In the present study, a total number of 20 eyes (right or left) [14] from 10 adult one-humped camels; 3-5 years old, 500 kg, prepared from Tabriz and Kerman slaughterhouse, were studied. First, the parts were anatomically studied for their appearance, dimensions, location and structure. Moreover, the dimensions were measured using calipers. Then for identifying their microscopic structure, fibrous tunic parts of the samples were cut and fixed in 10% formalin for at least 48 h. It is necessary to add that for complete fixation of the inner parts of the eye, 10% Formalin was injected into the eyeball with a syringe. The tissue samples were prepared, and their slides were produced using the routine methods of histology. The histological slides were stained by a general H&E staining (for the general study of the tissue), three types of Verhoeff special staining (for studying the elastic fibers), Trichrome masson's (for studying collagen fibers and muscles) and P.A.S. (for studying the existence of carbohydrates and tissue glycogen). They were later studied with a light microscope [15-17].

RESULTS

Anatomic Results

Cornea in a camel's eye is oval, but its medial angle is completely round, and its lateral angle is thinner and sharper *(Fig. 1a)*. In its normal state, cornea looks transparent and bright. The average cornea length (lateral-medial), width (dorsal-ventral), and thickness were respectively measured as 2.91±0.05, 2.07±0.04, and 0.12±0.05 cm. Moreover, the average cornea projection from the eyeball surface was 0.84±0.02 cm, and the average width of the brown layer or the width of black limbus layer was obtained as 0.4±0.0 cm. The eyeball that is seen in the form of a globe is made up of three layers, and it has vertical (dorsal-ventral), longitudinal (medial-lateral) and axial (anterior-posterior) diameters. Sclera is the thickest and most external layer of the eyeball and is basically white. Choroid and retina which are very fragrant, are packed together and located inside the sclera. The boundary between sclera and cornea is called limbus which has a black ring in its external surface; it is rather black in the parts adjacent to the cornea, but in the sclera area the blackness decreases *(Fig. 1a)*. In camels, the thickest part of the sclera is along the eye axis, and it is thinner in limbus areas *(Fig. 1b)*. In the limbus area, there is a projection of almost 0.2 cm towards the iris. The average dorsal-ventral, medial-lateral, and anterior-posterior thickness of the eyeball was respectively measured as 4.5±0.05, 4.52±0.05, and 3.32±0.03 cm. The thickness of the eye's three layers was measured as 0.1 cm.

Histology Results

The anterior epithelium of the cornea consists of non-

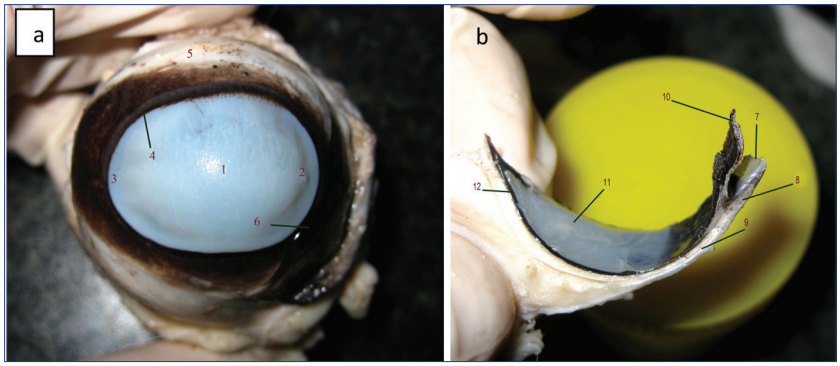

Fig 1. Gross structures of right eye of the camel (a) and anatomical section of the camel eye (b)

1- cornea, 2- medial angle, 3- lateral angle, 4- limbus, 5- sclera, 6- third eyelid, 7- cornea, 8- limbus, 9- sclera, 10- iris, 11- retina, 12- choroid

keratinised stratified squamous epithelium in which 2 or 3 layers of the surface cells are squamous in shape, and the lower layers include cells with round nucleus which are condensed to each other *(Fig. 2a)*. In the stroma of the cornea, the nucleus of the fibrocytesis located in a thin form between the layers containing collagen fibers parallel to the corneal surface *(Fig. 2b)*. Using the rows of the nucleus of the fibrocytes, the layers of the collagen fibers can be counted; in the present study 12 to 14 collagen layers were observed. The collagen fibers were observed in longitudinal and transversecross sections. Descemet's membrane (posterior lamina) includes a nearly continuous thick shapeless layer with a dark pink color the thickness of which is 15 to 20 µm *(Fig. 2b)*. The cornea's posterior epithelium includes a single layer of flattened cells that is separate from the posterior lamina. The intercellular borders of this epithelial layer are not clear. The nearest epithelium layer to the surface contains a thin positive P.A.S. reaction *(Fig. 2c)*. Inside the cytoplasm of the epithelial cells under the nucleus, the crescent shaped parts of the cytoplasm shows positive P.A.S. reaction. These areas show remarkable increase in the depth of the epithelium, but in the basal layer (the first cell layer from the epithelium) the cytoplasm does not have the areas reacting to P.A.S. The basement membrane is observed with a considerable thickness under the anterior epithelium of cornea in the form of positive P.A.S. red band *(Fig. 2c)*. In the corneal stroma, there are few areas reacting to P.A.S. coloring and are characterized by thin bands on collagen fibers. Descemet's membrane has the characteristic of high positive P.A.S. reaction and is observable in dark red *(Fig. 2b)*. The posterior epithelium of the cornea does not have the characteristic of positive P.A.S. reaction and is observed in the form of a thin band attached to descemet's membrane (light brown). In this coloring, the posterior endothelium is observed more distinctly. In Verhoeff coloring, the elastic fibers were observed among the collagen fibers of stroma *(Fig. 2d)*. In the posterior part of the eyeball (from inside to outside), the pigmented epithelium (containing melanin particles) forms the nearest layer to the surface. The thickness of epithelium increases in the anterior part of the eye ball, and in this part a non-pigment cell layer is added. The non-pigment epithelium is in the shape of simple columnar at the beginning, but later it turns into simple cubic form. This two-layered epithelium continues with more folds in the anterior part. Under epithelium, the collagen fibers are located parallel to the epithelium, so in some parts of this thickness the blood veins are scattered in fewer numbers (Choroid layer). After that there is the structure of sclera in which collagen is considerably thick, and the longitudinal and transversal cross sections are observed in the form of alternating layers *(Fig. 3a)*. Outside the sclera, connective tissue is loosed outwards and is covered with stratified cuboidal epithelium containing mucosal cells (conjunctiva) *(Fig. 3a)*. The conjunctiva epithelium has more mucosal cells in the posterior part, and the anterior part is observed in stratified columnar or transitional form. The connective tissue under the choroid, especially a striped area just below the pigment epithelium, shows greater P.A.S. state. In sclera, the collagen fibers have less positive P.A.S. reaction *(Fig. 3b)* while greater P.A.S. reaction is observed between the eyeball's skeletal muscles and the connective tissue of the conjunctiva; the conjunctiva epithelium also has positive P.A.S. reaction.

There are fewer elastic fibers in the eyeball, and they are observed in fewer numbers only in the depth of

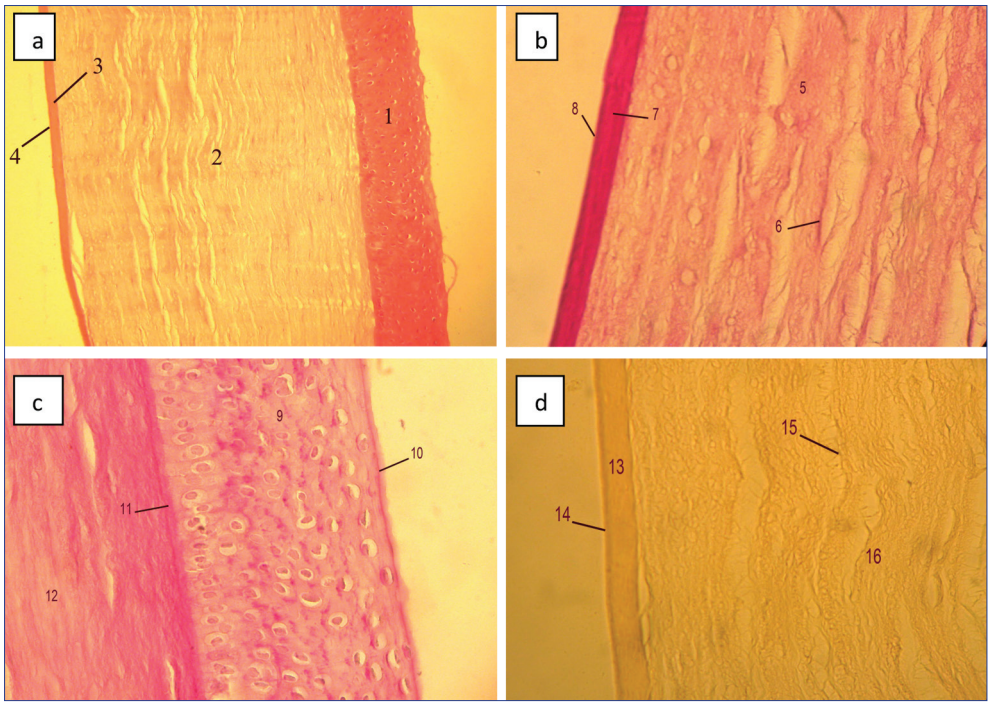

Fig 2. Photomicrograph of histological section of cornea in camel eye (H&E, ×100; a), (H&E, ×400; b), (P.A.S., ×400; c) and (Verhoeff , ×400; d)

1- Anterior epithelium, 2- stroma, 3- descemet's membrane, 4- posterior epithelium, 5-stroma, 6- fibrocyte, 7- descemet's membrane, 8- posterior epithelium, 9- anterior epithelium, 10- squamous cells, 11- basement membrane, 12- stroma, 13-descemet's membrane, 14- posterior epithelium, 15- elastic fibers, 16- stroma

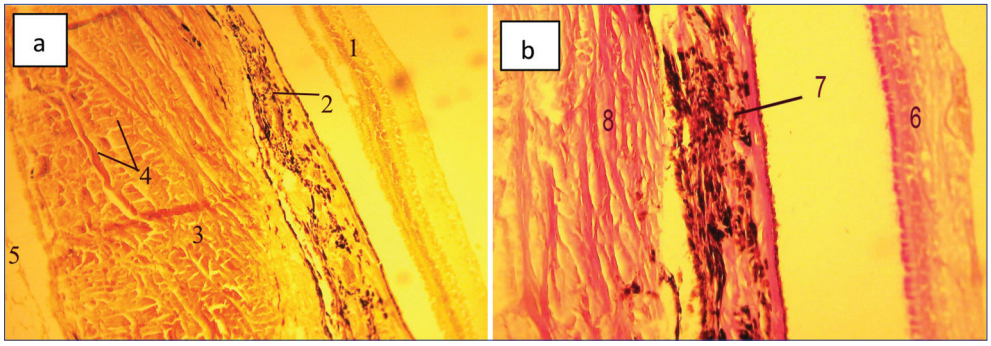

Fig 3. Photomicrograph of histological section of the eye in camel (Trichromemasson's, ×400; a), (P.A.S., ×400; b)

1- retina, 2- choroid, 3- sclera, 4- collagen fibers, 5- loose connective tissue, 6-retina, 7- choroid, 8- sclera

conjunctiva in the form of very thin fibers parallel to the collagen fibers. Choroid is located inside the sclera and contains pigment cells and blood vessels. The collagen fibers were observed in sclera and choroid, but they were not observed in retina *(Fig. 3a)*. Moreover, the camel's sclera has less elastic fibers which are observed in the form of very thin fibers and are different from the collagen fibers.

DISCUSSION

Anatomic Discussion

According to the research results, the present study, conducted on the eye of camel, revealed that the cornea in camel's eye is oval in shape; the only difference is the fact that its medial angle is completely round, and its lateral angle is thinner and rather sharp. In equines cornea makes up one fifth of the eye's fibrous tunic, and it is transparent, colorless and without any blood vessels [17]. Moreover, in horse the cornea is oval, and its medial angle is flatter than its lateral angle [8]. The egg-like shape of cornea has also been reported in cow [2,5]. The average transverse diameter (dorsal, ventral) of cornea in camel's eye is 2.07±0.04 cm while in cow it has been reported to be 2.5 cm [5,18]. In carnivores the thickness of the middle part of cornea has been reported to be more than the side parts (In the middle, the thickness is 0.1 cm, and in the sides it is 0.07 cm) [17]. As for other animals, cornea projection from the surface of eye ball is observed in camel too [8]. Corneal thickness**,** lens thickness and scleroretinal rim thickness increase with the advance of age in both buffaloes and camels [14,19].

The results show that the eyeballs of a camel are compressed in the anteroposterior axis. In other words, the length of the dorsoventral diameter of the eyeball is more than that of the anterioposterior diameter [15]. The same state has been reported in equine and large ruminant while in the carnivores the eye ball is completely global in shape [17]. In equine, four fifth of the eye ball is made up of sclera [17]. In cats and dogs, sclera is less thick along the eye axis, and is thicker in limbus areas or at the equator of the globe while in ungulates the thickest part of sclera is in the region of the optic nerve [17]. In dogs the average dorsal-ventral and medial-lateral diameter have been reported as 2.1 and 2.0 cm respectively [12,17]. Even some studies have reported that the anterior-posterior diameter of the eyeball in carnivorous is more than the vertical diameter [17]. Generally, the eyeball of the birds is bigger than the other animals, though the birds have smaller bodies [8,17]. This was true of the adaptation to underground seen in some rodents which involved the thickness of the cornea, sclera and choroids [20].

Histologic Discussion

The main body of sclera in other animals looks like the dermis of the skin that includes a great amount of irregular dens connective tissue [1,16,17]. Scleral tissue was composed of compacted bundless of collagen fibers. These fibers tended to be rounder in outer regions of sheep eyes compared to inner regions [22]. In most of the animals the outer layer of sclera or episclera also consists of loss connective tissues that are very vascular and contain neural fibers [15]. The camel has the longest dorsal and ventral shelf followed by donkey, buffalo and cow. On the other hand, the medial and lateral shelves are longest in donkey and smallest in camel [23]. In most of the animals, at the point where the optic nerve passes through the sclera it becomes sieve like and is known as the scleral lamina cribrosa [8]. Abnormal tension in this region due to glaucoma results in the disruption of axoplasmic flow in individual nerve fibers of the optic nerve [17]. Such a cribrosal sheet is also observed in camels. Only the blood vessels feeding the retina are located in limbus area, and the retina, by itself, has no vessels [8,24]. Besides dens connective tissue, the sclera can be composed of cartilage as in birds [17]. When cartilage is found in the sclera, it usually forms a complete cup that extends to a ring of boney plates or scleral ossicles [17]. Although birds and reptiles posses this structure, the ossicle is believed to have originated from fish and were eventually passed on to amphibian [17]. Birds such as the kingfisher and other diving birds have larger and potentially more powerful ossicles than those species that tend to be more confined to land [17]. Owl and hawks have used them to produce elongated and cone-shaped eyes that have resulted in remarkable differences in the radii of curvatures between the cornea and globe [15,17]. In a functional sense ossicles are believed to have been devised fore retaining ocular rigidity [8,17]. The bones have developed for strengthening

the eyeball, but this type of bones was not observed in the camel. In the other animals, the cornea epithelium has been reported to be of nonkeratinized stratified squamous type, containing 4-12 cell layers [8,15]. The epithelial tissue of cornea in dogs, cats, and birds consists of one layer of basal cells with two or three layers of polyhedral (wing) or coin cells and 2 or 3 layers of nonkeratinized squamous cells [21]. The bigger animals have more layers of wing and squamous layers [21]. The anterior epithelium of cornea in carnivores is 25-40 nanometers, and in big domestic animals it is 2 to 4 times thicker [1,8]. The cornea epithelium is thicker in the edges in comparison to the central parts, but in limbus area, it suddenly becomes thin [17]. In primates, the Bowman's membrane or the anterior limiting lamina does not have any cells, and it is even with a thickness of 10-16 nanometers in human eye [16]. It contains collagen fibers dispersed in different dimensions, and has an oblique path in the adjacency of lower segment [25]. The Bowman's membrane is not formed in most animals, occurring in avian and human cornea as well as some cetaceans and the giraffe [17]. Though it is considered part of the corneal stroma, it is formed by the anterior epithelium and is 10 to 15 nanometers thick, acellular, and composed of small highly organized collagen fibers [17,25].

In camels, the epithelium constituted 36% of the camel cornea whereas corneal stroma constituted 62% of the corneal thickness [25,26]. In most of the animals, the corneal stroma comprises 90% of the thickness of the cornea [8]. It is ligamentous-like, consisting of transparent lamellae of dense regular connective tissue [15]. In some animals, at the fine structural level, the descemet's membrane consists three regions: an anterior unbanded zone, an anterior banded zone, and a posterior unbanded zone has been reported [15,26]. Descemet's membrane, is a homogeneous acellular membrane that is 10 to 15 µm thick in the dog and up to 30 µm thick in the horse [17]. In most of the animals, this membrane is eosinophilic when stained with H&E and with PAS it stains brightly [15]. This layer is a true basement membrane that is formed by endothelium membrane [8]. In the other animals, the corneal endothelium contains a layer with cuboidal or polygonal cells and has covered the posterior surface of the cornea [17]. In some animals the existence of hexagonal cells of this layer has been reported [15].

In anatomy, except for some differences, the cornea and sclera of the camel are similar to those of the other ungulates. Except for a little differences in Bowman's layer and descemet's membrane, it is similar to the fibrous tunic of the other animals.

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Research Article

The Effect of Ticagrelor on Ischemia-Reperfusion Injury of Kidney: Is the Pleiotropic Effect a Valid Factor?

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Abstract

The goal of this study was to determine the protective effect of ticagrelor against ischemia-reperfusion injury in the kidney of rats via histological examination, biochemical parameters, and immunohistochemical analysis. Thirty male rats were randomized into five groups. The animals received ticagrelor 5 mg/kg, 10 mg/kg and 20 mg/kg or normal saline 0.1 mL/kg (control) orally before the procedure. The shame group only had laparatomy. An ischemia-reperfusion injury was done by clamping the renal hilus. There was less malondealdehyde assay (MDA) in ticagrelor groups than the control group, and this decrease was statistically significant (P=0.001 in all ticagrelor received groups). The glutathione peroxidase (GPx) and glutathione reductase (GR) were elevated in ticagrelor groups at all doses. Similar findings were observed in all treatment groups. The 5 mg ticagrelor treated group had no changes in glomerules and tubules relative to the control group via histology. Dilated tubular structures were similar to the sham group-both at 10 and 20 mg ticagrelor. Caspas-3 and NF-KB activity was similar in ticagrelor treated groups to sham group. Our study showed that ticagrelor is an effective agent to protect kidney from renal ischemia-reperfusion injury. Ticagrelor may protect the tissues by reducing the concentration of reactive oxygen species and inducing the antioxidant system-especially with the pleiotropic effect.

Keywords: *Kidney, Ischemia, Reperfusion, Injury, Ticagrelor*

Böbrekte İskemi-Reperfüzyon Hasarında Ticagrelor'un Etkisi: Pleiotropik Etki Geçerli Bir Faktör mü?

Özet

Bu çalışmada, ticagrelor'un ratların böbreğinde oluşturulan iskemi-reperfüzyon hasarına karşı koruyucu etkisinin histolojik, biyokimyasal ve immünohistokimyasal yöntemler kullanılarak tanımlanması amaçlanmıştır. Bu amaçla, 30 erkek rat rastgele olarak 5 gruba ayırılmıştır. Bu hayvanlara, yapılacak işlemlerden önce ticagrelor 5 mg/kg, 10 mg/kg ve 20 mg/kg dozlarında, kontrol grubuna da 0.1 mL/kg serum fizyolojik oral olarak verilmiştir. Sham grubunda ise sadece laparatomi yapılmıştır. İskemi-reperfüzyon hasarı renal hilusun klemplenmesi yoluyla yapılmıştır. Ticagrelor verilen gruplarda saptanan malondialdehit (MDA) düzeyinin kontrol grubundan daha az olduğu ve bu düşüklüğün istatistiksel olarak anlamlı olduğu tespit edildi (tüm ticagrelor grupları için P=0.001). Glutatyonperoksidaz (GPx) ve glutatyonredüktaz (GR) düzeyleride ticagrelor verilen tüm gruplarda artmış olarak bulundu. Histopatolojik incelemelerde, tedavi verilen tüm gruplarda benzer sonuçlar gözlendi. Ancak 5 mg ticagrelor verilen grupta glomerul ve tübül yapısında kontrol grubu arasında fark olmadığı belirlendi. 10 ve 20 mg ticagrelor verilen gruplarda, sham grubuna benzer özellikte dilate tübüler yapılar olduğu gözlendi. Caspas-3 ve NF-KB aktiviteleri ticagrelor verilen gruplarda sham grubuna benzer özelliklerdeydi. Çalışmamız, ticagrelorun böbreğin iskemi-reperfüzyon hasarına karşı korunmasında etkili bir ajan olduğunu göstermiştir. Ticagrelor, pleiotropik etkisiyle reaktif oksijen türlerinin konsantrasyonunu azaltarak ve antioksidan sistemi aktive ederek dokuları koruyabilir.

Anahtar sözcükler: *Böbrek, İskemi, Reperfüzyon, Hasar, Ticagrelor*

INTRODUCTION

Partial nephrectomy is a complex procedure performed in the practice of urologic oncology. The most important part of this procedure is cross-clamping of the renal vessels. This maneuver results in ischemia, while declamping after completing the procedure causes reperfusion. The rapid release of oxygen free radicals and inflammatory mediators-especially polymorphonuclear leukocytes-induce both systemic and local damage with reperfusion [1,2]. These

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products may lead to complications due to systemic inflammatory response such as multiorgan failure and even death [3]. The lungs, heart, and kidneys are distant organs that are especially damaged after ischemia and reperfusion.

Ticagrelor is one of the second group of non-thienopyridine derivatives such as elinogrel and cangrelor. It is a direct-acting antagonist of $P2Y_{12}$, which is a purinergic receptor for adenosine diphosphate (ADP) expressed by platelets. $P2Y_{12}$ plays an important role in thrombosis and homeostasis and defects in $P2Y_{12}$ to result in bleeding due to its role in ADP-induced platelet aggregation [4-6]. Due to these effects, ticagrelor is widely used to prevent stroke and acute coronary syndrome [7].

There is a significant debate that adenosine-related complexes also have extra-platelet effects, i.e., pleiotropic effects. The best explanation is that unlike platelets, $P2Y_{12}$ receptors are found in many cells including leukocytes, vascular smooth muscle cells, macrophages, microglial, specific subregions of the brain, and dendritic cells. This increases the number of potential effects of ticagrelor [8-12]. Furthermore, the "pleiotropic effects" of ticagrelor may also occur due to different mechanisms other than interaction with the $P2Y_{12}$ receptor [13].

ADP plasma levels increase after injury, inflammation, and ischemia-reperfusion [14]. Ticagrelor inhibits uptake of adenosine into the cell, and subsequent to this, elevated levels of endogenous adenosine decrease levels of inflammatory markers [15,16].

To the best of our knowledge, there is no previous study in the literature about the protective effects of ticagrelor on renal injury. The aim of our study was to determine the protective effect of ticagrelor against ischemia-reperfusion injury in the kidney of rats via histological examination and biochemical parameters.

MATERIAL and METHODS

Our study was approved by the Animal Experiments Local Ethics Committee at Kafkas University (KAÜ-HADYEK 2015-072). Thirty, male Spraque-Dawley rats weighing 350-400 g were randomly divided into five groups. The animals were initially anesthetized with intraperitoneal ketamine HCl 20 mg/kg (Ketalar, Parke-Davis, Detroit, USA), then propofol 80 mg/kg induction, and 3 mg/kg/min propofol infusion was done, and administered ticagrelor (BRILINTA, Astra Zeneca, Södertalje, Sweden) at doses of 5 mg/kg, 10 mg/kg, and 20 mg/kg or 0.1 mL/kg normal saline orally via gastric gavage before the procedure. All groups had laparotomy: a ischemia-reperfusion injury was induced by clamping the renal hilus through a midline incision on the approach to the left kidney. Then, atraumatic vascular clamps were applied to the renal vessels for 2 h. The clamps were removed afterwards for 4 h of reperfusion. At the end of these procedures, the animals were sacrificed with lethal injection of thiopental (Pentothal Sodium, Fako, Turkey). After sacrifice, the left kidneys of the animals were extracted and washed with 0.9% saline solution before both histopathological and biochemical analysis (malondealdehyde assay (MDA), glutathione reductase (GR) and glutathione peroxidase assays (GPx)).

Biochemical Analysis

Liquid nitrogen was used to freeze kidney tissues, and the tissues were stored at -80°C. Tissue sections were prepared and dried under vacuum overnight at 20°C. Freeze-dried sections were stored at -20°C until biochemical analysis. MDA, GPx, and GR levels were performed with enzyme-linked immunosorbant assays. The levels of these oxidant and antioxidant enzymes in renal tissues were measured using a Biotin double antibody sandwich technology (Bioassay Technology Laboratory, Shanghai, China). The MDA concentrations were expressed as nmol/mL, and the concentrations of GPx and GR were expressed as ng/mL.

Histological Analysis

Histopathological and immunohistochemical examination of kidney tissue samples were completed after being fixed in 10% tamponed formaline for 72 h. Sections were prepared after fixation with bright field microscopic techniques, and a microtome (Leica RM2125RT) was used to obtain 5-micron-thick kidney section. The sections were stained with PAS and Masson triple coating.

The scores for the kidney were measured semi-quantitatively. Parameters including histopathological changes such as tubular dilatation, cast formation, loss of tubular brush border, congestion, cellular degeneration, and atrophic tubules were used for scoring. Evaluation was done at 400X magnification. The parameters were evaluated in five randomly selected areas in each section of the kidney. Five assessments were made for each selected area for each parameter and averaged to obtain scores from 0 to 4. The scoring was (0) normal, (1) mild, (2) moderate, (3) severe, and (4) extremely severe.

Immunohistochemical Analysis

To observe the nephroprotective effects, caspase-3 levels and NF-KB (p65) were analyzed with immunohistochemistry. Immunohistochemical staining for NF-KB and caspase-3 was performed with a VENTANA BenchMark GX System (Ventana Medical Systems, Inc.), which is a Universal DAB Detection Kit with ultraview. The antibody for caspase-3 staining was a rabbit polyclonal caspase-3 primary antibody (ab4051; abcam) used at 1:50 for 32 min at 37°C. The primary antibody for NF-KB staining was a mouse monoclonal NF-KB (p65) (sc-8008; Santa Cruz) used at 1:80 dilution at the same time and temperature. After the slides were incubated with the diluted antibody,

they were exposed to Ventana. DAB was used as the chromogen, and hematoxylin was the counter stain. These sections were photographed with a light photomicroscope (Olympus BX 43) and digital camera (Olympus, DP21) for light microscopy studies. The staining intensity of immunopositive cells was examined in five random high-power fields (200X) per section with cellSens software (Olympus).

Semi-quantitative scoring for NF-KB and caspase-3 activities was made according to several parameters. These were evaluated according to the degree of nuclear and cytoplasmic staining in the proximal, distal, and medulla collecting tubules as well as the glomerule. According to the staining intensity, the sections were graded as follows: 0 (none), 1 (mild), 2 (moderate), 3 (severe), or 4 (extremely severe).

Statistical Analysis

All data were statistically evaluated using the Mann-Whitney U test. The significance of the differences was accepted as p at the 5% level.

RESULTS

Biochemical Results

MDA is a marker of free-radical-mediated lipid peroxidation. There was less lipid peroxidation product in the animals that received ticagrelor than the control group, and this decrease was statistically significant (P=0.001 in all 5 mg/ kg, 10 mg/kg, and 20 mg/kg groups). *Fig. 1* presents the results for MDA assays in renal tissues. GPx and GR are antioxidant enzymes, and our results showed that both GPx and GR were elevated in all groups that received ticagrelor (P=0.000 in 5 mg/kg, 10 mg/kg, and 20 mg/kg groups). *Fig. 2* and *Fig. 3* show the GPx and GR assays in renal tissues. MDA, GPx, and GR levels were not significantly different among the animals administered ticagrelor at 5 mg/kg, 10 mg/kg, and 20 mg/kg *(Table 1)*.

Histological Results

In the control group, minimal cortical and medullary congestion was observed while no obvious morphological changes were observed *(Fig. 4C, Fig. 5C)* in the sham group.

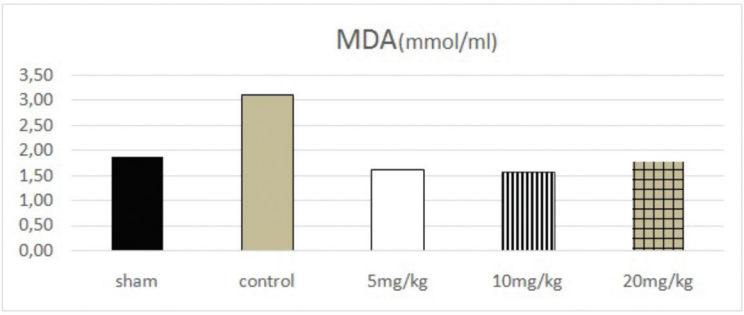
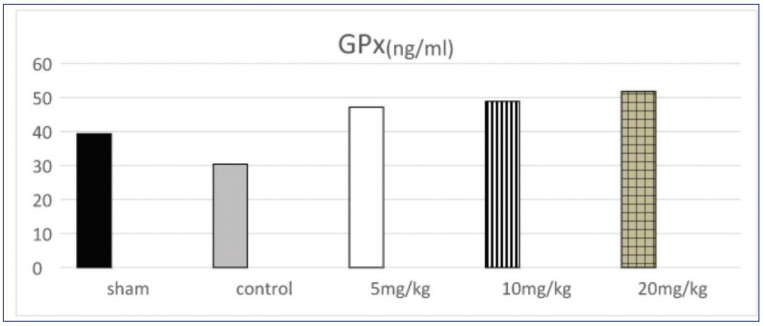


Fig 1. The effect of ticagrelor pretreatment on MDA levels after renal vessel clamping and renal ischemia reperfusion injury in rats. The animals received 0.9% saline solution (control), ticagrelor 5 mg/kg, 10 mg/kg and 20 mg/kg; sham group represents the manipulated animals

Fig 2. GPx. Effect of pretreatment with ticagrelor on renal injury after abdominal aorta ischemia reperfusion injury in rats. The animals received 0.9% saline solution (control), ticagrelor 5 mg/kg, 10 mg/kg, and 20 mg/kg; sham group represents the manipulated animals



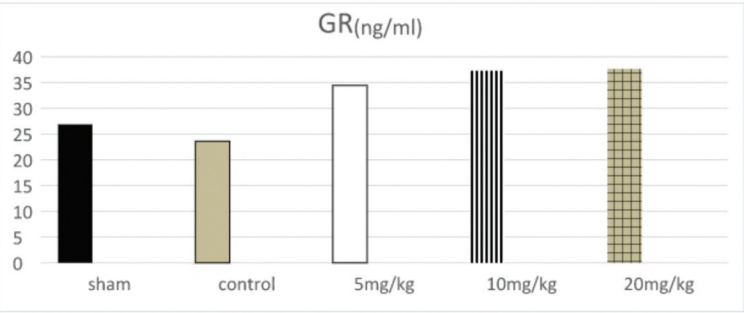


Fig 3. GR levels. Effect of pretreatment with ticagrelor on renal injury after abdominal aorta ischemia reperfusion injury in rats. The animals received 0.9% saline solution (control), ticagrelor 5 mg/kg, 10 mg/kg, and 20 mg/kg; sham group represents the manipulated animals

Table 1. *Effect of different doses of ticagrelor on MDA, GPx, and GR levels*

Parameters	Ticagrelor Doses			P
	5 mg/kg	10 mg/kg	20 mg/kg	
MDA (nmol/mL)	1.61±0.60	1.58±0.41	1.74±0.49	0.926[a] 0.680[b] 0.547[c]
GPx (ng/mL)	47.16±2.91	48.82±2.38	51.79±4.84	0.306[a] 0.073[b] 0.208[c]
GR (ng/mL)	34.43±2.55	37.29±5.08	37.65±4.03	0.245[a] 0.130[b] 0.897[c]

[a] *level of significance between 5 mg/kg dose of ticagrelor and 10 mg/kg dose,*
[b] *level of significance between 5 mg/kg dose of ticagrelor and 20 mg/kg dose,*
[c] *level of significance between 10 mg/kg dose of ticagrelor and 20 mg/kg dose*

groups, although the 5 mg ticagrelor treated group *(Fig. 4D, Fig. 5D)* showed no changes in glomerules and/or tubules compared to the control group. Dilated tubular structures were similar to the sham group and were noted in both 10 and 20 mg ticagrelor treatment groups *(Fig. 4, Fig. 5)*

Immunohistochemical Results

When caspase-3 activity was examined in the control group's renal cortex, the proximal and distal tubule cells had a nuclear immune reaction. The caspase-3 activity was more intense in the nucleus of proximal tubule cells; it was moderate in the distal tubules. The cytoplasm of the proximal tubule cells had a mild immune reaction; there was no immune reaction in the distal tubules.

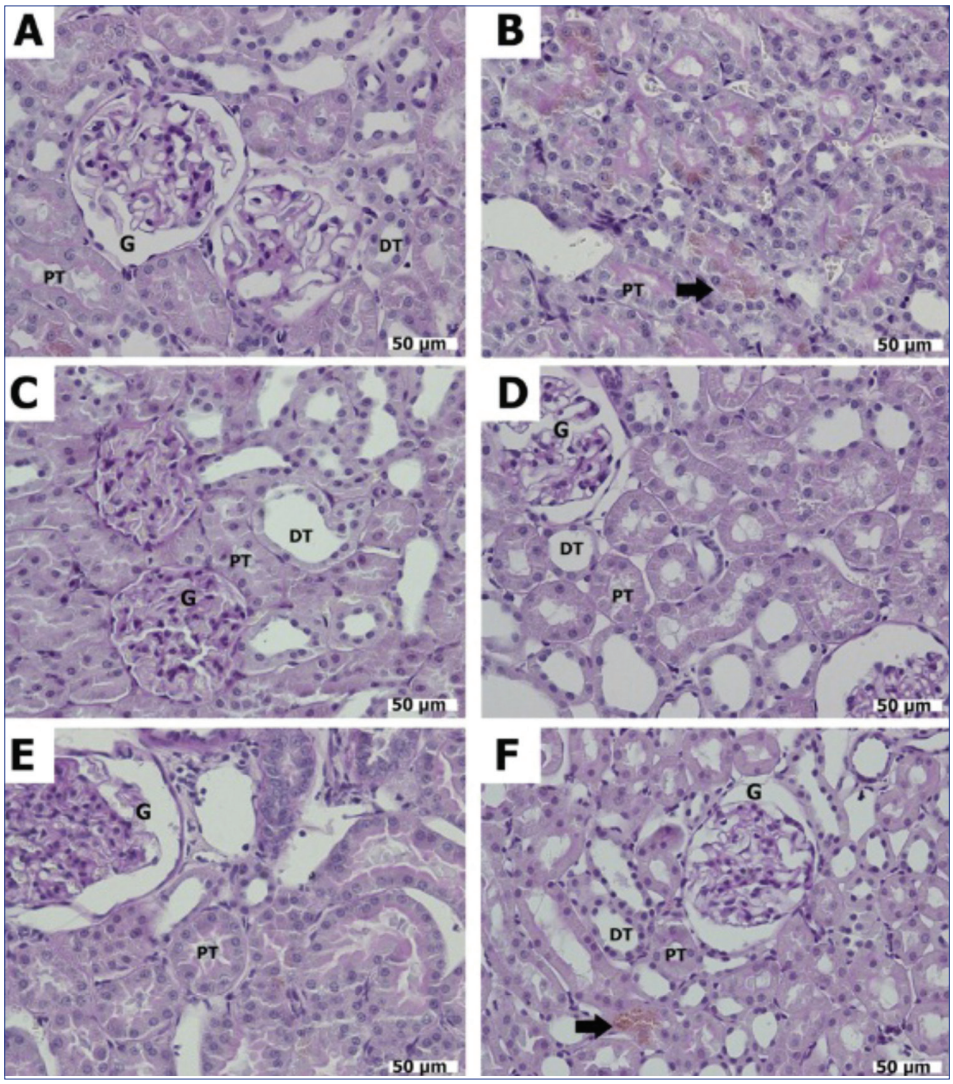


Fig 4. The histological appearance of renal tissue obtained from subjects belonging to the experimental group. While the view of kidney sections of group C was normal, the sham (A-B) group had areas with hemosiderin *(black arrows)* in kidney sections. In other treatment groups (D: 5 mg ticagrelor, E: 10 mg ticagrelor, F: 20 mg ticagrelor) dilatations in the Bowman capsule were observed (Dye: PAS; Mag: 40X, A-B: Sham Group, C: Control Group, D: 5 mg Group, E: 10 mg Group, and F: 20 mg Group; G: Glomerules, DT: Distal Tubule, PT: Proximal Tubule, *black arrow:* hemosiderin area)

In the sham group, we observed a higher filtration range *(Fig. 4, Fig. 5B)*. Both glomerular capillaries and Bowman ranges showed significant dilatation. Cast formation *(Fig. 5B)* was most intense in this group. Based on these evaluation criteria, some effects were found and shown in *Table 2*. Similar findings were observed in all treatment

Collector tubules in the medulla were slightly positive. Glomerular, vascular smooth muscle, and transitional epithelium showed no immune reactions. The immune activity was more intense-especially in neighboring regions of the cortex through the medulla *(Fig. 6A)*. In general, other groups have similar findings as the control group,

and we indicate the differences between the groups as follows *(Table 3)*.

In the sham group, the medullar area with collecting tubules was slightly positive. The 5, 10, and 20 mg groups were similar to the sham group *(Fig. 6)*. The NF-KB activity in the control group was observed differently in the renal cortex, proximal, and distal tubules. The proximal tubule had a granular appearance, and there was a severe cytoplasmic view. Distal tubule immunostaining observed homogeneity and mild intensity in the cytoplasm.

Collector tubules in the medulla area were slightly positive *(Fig. 7C)*. The sham group and control groups had a positive immune response *(Fig. 7A,B)*. Some distal tubules had obvious cytoplasmic NF-KB activity. In the 5 mg group, while NF-KB activity was observed in the glomeruli, some DT cytoplasmic NF-KB activity was also seen. The 5, 10 and 20 mg groups were similar to the sham group *(Fig. 7)*; immunohistochemical values are given as intensity scores *(Table 3)*.

Table 2. *Histopathological scores of groups ((0) normal, (1) mild, (2) moderate, (3) severe, (4) extremely severe)*

PAS & Triple	Control Group	Sham Group	5 mg Ticagrelor	10 mg Ticagrelor	20 mg Ticagrelor
Loss of tubular brush border	1	2	1	2	2
Tubular dilatation	1	2	2	2	2
Cellular degeneration	1	2	1	1	2
Congestion	0	1	1	1	1
Cast formation	0	3	1	1	1
Atrophic tubules	0	1	0	1	1
Total score	3	11	6	7	9

DISCUSSION

Free oxygen radicals and reactive oxygen species cause ischemia-reperfusion injury and the effects on many tissues

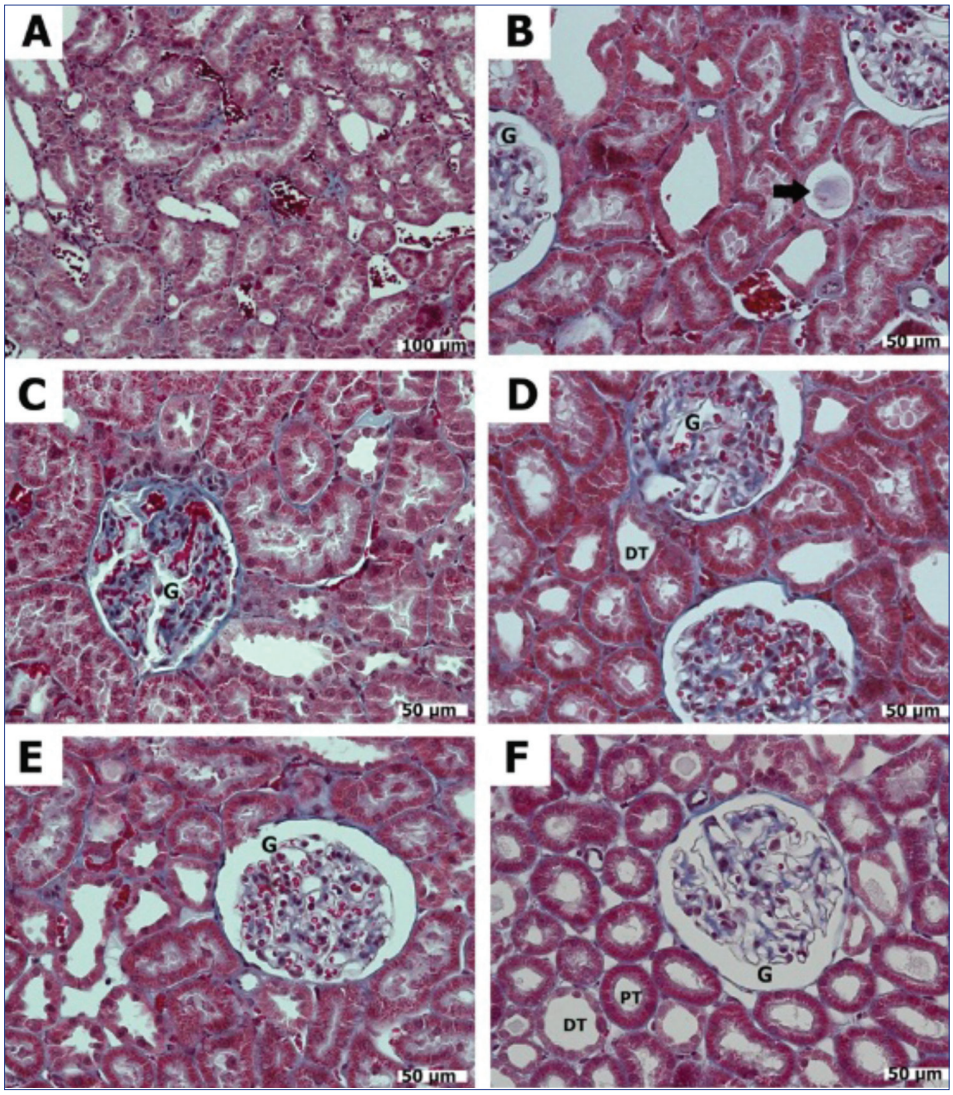


Fig 5. The histological appearance of renal tissue obtained from subjects belonging to the experimental group. Dilatations were observed in the filtration range of kidney sections, additionally cast formations were observed *(black arrow)* in the sham group (A-B). While control group (C) had normal appearance, 5 mg (D), 10 mg (E) and 20 mg (F) ticagrelor treatment groups had ischemic results such as loss of tubulin in the microvilli brushes, and dilated tubules observed (Dye: TRIPLE; A-B: Sham Group, C: Control Group, D: 5 mg Group, E: 10 mg Group, and F: 20 mg Group, G: Glomerules, DT: Distal Tubule, PT: Proximal Tubule, *black arrow:* cast formation)

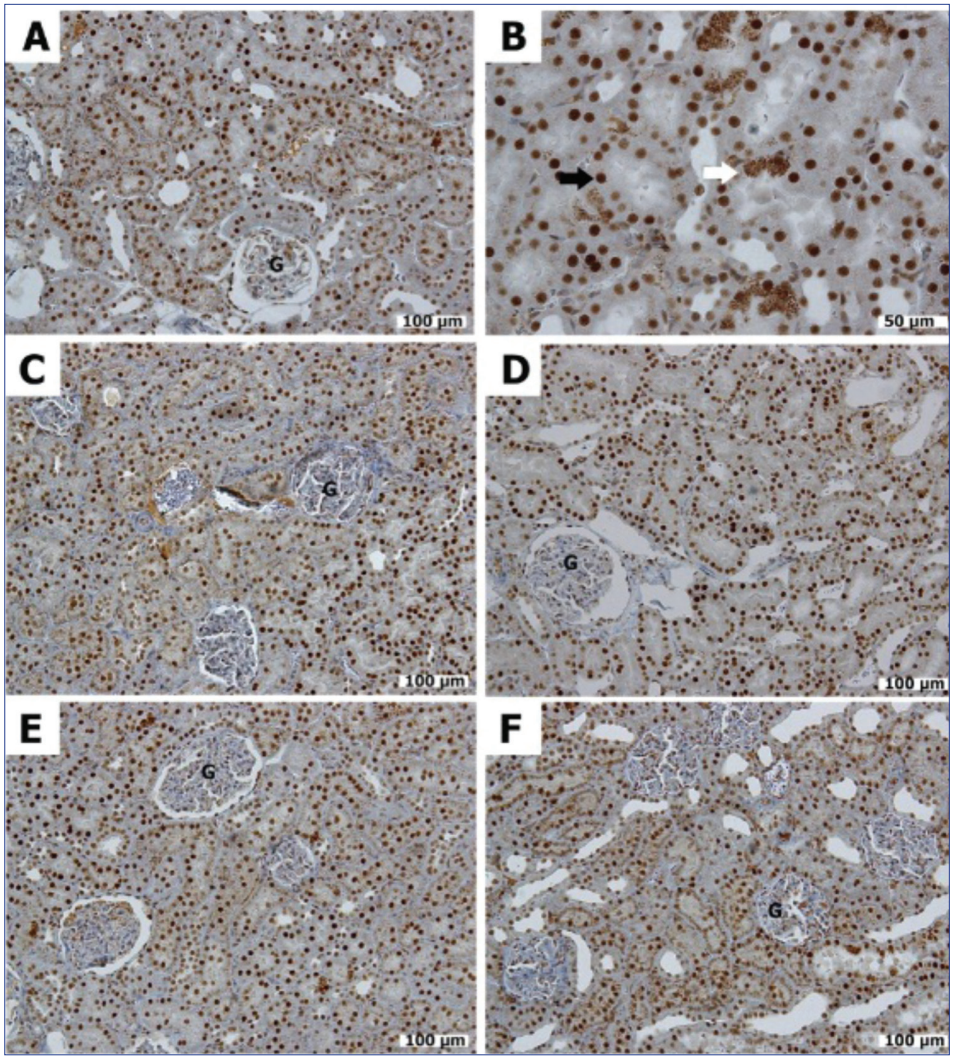


Fig 6. View of caspase-3 immune activity in kidneys. In all groups, the tubules' nuclear caspase activity is seen with different concentrations *(black arrow)*;sham *(white arrow)* and 25 mg group intensive cyto-plasmic reactions *(white arrow)* were also observed (Dye: Caspase-3; A-B: Sham Group, C: Control Group, D: 5 mg Group, E: 10 mg Group, and F: 20 mg Group, *black arrow:* nuclear Caspase-3 activity, white arrow: cytoplasmic Caspase-3 activity, G: Glomerules)

Table 3. *Results of Caspase-3 and NF-KB activation ((0) normal, (1) mild, (2) moderate, (3) severe, (4) extremely severe)*

Caspase-3 Staining	Control Group	Sham Group	5 mg Ticagrelor	10 mg Ticagrelor	20 mg Ticagrelor
Proximal tubules	4	3	4	4	3
Distal tubules	1	1	1	1	1
Glomerule	0	1	0	0	1
Medulla	1	2	1	1	2
Total score	6	7	6	6	7
NF-KB					
Proximal tubules	2	1	1	1	0
Distal tubules	1	2	1	1	2
Glomerule	1	3	2	3	3
Medulla	2	2	2	2	2
Total score	6	8	6	7	7

are well-known [17,18]. These effects include changes in the level of ATP depletion, oxidative phosphorylation, increases in the level of intracellular calcium, and protein kinase activation. The release of nucleases, lipase proteases, and phosphatases can damage the cell's integrity and function [19].

Reperfusion of ischemic tissue can stimulate the production of reactive oxygen species [20], which can lead to pre-oxidation of cellular structures and components such as nucleic acids, proteins, and lipids [21]. Kidney ischemia-reperfusion injury -together with a reduction in anti-oxidant defense- is associated with oxidative stress in the kidney [22-24].

Nitric oxide synthase inhibitors, statins, betacarotene, erythropoietin, ascorbic acid, phenolic acid, antioxidants, and antioxidant enzyme mimetics have all been shown to have potential positive effects that protect renal function and reduce kidney ischemia-reperfusion injury [23-26].

The use of ticagrelor increases adenosine levels. Adenosine accumulation during ischemia and inflammation protects tissue from damage. In ischemic tissue, adenosine accumulates because of inhibited adenosine kinase.In inflamed tissue, adenosine consists of adenine nucleotides that are delivered from numerous cells including mast

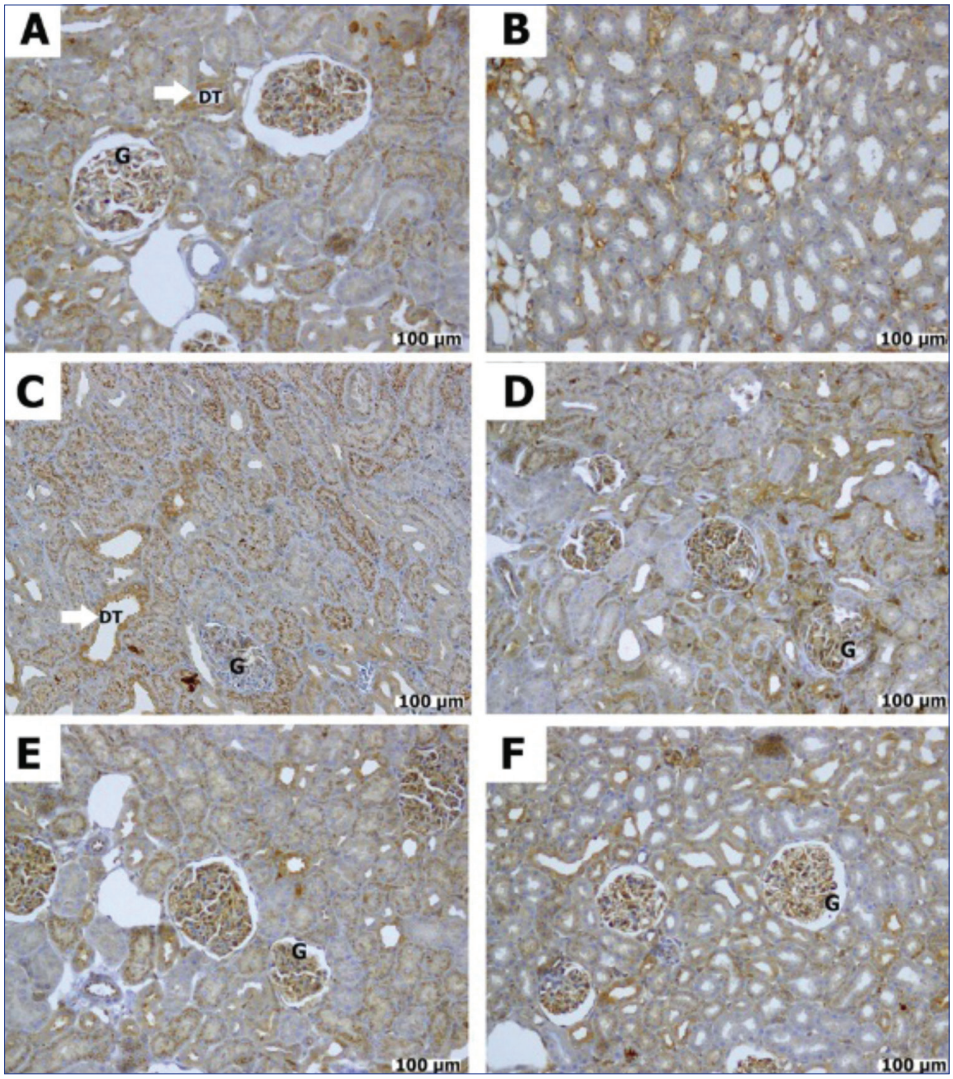


Fig 7. Kidney tissue NF-KB immune activity. No immune glomerular activity was seen in the control (c) group, otherwise severe NF-KB activity was observed in the sham (A-B) and other treatment groups (D-F) (Dye: NF-KB; A-B: Sham Group, C: Control Group, D: 5 mg Group, E: 10 mg Group, and F: 20 mg Group, Mag: 20x, *white arrow:* cytoplasmic Caspase-3 activity, G: Glomerule, DT: Distal Tubule)

cells, endothelium, and platelets. This is only one of the pleiotropic effects of this drug. Thus, it is better to indicate the pleiotropic effects of ticagrelor [27].

The $P2Y_{12}$ receptor antagonists exhibit a wide range of actions beyond their primary anti-aggregation functions that is the primary indication [28]. They modulate inflammation, improve endothelial function, and prevent vasoconstriction while offering adenosine-likeand post-conditioning-like effects. This offers an extra gain benefit for patients treated with the $P2Y_{12}$ blocker ticagrelor. This motivated us to use ticagrelor to prevent or reverse ischemia-reperfusion kidney damage. To the best of our knowledge, this is the first study to evaluate ticagrelor in kidney injury. Thus, we can not compare it to other studies. There are many well-known studies of other drugs and their effects on ischemia-reperfusion injuries.

In our study, the animals that received ticagrelor had significantly less MDA than the control group. The antioxidant enzymes of both GPx and GR were elevated in groups administered ticagrelor at all doses (P=0.000 in 5 mg/kg, 10 mg/kg and 20 mg/kg groups). This shows that ticagrelor protects kidney tissue from ischemia-reperfusion injury and oxidative stress. MDA, GPx, and GR levels were not significantly different between the ticagrelor animals at 3 different doses. In our study, the protective effect of ticagrelor was independent of the doses in terms of biochemistry.

The histopathological findings have a limited similarity with the degree of lipid peroxidation and biochemical results. Tubular dilatation, which was observed in the ticagrelor treatment groups, may stem from precipitated proteins that obstruct tubules and raise the intratubular pressure. This is consistent with the results from animal models that showed increased intratubular pressure in ischemic injury [29]. Histopathological findings also confirmed the protective action of ticagrelor, but this was dose-dependent. In the ticagrelor group, the damage was less severe than in the control group. The immunohistochemical results also showed the nephroprotective effect of this drug. Ticagrelor may protect renal tissue with its pleiotropic effect or through some other mechanism. Accordingly, our biochemical findings showed that the administration of

ticagrelor protects kidneys from ischemia-reperfusion injuries.

Our study showed that ticagreloris an effective agent to protect kidneys from renal ischemia-reperfusion injury. Ticagrelor may protect the tissues by reducing the concentration of reactive oxygen species and inducing the antioxidant system especially with its pleiotropic effects. However, further studies are needed to understand the possible mechanisms by which ticagrelor can prevent kidney ischemia-reperfusion injury.

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Research Article

Development of A Multiplex PCR Method for Direct Detection of Common Mastitis Pathogens in Bovine Milk Samples [1] [2]

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Abstract

The aim of this study was to evaluate a simple and rapid DNA extraction method combined with a multiplex polymerase chain reaction (mPCR) for the identification of the major mastitis pathogens *(Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli* and *Mycoplasma bovis)* from milk samples. Of the 200 California Mastitis Test (CMT) positive milk samples, 45 (22.5%), 21 (10.5%) and 11 (5.5%) were detected as positive for the presence of *S. aureus, S. agalactiae* and *E. coli* by culture, respectively. In mPCR by DNA isolation method optimised here, *S. aureus, S. agalactiae* and *E. coli* were detected in 26.5% (53/200), 12% (24/200) and 6% (12/200) of the milk samples, respectively. The abovementioned agents were observed in similar proportions when the samples were analysed by a commercial DNA isolation kit. On the other hand, *M. bovis* was not detected in any of the milk samples by either culture or mPCR methods. A significant difference was determined between the results of culture and mPCRs ($P<0.001$). Diagnostic sensitivity and specificity of the optimised mPCR were calculated as 100% and 89.2% respectively, when culture results were considered as reference. The results suggest that the mPCR assay employed in this study could be used as an alternative routine diagnostic method for rapid, sensitive, and specific simultaneous detection of major mastitis agents in bovine milk samples.

Keywords: *Mastitis, Major Pathogens, DNA isolation, Multiplex PCR*

Mastitisli İnek Sütlerinde Önemli Patojenlerin Direkt Tespiti İçin Bir Multipleks PCR Yönteminin Geliştirilmesi

Özet

Bu çalışmanın amacı, inek sütlerinde major mastitis patojenlerinin *(Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli* ve *Mycoplasma bovis)* saptanabilmesi için hızlı, basit ve spesifik bir DNA ekstraksiyon ve multipleks PCR yöntemi geliştirmektir. Kaliforniya mastitis testi (CMT) pozitif 200 süt örneğinin kültürü sonrasında *S. aureus, S. agalactiae* ve *E. coli* varlığı sırasıyla 45 (%22.5), 21 (%10.5) ve 11 (%5.5) örnekte saptandı. Çalışmada optimizasyonu yapılan DNA ekstraksiyon metodu ile elde edilen DNA örneklerinin mPCR analizi neticesinde *S. aureus, S. agalactiae* ve *E. coli* varlığı sırasıyla %26.5 (53/200), %12 (24/200) ve %6 (12/200) olarak belirlendi. Ticari DNA izolasyon kitiyle elde edilen DNA örneklerinde de yukarıdaki etkenlerin varlığı benzer oranda bulundu. Öte yandan süt örneklerinin hiçbirinde kültür veya mPCR ile *M. bovis* tespit edilmedi. Kültür ve mPCR sonuçları arasındaki fark istatistiki olarak önemli bulundu ($P<0.001$). Kültür sonuçları referens olarak kabul edildiğinde, optimize edilen mPCR'ın sensitivitesi ve spesifisitesi sırasıyla %100 ve %89.2 olarak hesaplandı. Bu sonuçlara göre; çalışmada kullanılan mPCR yöntemi sığır süt örneklerinde majör mastitis etkenlerinin hızlı, duyarlı, spesifik ve eş zamanlı tespit edilmesinde alternatif rutin teşhis metodu olarak kullanılabilir.

Anahtar sözcükler: *Mastitis, Majör patojenler, DNA izolasyonu, Multipleks PCR*

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INTRODUCTION

Mastitis is a frequent intramamary infection seen in dairy herds and leads to significant economic losses (estimated at $150-300/cow, per year) in dairy industry all over the world [1-4]. It constitutes the 26% of cattle diseases and losses due to mastitis in the USA have been recorded as two billion dollars annually [3]. Studies conducted in dairy herds in many countries represented the prevalence of disease in cattle as 50% and the ratio of infection in udder quarters as 25% [5].

The most common pathogens that cause mastitis are known as contagious agents including *S. aureus*, *S. agalactiae*, *Mycoplasma* sp. and, environmental agents including *E. coli*, *S. dysgalactiae*, *S. uberis* [6-10]. It is stated that eleven different *Mycoplasma* species induce mastitis [11]. Losses due to *M. bovis* mastitis in the USA have been estimated as 108 million/year dollars and the proportion of disease in infected herds has been reported over the 70% [12]. Recent studies indicated that the prevalence of mycoplasmal mastitis has increased in most regions of the world in last years [13,14].

Although the culture method has been regarded as a gold standard for identification of mastitis pathogens, it is labor-intensive and time-consuming [15]. The more sensitive and specific PCR assays allow examining a wide range of samples in a short time. Direct DNA extraction from samples, without the need for cultivation of agents, provides to PCR to accomplish a reliable direct identification [16]. mPCR facilitates the simultaneous identification of different bacterial pathogens in a few hour time, although conventional culture methods require several days. So far, in order to determine the causes of mastitis in milk samples directly, either single bacterium (by PCR) or most common species such as *S. aureus* and *S. agalactiae* (together by mPCR) have been studied in researches carried out in several regions of the world [17-20]. However, mycoplasmas have been neglected in mastitis cases in recent years, due to the labor-intensive and time-consuming characteristics and owing to presence of other major agents such as contagious and environmental agents at high proportions in milk.

The purpose of this study was to develop rapid, simple, sensitive and specific DNA extraction and mPCR method for the detection of major mastitis pathogens (*S. aureus*, *S. agalactiae*, *M. bovis* and *E. coli*) in bovine milk samples, without the need for culture and biochemical identification.

MATERIAL and METHODS

Milk Samples

CMT positive milk samples were collected from 200 dairy cattle in 25 different farms located in Elazig province, in eastern Turkey. Approximately 10 mL of milk sample was taken into sterile tubes from udders aseptically and transferred to labortory in cold chain conditions. In addition, 19 *M. bovis* positive milk samples provided from a previous study [14] were included for use in mPCR tests.

Culture and Isolation

Milk samples were inoculated on blood agar base (Merck) supplemented with 5% defibrinated sheep blood and incubated aerobically for 48 h at 37°C for the isolation of *S. aureus*, *S. agalactiae* and *E. coli*. Also, milk samples were inoculated onto Edwards Medium Modified Agar (Oxoid) for *S. agalactiae* and Eosine Methylene Blue Agar (Oxoid) for *E. coli*. Additionally, samples were plated onto supplement G including Mycoplasma Agar (Oxoid) and incubated at 37°C in 5% CO_2 for 7 to 10 days for *M. bovis*. After biochemical identification, suspected isolates were subjected to DNA extraction and PCR. *S. aureus* (ATCC 25923), *S. agalactiae* (ATCC 13813), *E. coli* (ATCC 25922) and *M. bovis* (provided from Department of Microbiology, Faculty of Veterinary Medicine, Firat University) reference strains were used as positive controls in all assays.

Direct DNA Extraction from Milk Samples

Two different extraction procedures were employed for direct DNA extraction from milk samples. In the first, a commercial product named as Milk Bacterial DNA Isolation Kit (Norgen Biotek, Canada) was used to extract the bacterial DNA from milk samples. The extraction was carried out as described by the manufacturer.

In the second, one mL of milk sample was transferred into an eppendorf tube and was mixed with the same volume of Tris-EDTA buffer for washing. Then, the mixture was centrifuged at 11.600 g for one min. The washing process was repeated until a clear suspension was seen. Clear suspension was centrifuged at 11.600 g for 1 min, the pellet was resuspended in 300 µL distilled water and was used for DNA extraction. The spiked milk suspension was treated with 300 µL TNES buffer (20mM Tris, pH 8.0, 150mM sodium chloride, 10mM EDTA, 0.2% sodium dodecyl sulphate) and 200 µg/mL proteinase K, and was incubated at 56°C for one hour. Then, 5 µL Lysostaphin (Sigma, 100 µg/mL) and 10 µL Mutanolysin (Sigma, 5U/µL) were added onto this suspension that was re-incubated at 37°C for 1 h. The suspension was then heated at 95°C for 10 min to inactivate the enzymes, followed by centrifugation for 10 min at 11.600 g. Finally, the suspension was removed; the pellet was dried and resuspended in 100 µL sterile distilled water.

Limit of Detection

S. aureus ATCC 25923 was used for evaluating the second DNA extraction method which was developed in this study. Several *S. aureus* colonies were suspended in sterile phosphate-buffered saline (PBS) and tenfold dilution series containing $6x10^7$ to $6x10^0$ CFU per mL were prepared. All the tubes were centrifuged and the suspension was

removed. One ml sterile milk was added to each tube and tubes were gently vortexed until cells were resuspended. Finally, DNA extraction procedure described above was applied and the detection limit was calculated.

Multiplex PCR Method

DNA samples of *S. aureus*, *S. agalactiae*, *E. coli* and *M. bovis* reference strains were subjected to amplification alone and in combinations in order to optimize mPCR. Appropriate PCR mix was arranged for mPCR conditions. The mPCRs were performed in a total reaction volume of 50 µL, containing 5 µL 10x PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 8 µL 25 mM $MgCl_2$ 200µM of each deoxynucleotide triphosphate, 2 U Taq DNA Polymerase (MBI Fermentas), 20 pmol of each species specific primer *(Table 1)* and 5 µL of template DNA. Techne 512 Gradient PCR (Techne, England) was used to optimize the best annealing temperature for all primer pairs. The optimal annealing temperature (58°C) was achieved by using an automated program ranging from 50°C to 62°C. The conditions for the mPCR were an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min (denaturation), 58°C for 1 min (primer annealing), 72°C for 1 min (extension) and a final extension step at 72°C for 5 min. The amplified products were detected by ethidium bromide (10 mg/mL) staining after electrophoresis at 80 V for two hours in 2% agarose gels.

Statistical Analyses

Comparison of the methods (culture and mPCRs) with regard to their rate of positivity in milk samples was carried out by the Cochran Q test, followed by pairwise comparisons applying McNemar tests. In all statistical tests, $P<0.05$ value was considered statistically significant. Analyses were performed using the commercial software SPSS 14.0.

RESULTS

Bacteriological Findings

After the incubation period, suspected colonies grown on specific media were examined and identified by primary identification tests. Of the 200 milk samples 114 (57%) were found to be positive by culture. When the culture results were assessed at bacterial species level, 45 (22.5%), 21 (10.5%) and 11 (5.5%) of the milk samples were detected as positive for the presence of *S. aureus, S. agalactiae* and *E. coli,* respectively. The isolation percentages of mixed infections of *S. aureus* + *S. agalactiae* and *S. aureus* + *E. coli* were calculated as 3.5% (7/200) and 2.5% (5/200), respectively. Other bacteria which were detected in 25 samples were beyond the scope of this study. On the other hand, none of the milk samples were positive for *M. bovis*. The overall isolation percentage of the major pathogens examined in this study was determined as 44.5% (89/200) *(Table 2)*.

mPCR Findings

DNAs obtained from milk samples by commercial DNA isolation kit were subjected to optimized mPCR combined with species specific primer pairs of *S. aureus*, *S. agalactiae*, *E. coli* and *M. bovis*. Amplification products at the molecular sizes of approximately 232, 447, 586 and 1318 bp which were considered indicative for *E. coli*, *M. bovis*, *S. agalactiae* and *S. aureus,* respectively, were detected *(Fig. 1)*. This indicated that the mPCR assay optimised in this study was working well.

Similar results were observed in the mPCR analysis of DNAs extracted from CMT positive milk samples by both commercial DNA isolation kit (mPCR-1) and optimised direct isolation method (mPCR-2). In total, 104 (52%) and 101 (50.5%) of milk samples were found positive for either of the mastitis agents examined here by the commercial kit and optimised method, respectively. In mPCR analysis of milk samples by optimised DNA isolation method (mPCR-2), specific amplification products that were considered indicative for *S. aureus*, *S. agalactiae* and *E. coli* were detected in 26.5% (53/200), 12% (24/200) and 6% (12/200) of the milk samples respectively. In addition, mixed infections of *S. aureus* + *S. agalactiae* (7/200) and *S. aureus* + *E. coli* (5/200) were identified by mPCR examination. *M. bovis* was not detected in mPCR analysis, either. On the other hand, detection rates of *S. aureus*, *S. agalactiae* and *E. coli* were estimated as 27% (54/200), 12.5% (25/200) and 6% (12/200) respectively in mPCR analysis of milk samples

Table 1. *Primer sequences and lengths of mPCR amplification products*

Primer	Specificity	Sequences (5'-3')	Fragment Size (bp)	References
Sau327	*S. aureus* (f)	GGACGACATTAGACGAATCA	1318	[16]
Sau1645	*S. aureus* (r)	CGGGCACCTATTTTCTATCT		
Sag432	*S. agalactiae* (f)	CGTTGGTAGGAGTGGAAAAT	586	[16]
Sag1018	*S. agalactiae* (r)	CTGCTCCGAAGAGAAAGCCT		
Eco223	*E. coli* (f)	ATCAACCGAGATTCCCCCAGT	232	[16]
Eco455	*E. coli* (r)	TCACTATCGGTCAGTCAGGAG		
Mb-Mp1	*M. bovis* (f)	TATTGGATCAACTGCTGGAT	447	[21]
Mb-Mp2	*M. bovis* (r)	AGATGCTCCACTTATCTTAG		

Table 2. *Bacteriological and mPCR findings obtained from bovine milk samples*

Bacterial strains	Culture	mPCR-1*	mPCR-2**
S. aureus	45 (22.5%)	54 (27%)	53 (26.5%)
S. agalactiae	21 (10.5%)	25 (12.5%)	24 (12%)
E. coli	11(5.5%)	12 (6%)	12 (6%)
S. aureus + *E. coli*	5 (2.5%)	5 (2.5%)	5 (2.5%)
S. aureus + *S. agalactiae*	7 (3.5%)	8 (4%)	7 (3.5%)
M. bovis	-	-	-
Other bacteria	25 (12.5%)	ND	ND
Total	**114 (57%)***[a]**	**104 (52%)[b]**	**101 (50.5%)[b]**

** Commercial Milk Bacterial DNA Isolation Kit (Norgen Biotek, Canada); ** Optimized Direct DNA Isolation Method; *** Statistical tests applied for S. aureus, S. agalactiae and E. coli isolation ratio (44.5%); [a,b] different letters represents significancy of difference between groups;* ***ND:*** *Not determined*

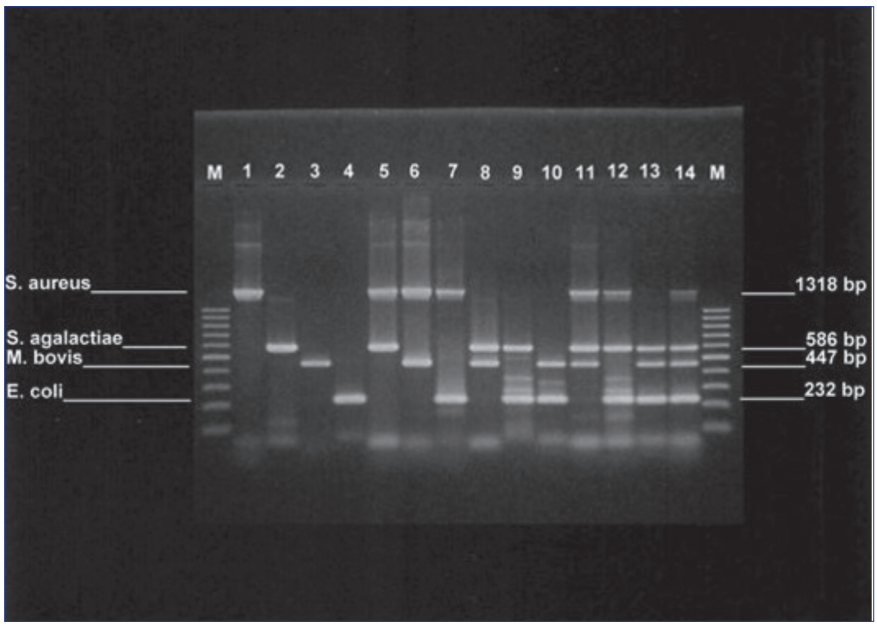
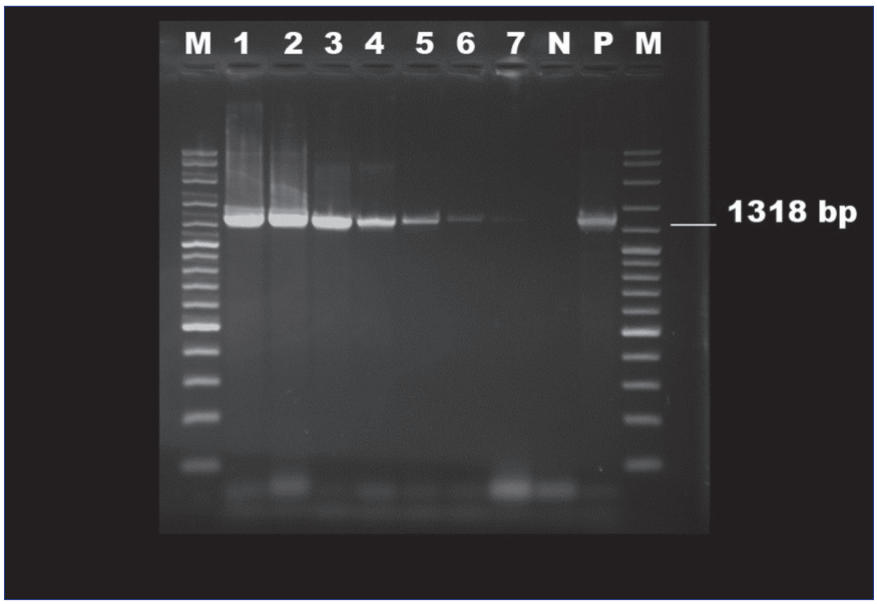


Fig 1. Agarose gel electrophoresis of products of *S. aureus*, *S. agalactiae*, *E. coli* and *M. bovis* isolates determined by optimized multiplex polymerase chain reaction M: 100 bp DNA Ladder (Fermentas, SM 0241). Lane 1: *S. aureus*, Lane 2: *S. agalactiae*, Lane 3: *M. bovis*, Lane 4: *E. coli*, Lane 5: *S. aureus* + *S. agalactiae*, Lane 6: *S. aureus* + *M. bovis*, Lane 7: *S. aureus* + *E. coli*, Lane 8: *S. agalactiae* + *M. bovis*, Lane 9: *S. agalactiae* + *E. coli*, Lane 10: *M. bovis* + *E. coli*, Lane 11: *S. aureus* + *S. agalactiae* + *M. bovis*, Lane 12: *S. aureus* + *S. agalactiae* + *E. coli*, Lane 13: *S. agalactiae* + *M. bovis* + *E. coli*, Lane 14: *S. aureus* + *S. agalactiae* + *M. bovis* + *E. coli*

Fig 2. Agarose gel electrophoresis representing detection limit of optimized DNA extraction method. M: 100 bp DNA Ladder (Fermentas, SM 0321) Lane 1: 6 x 10^7 bacteria/mL milk, Lane 2: 6 x 10^6 bacteria/mL milk, Lane 3: 6 x 10^5 bacteria/mL milk, Lane 4: 6 x 10^4 bacteria/mL milk, Lane 5: 6 x 10^3 bacteria/mL milk, Lane 6: 6 x 10^2 bacteria/mL milk, Lane 7: 6 x 10^1 bacteria/mL milk, N: 6 x 10^0 bacteria/mL milk, P: *S. aureus* ATCC 25923



by commercial DNA isolation kit (mPCR-1). Also, mixed infections of *S. aureus* + *S. agalactiae* (8/200) and *S. aureus* + *E. coli* (5/200) were identified by mPCR examination *(Table 2)*.

When the culture was considered as gold standard, the sensitivity and specificity of the mPCR-2 were calculated as 100% (89/89) and 89.2% (99/111), respectively. On the other hand, when mPCR assay combined with the commercial isolation kit was considered as gold standard, the sensitivity and specificity of the mPCR-2 were evaluated as 97% and 100%, respectively. The isolation percentages of the major mastitis pathogens by culture, mPCR-1 and mPCR-2 were compared with Cochran's Q statistical test and the difference was significant ($P<0.001$). The McNemar test indicated a significant difference between the culture and two mPCR methods ($P<0.001$). However, the difference between the mPCR-1 and mPCR-2 was not significant ($P>0.05$).

Although no positive results were obtained for *M. bovis* in the analysis of 200 milk samples by both culture and molecular tests, mPCR analysis of DNA samples extracted by both procedures from 19 *M. bovis* positive cultures which were provided from a previous study yielded amplification products at the molecular size of approximately 447 bp, which was indicative for the presence of *M. bovis*.

The Limit of Detection

DNA samples obtained from tenfold dilution series of spiked milk samples containing *S. aureus* ATCC 25923 ($6x10^7$ to $6x10^0$ CFU/mL) were tested in order to compare detection limit of the optimized DNA extraction method with the commercial DNA isolation kit. The minimum detection limit of the optimized method was estimated as $6x10^1$ CFU/mL with the specific band at the molecular size of approximately 1318 bp *(Fig. 2)*. On the other hand, the commercial DNA isolation kit has been declared to detect as few as 10 CFU/mL in milk samples by the manufacturer.

DISCUSSION

Mastitis is a disease of dairy cattle and cause significant economic losses in milk industries [22]. In order to develop control strategies in dairy farms, rapid and reliable identification of pathogens that cause mastitis is very important [23]. Identification of bacteria by culture methods requires 2 to 10 days and mix infections or closer species cannot be distinguished by biochemical assays. Although the culture method has been regarded as a gold standard for identification of pathogens of mammary gland, it is arduous, time-consuming and sometimes incapable [15,24]. In order to overcome identification problems such as growth failure of bacteria, a mPCR in combination with direct bacterial DNA isolation from milk samples was employed to detect the most common mastitis pathogens *(S. aureus*, *S. agalactiae*, *M. bovis* and *E. coli)*. This is the first study conducted in Turkey for direct identification of four mastitis agents in bovine milk simultaneously.

Bacterial growth was observed in 44.5% of 200 CMT positive milk samples in the present study. This rate was lower than those obtained in mPCR assays (mPCR-1: 52% and mPCR-2: 50.5%) combined with both extraction procedures indicating that mPCR assays were superior to culture in terms of sensitivity. Multiple factors may influence the performance of cultivation results. The absence of bacterial growth has been reported as 20-30% of milk samples in clinical mastitis [25-28]. Besides, in Canada 43.9% of milk samples have been declared as culture negative [29]. In a study conducted in subclinical-clinical mastitis (not separated), no growth was observed in almost half of the samples [24]. It was mentioned that failure of bacterial growth in milk samples may be due to low bacterial concentration, pathogens not growing on standard medium or existence of substances that suppress the observation [30,31]. Milk samples may contain external inhibitor substances such as antimicrobial or disinfectant residues due to treatment of udders before sampling and this situation may suppress the bacterial growth [32].

The mPCR assays were compared with conventional culture and a statistically significant difference between the tests was noted. This is not surprising because milk harbor several organisms that can be difficult to culture, particularly when samples are not plated immediately. The sensitivity and the specifity of the optimised mPCR were calculated as 100% and 89.2% respectively, when culture results were considered as gold standard. Amplification products for at least one of the mastitis agents studied here were obtained in twelve milk samples from which no growth was observed.

The PCR facilitates sensitive, rapid, reliable, objective and user-independent detection of bacterial and antimicrobial resistance genes. mPCR ensures rapid identification of many species simultaneously and reduces false negative results, when used in addition to conventional methods in mastitis control programs [33,34]. In this study, identification of pathogens from milk samples was accomplished in a short time (six hours) by mPCR, without a bacterial culture step. It has been reported that rapid detection of mastitis reduces the treatment time, enhances the cure rates and decreases inappropriate and redundant antimicrobial usage [35,36].

Although the detection of DNA in milk samples is not sufficient to indicate the presence of the disease, identification of pathogens by mPCR could be helpful to get rapid information and to develop control strategies. Despite the fact that molecular methods are regarded as integrant or alternative for conventional methods [16], for direct detection of milk pathogens, milk samples may contain PCR inhibitor substances. In the examination of mastitis, highly qualified DNA extraction protocols and reagents should be implemented to milk samples to obtain decisive results.

Many commercial extraction kits were developed and marketed for this purpose but they are costly. In this study, a DNA extraction method was developed and compared with a commercial kit. The direct DNA extraction method from milk was evaluated to detect as few as 6 x 10^1 CFU/mL, while the minimum detection limit of the commercial DNA isolation kit has been declared as 10 bacteria/mL by the manufacturer. This slight difference might be overlooked when the cost is taken into consideration. Commercial kit requires enzyme (lysostaphin) addition for isolating DNA from Gram positive (especially *S. aureus*) bacteria. Besides, isolation percentages of two methods were compared and the difference between the mPCR1 and mPCR 2 was not significant (P>0.05). The new optimised DNA extraction method can therefore be suggested as an alternative to commercial kits owing to the fact that it could easily be modified and performed in many routine laboratories and the cost of the extraction per sample is cheaper than commercial kits.

S. aureus and *S. agalactiae* are regarded as major mastitis agents. In previous studies, mastitis pathogens have been studied merely by PCR or at most two agents by mPCR. Thus other mastitis pathogens and especially labor-intensive mycoplasmas which require adequate laboratory facilities, experienced personnel were ignored [37]. Four different bacteria including *M. bovis* were successfully identified in spiked milk samples by mPCR method that was developed in the current study. This may lead to clarify mix infections in mastitis. Additionally, both the commercial kit and optimized DNA extraction method were tested with 19 milk samples submitted to our laboratory which were indicated as positive for *M. bovis* by culture and PCR. Direct DNA extraction from these milk samples by both methods followed by mPCR produced positive results for *M. bovis* which requires 7-10 days for cultivation.

It can be concluded that DNA isolation and mPCR methods developed in this study are more sensitive and faster than conventional culture, and can be easily applied for detection of *S. aureus*, *S. agalactiae*, *E. coli* and *M. bovis* in milk samples simultaneously. It can therefore be used as alternative to conventional culture method in the routine diagnosis. Early detection of mastitis by mPCR may contribute to surveillance programs, and to planning prevention and control strategies. Also this study would be basis of further studies that aim to optimize and perform new processes for examining other bacterial agents that cause mastitis.

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Research Article

Investigation of Oxidative Stress Index and Lipid Profile in Cattle with Brucellosis

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Abstract

The aim of this study was to define the oxidative-antioxidative capacity and lipid profile in cattle with brucellosis. A total of 32 cattle of Simmental breed were used in the study, among those, 22 demonstrated microbiologically positive for brucellosis while 10 were negative. Biochemical analysis included total oxidant capacity (TOC), total antioxidant capacity (TAC), oxidative stress index (OSI), triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), very low-density lipoprotein-cholesterol (VLDL-C) and low-density lipoprotein-cholesterol (LDL-C). TAC value was found to be statistically significantly lower (P=0.007), and TOC and OSI values were found to be significantly higher [respectively (P=0.013) and (P=0.002)] in cattle with brucellosis compared to the control group. TC (P=0.012) and LDL-C (P=0.0004) values were significantly increased, and TG (P=0.004), HDL-C (P=0.023) and VLDL-C (P=0.004) values were significantly decreased compared to the control group. As a conclusion, it was determined that Brucella infection leads to important changes in the oxidative-antioxidative capacity and lipid profile in cattle. These data may contribute to the diagnosis of the disease and especially to the determination of inflammation severity.

Keywords: *Brucellosis, Cattle, HDL, LDL, Total antioxidant capacity (TAC), Total oxidant capacity (TOC)*

Brusellozisli Sığırlarda Oksidatif Stres İndeksi ve Lipid Profilinin İncelenmesi

Özet

Bu çalışmanın amacı, brusellozisli sığırlarda oksidatif-antioksidatif kapasite ve lipid profilinin belirlenmesidir. Çalışmada mikrobiyolojik analiz sonrası brusellozis tespit edilen 22 ve negatif olan 10, toplamda 32 Simental ırkı inek kullanıldı. Biyokimyasal olarak total oksidan kapasite (TOK), total antioksidan kapasite (TAK), oksidatif stres indeksi (OSİ), trigliserid (TG), total kolesterol (TC), yüksek yoğunluklu lipoprotein-kolesterol (HDL-C), çok düşük yoğunluklu lipoprotein-kolesterol (VLDL-C) ve düşük yoğunluklu lipoprotein-kolesterol (LDL-C) değerleri belirlendi. Brusellozisli sığırlar ile kontrol grubu karşılaştırıldığında TAK değerinin istatiksel olarak anlamlı bir şekilde düştüğü (P=0.007), TOK ve OSİ değerlerinin ise istatistiksel olarak anlamlı bir şekilde artmış olduğu, [sırasıyla (P=0.013) ve (P=0.002)] tespit edildi. Yine TC (P=0.012) ve LDL-C (P=0.0004) değerlerinin kontrol grubuna göre istatistiksel olarak anlamlı arttığı, TG (P=0.004), HDL-C (P=0.023) ve VLDL-C (P=0.004) değerlerinin istatistiksel olarak azalmış olduğu belirlendi. Sonuç olarak, brusella enfeksiyonunun sığırlarda oksidatif-antioksidatif kapasitede ve lipit profilinde önemli değişikliklere neden olduğu belirlendi. Elde edilen bu bulgular özellikle hastalıktaki yangı şiddetinin belirlenmesine ve diagnozuna katkıda bulunabilir.

Anahtar sözcükler: *Brusellozis, HDL, LDL, Sığır, Total antioksidan kapasite (TAK), Total oksidan kapasite (TOK)*

INTRODUCTION

Brucellosis is an infectious disease of farm animals that leads to important economic losses, characterized by chronic inflammation of various organs and abortion [1]. In cattle, disease agent is a gram negative, facultative intracellular bacterium, *B. abortus*. Cases originated from *B. melitensis* have been also reported scarcely [2]. Veterinarians, farm workers, butchers and shepherds are under risk since they are in close contact with the animals [3]. Infection is caught

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through the digestive system, conjunctiva, respiratory system or through the skin, and is carried to the regional lymph nodes, then to the spleen, liver, bone marrow, central nervous system and urogenital organs via lymph and blood circulation [4]. Although brucellosis is a multi-systemic disease, most common symptoms are abortion, retentio secundinarum, metritis-endometritis, reduced milk yield, weak and/or dead infant birth, infertility, fever, arthritis, bursitis, epididymitis and orchitis [2]. The most risky situation currently is caused by animals infected with *Brucella*. Contaminated food obtained especially from infected ruminants (milk, cheese, etc) forms risk for human health [1,4].

Free oxygen radicals lead to damage in biological molecules (lipid, proteins and DNA), prevent normal functioning of the cells and lead to increased level of various metabolites which are final products of lipid peroxidation [5,6]. Cells and tissues have antioxidant systems that inhibit radical products and reactions. Studies have demonstrated altered total oxidant (TOC) and total antioxidant capacity (TAC), or oxidative stress index (OSI) in case of local and systemic inflammation or infection [5,7,8].

Lipids contribute to the formation of energy, steroid hormones and biliary acids. They are formed of lipoproteins, esterified/non-esterified cholesterol, triglycerides (TG), phospholipids, lipids and proteins. Lipoproteins such as very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) have various functions in the organism. These lipoproteins carry cholesterol and triglycerides at different degrees. Cholesterol is mostly carried by LDL and triglyceride is mostly carried by VLDL [9]. LDL and HDL hydrolyse lipid peroxide deposits and have an important role in preventing against oxidation. Furthermore, this effect has been reported to be inversely related to oxidative stress [10]. Moreover, various enzyme activities related to lipid profile have been known to reduce during infection and inflammation, and acute phase HDL has been known to be unable to protect LDL against oxidation [11].

The aim of this study was to determine the oxidative-antioxidative capacity and lipid profile in cattle with brucellosis. For this purpose, concentrations of serum TAC, TOC, OSI, TG, total cholesterol (TC), HDL-cholesterol (HDL-C), VLDL-cholesterol (VLDL-C) and LDL-cholesterol (LDL-C), and the correlative relationship between each other were determined.

MATERIAL and METHODS

This study has been conducted in Kars between September and December 2015, after approval by Kafkas University Animal Experiments Local Ethical Committee (KAU-HADYEK-2015/59). The region was 1756 m high, and the gps coordinates were, 40°36'4.8132" North and 43°5'50.8344" East. Material of the study was formed by 270 Simmental breed cattle with brucellosis-like clinical symptoms and reproductive problems such as abortion, retentio secundinarum, and infertility within the farms of Kars. Biochemical analyses were performed in 22 serum samples with microbiologically detected brucellosis. Control group included 10 Simmental breed cattle with normal findings in the general clinical examination (body temperature, respiration, pulse, etc), demonstrating no genital system infection and were confirmed to be *Brucella* negative in the microbiological examination. Animals included in the study had no history of vaccination against *Brucella* agents. Cattle were between 2 and 3 years of age, and their body condition scores varied between 2.5-3.25 according to 1-5 scoring system that increase by 0.25 [12]. Animals fed similarly in similar farm conditions were selected for the study. Blood samples of the animals were collected from *vena coccygea* into 10 mL sterile vacuum tubes without anticoagulant, using holder needles. Blood samples were separated from the serum by centrifugation at 3000 rpm for 15 min. Serum samples were kept at -20ºC until analysis.

Biochemical Analysis

Total oxidant capacity (TOC), total antioxidant capacity (TAC) were analyzed using commercial test kits (Rel Assay Diagnostics, Turkey) as previously described in [13]. Briefly, hydrogen peroxide and trolox were used as standards to calculate for TOC and TAC, respectively. Oxidative stress index (OSI) which is the indicator of the degree of oxidative stress was calculated by using (Arbitrary Unit) = [TOC (mmol Trolox equivalent/L)/10xTAC (μmol H_2O_2 equivalent/L)] formula [13].

Colorimetric analysis (Epoch®, Biotek, USA) was performed for the detection of TG, TC and HDL-C levels in the serum samples examined for lipid profile, using commercial kits (IBL®, Turkey). VLDL-C and LDL-C levels were calculated according to the formula of Friedewald et al.[14]: LDL-C = (TC) – (HDL) – (TG/5) [If TG (mg/dL) <400 mg/dL, VLDL (mg/dL) = TG (mg/dL)/5].

Detection of Brucella Antibodies

Presence of *Brucella* specific antibodies in serum samples was investigated via Rose Bengal Plate Test (RBPT) and Serum Tube Agglutiantion Test (SAT), and the tests were performed in company with Brucella positive and negative control sera. Test antigens were provided from Pendik Veterinary Control and Investigation Institute. RBPT and SAT tests were performed according to the method described by Alton et al.[15]. Equal amounts of (20 μL) antigen and serum samples were mixed on a slide for the RBPT test, and the agglutination observed within 3-4 min was assessed as positive. In the SAT test, serial dilutions of 0.5 mL antigen was performed between 1:10 and 1:320, and was added onto tubes including 0.5 mL serum dilutions, overnight incubated at 37ºC, the lace-like agglutinations at the bottom of the tubes were assessed

as positive outcome. (++) or more severe reactions in RBPT, and positive samples in dilutions of 1:40 or more in SAT after analysis were evaluated as diagnostic dilutions [16,17].

Statistical Analysis

Statistical analysis was performed using SPSS® (SPSS 18.0, Chicago, IL, USA) program package. Distribution of the data within groups was evaluated using Shapiro-Wilk test. Parametrically distributed groups were compared using T test (Independent-Samples T-Test). Correlations between variables were determined using Pearson correlation test. Obtained values were expressed as mean ± standard error of mean (SEM). P≤0.05 was accepted as statistically significant.

RESULTS

RBPT and SAT Results

In 22 of 270 serum samples in the study group, (++++) positivity was obtained with RBPT, and in all of these 22 samples, *Brucella* positivity was detected at 1:320 dilution with SAT. All 10 samples within the control group were detected to be RBPT negative. In these samples, non-specific antibodies for *Brucella* and negative reactions were detected for 1:10 or lower dilutions in SAT.

Clinical Examination

While the mean temperature, heart rate and respiratory rate were 39.27±0.25°C, 80.0±4.0 bpm and 29.0±1.00 bpm, respectively, in the clinical examination of brucella-infected cattle, these values were found to be 38.6±0.02°C, 64.0±1.2 bpm and 21.0±0.30 bpm, respectively, in healthy animals.

Biochemical Results

Oxidant-antioxidant capacity was compared between cattle with brucellosis and the control group, and TAC value was determined to be statistically significantly reduced (P=0.007), whereas TOC and OSI values were found to be significantly increased (P=0.013) and (P=0.002), respectively *(Table 1)*.

The lipid profile revealed statistically significantly higher TC and LDL-C values compared to the control group (P=0.012) and (P=0.0004), respectively; and significantly lower TG (P=0.004), HDL-C (P=0.023) and VLDL-C (P=0.004) values *(Table 2)*.

A significantly high positive correlation was detected between TOC and OSI (r = 0.78, P<0.001) and TG and VLDL-C (r = 1.00, P<0.001) in the control group, and a significantly negative correlation was determined between TAC and HDL-C (r = -0.66, P<0.05), and LDL-C and HDL-C (r = -0.84, P<0.01). A significantly positive correlation was detected between TOC and OSI (r= 0.71, P<0.001), TG and VLDL-C (r= 1.00, P<0.001), and TC and LDL-C (r= 0.60, P<0.01), and significantly negative correlation was determined between TAC and OSI (r= -0.68, P<0.001), TG and OSI (r= -0.47, P<0.05), VLDL-C and OSI (r = -0.48, P<0.05), and HDL-C and LDL-C (r = -0.76, P<0.001) in the *Brucella* group *(Table 3)*.

Table 1. *Changes in TAC, TOC and OSI concentrations in cattle with brucellosis and healthy animals (mean ± SEM)*

Parameters	Control (n=10)	Brucellosis (n=22)	P value
TAC (mmol Trolox Eq/L)	1.23±0.19	0.77±0.06	0.007
TOC (μmol H_2O_2 Eq/L)	26.41±4.57	41.47±3.33	0.013
OSI (Arbitrary Unit)	2.57±0.60	6.16±0.69	0.002

TOC: *Total oxidant capacity,* ***TAC:*** *Total antioxidant capacity,* ***OSI:*** *Oxidative stress index*

Table 2. *Lipid profile concentrations in cattle with brucellosis and healthy animals (mean ± SEM)*

Parameters	Control (n=10)	Brucellosis (n=22)	P value
TG (mg/dL)	15.12±0.89	11.60±0.64	0.004
TC (mg/dL)	92.49±1.33	97.34±1.08	0.012
HDL-C (mg/dL)	43.01±2.28	37.20±1.27	0.023
VLDL-C (mg/dL)	3.02±0.18	2.32±0.13	0.004
LDL-C (mg/dL)	46.47±2.23	57.82±1.63	0.0004

TG: *Triglyceride,* ***TC:*** *Total cholesterol,* ***HDL-C:*** *High-density lipoprotein-cholesterol,* ***VLDL-C:*** *Very low-density lipoprotein-cholesterol,* ***LDL-C:*** *Low-density lipoprotein-cholesterol*

DISCUSSION

In this study, we investigated the effect of brucellosis on changes in oxidative-antioxidative capacity and lipid profile in cattle. Brucellosis, which is a zoonotic disease with chronic progression, not only threatens public health but leads to economic loss of important extent by causing abortion, retentio secundinarum, postpartum infections and infertility [1]. While eradication of brucellosis has been an important economic value, it is important to determine its biochemical and cellular changes for treatment purposes [18,19]. This study determines the changes in TOC, TAC and lipid profile that may play a role in the pathogenesis of brucellosis. Although there are many studies in the medicine [10,11,20-22], the number of studies in the veterinarian field, especially on cattle is limited.

In the case of inflammation or various infections, anti-oxidant defense gets weakens, and increased reactive oxygen species react with macromolecules, including proteins, lipids and DNA; and ultimately, lead to oxidative damage [8,23,24]. In the current study, parameters of oxidative stress were measured to determine the severity of the cellular damage and the effectiveness of the treatment likewise reported formerly [5,6,25]. Oxidative stress parameters have been reported to be stimulated while the anti-oxidant concentration decreased in brucellosis, a chronic

Table 3. *Pearson coefficient of correlations between groups of brucellosis and control*

Groups	Parameters	TOC	OSI	TG	TC	HDL-C	VLDL-C	LDL-C
Brucellosis (n=22)	TAC	-0.101	**-0.680*****	0.437	0.120	-0.058	0.438	0.090
	TOC		**0.709*****	-0.211	-0.064	-0.165	-0.214	0.103
	OSI			**-0.482***	-0.231	0.088	**-0.484***	-0.183
	TG				0.178	0.303	**1.000*****	-0.196
	TC					0.064	0.178	**0.598****
	HDL-C						0.303	**-0.760*****
	VLDL-C							-0.197
Control (n=10)	TAC	0.034	-0.525	-0.400	-0.184	**-0.655***	-0.398	0.590
	TOC		**0.783****	0.445	-0.239	-0.254	0.444	0.082
	OSI			0.365	-0.192	0.298	0.362	-0.447
	TG				0.381	-0.091	**1.000*****	0.241
	TC					0.287	0.381	0.273
	HDL-C						-0.093	**-0.841****
	VLDL-C							0.243

** Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed); *** Correlation is significant at the 0.001 level (2-tailed)*

infection [7,24,26]. In studies conducted on patients with diagnosed brucellosis, serum TOC and OSI concentrations were reported to significantly increase, and TAC concentration was reported to decrease [10,11,21]. In this study, we found that *Brucella* significantly increase TOC (P=0.013) and OSI (P=0.002) concentrations in cattle. TAC concentration, on the other hand, was observed to decrease (P=0.007) which is in parallel with the previous studies. These findings indicate that *Brucella* infection impairs the oxidant-antioxidant balance in cattle. Brucellosis may lead to severe inflammation and cellular damage, and the phagocytic activity that is formed against the infection may alter TOC and OSI concentrations as well. These reactions formed are tried to be compensated by the organism, and that may be the reason of reduction in TAC concentration. The correlation observed between the oxidant-antioxidant mechanisms is the evidence of this situation *(Table 3)*.

Infection and inflammations may lead to changes in lipid and lipoprotein metabolism similar to that of the cytokines. Therefore, serum TC, HDL-C, LDL-C and TG concentrations are affected as well [25,27,28]. Especially, after the introduction of an infection or inflammation agent into the host, serum oxidized lipids are increased and LDL oxidation is started [29]. Changes in cholesterol levels observed during infections are demonstrated. However, the underlying mechanisms of these changes are yet uncertain [11,25]. Brucella species are facultative, intracellular pathogens among the phagocytic cells that can stay alive and reproduce. Oxidative catabolism by polymorphonuclear leukocytes and macrophages plays the primary role in the elimination of brucella [11]. At the same time, these bacteria can prevent the apoptosis of macrophages and weaken the immune response of the host organism [10]. As a result, infection and inflammation are related to the reduction in HDL-C levels [11] because it is well known that acute phase HDL cannot protect LDL against oxidation during infection and inflammation [10,11]. In a study conducted on sheep infected with *B. melitensis*, serum cholesterol and LDL levels were detected to increase [9]. In human, brucellosis has been reported to cause an increase in TC and LDL levels and a reduction in HDL levels [10,11,20]. In our study, lipid profile was observed to change as a result of brucellosis that TG, HDL-C and VLDL-C levels were decreased, and that TC and LDL-C were increased in cattle. These outcomes were in accordance with the data in literature.

There are several enzyme activities that protect HDL and LDL against oxidation such as paraoxonase (PON1). These enzyme activities were detected to be inversely related to oxidative stress. PON1 is a HDL-related antioxidant enzyme and there is positive correlation between them [10,11]. Furthermore, especially LDL was detected to be in correlative relationship of LDL with oxidative stress markers [25]. In our study, negative correlation was observed between TG and OSI (r= -0.47), and between VLDL-C and OSI (r= -0.48) in the group with brucellosis (*Table 3*). These findings indicate that lipid metabolism is related to oxidative damage and oxidative stress markers.

In conclusion, *Brucella* infection was detected to cause important changes in the oxidative-antioxidative capacity and lipid profile in cattle. These data obtained may contribute to define the severity of the inflammation and to its diagnosis in particularly this disease.

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Research Article

The Effects of Diets with Different Protein Contents on Growth Performance and Digestibility, and on Some Ruminal Fermentation and Blood Parameters, in Bafra Lambs [1] [2]

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Abstract

The objectives of this study were to determine the effects of diet with increasing dietary levels of crude protein (CP) on digestibility, rumen pH, growth performance, volatile fatty acids (VFAs) and ammonia nitrogen (NH_3-N), and on several blood parameters (serum urea, glucose and total protein), in the finishing period of Bafra lambs. Thirty male Bafra lambs, which were 3-3.5 months of age and average live weight of 24±0.4 kg, were divided into three groups (n=10 lambs per group) in a completely randomized design experiment. The diets were composed of 80% compound feed containing 11, 14 or 17% CP and 20% vetch straw. Lambs were fed ad libitium twice each day during the feeding trial period of 60 days. In the last week of the feeding period, all feces were collected to determine digestibility. The rumen and blood samples were collected at the end of the feeding period. The average feed intake was similiar among all tested diets. The best feed conversion rate was recorded the 17% CP diet. Final live weight and average daily gain (ADG) of lambs were significantly higher in lambs fed the 17% CP diet (P<0.05). CP digestibility was highest in the 17% CP diet (P<0.05) but the dry matter (DM) and organic matter (OM) digestibility were not affected by protein level. The rumen fluid NH3-N level increased significantly (P<0.05), pH and butyric acid level were not significantly affected, ruminal acetic acid and acetate:propionate ratio increased significantly (P<0.05) and propionic acid level decreased significantly (P<0.05) with the increase in dietary CP level. No significant differences were observed between groups for serum glucose and albumin, while the serum urea levels in the 14% and 17% CP groups were higher than for 11% CP (P<0.05). The highest economic value was for the 17% CP at 187.97%. Bafra lambs fed a diet containing 17% CP and vetch straw achieved a significantly higher body weight, had the best FCR and yielded a higher net profit than the other groups. If larger scale, on-farm studies confirm the findings of this study, Bafra lamb producers should be encouraged to maximise potential net profit by adopting its feeding regime, including 17% CP.

Keywords: *Bafra lambs, Crude protein level, Performance, Digestibility, Rumen fermentation, Blood parameters*

Farklı Düzeylerde Protein İçeren Konsantre Yem Karışımının Bafra Irkı Kuzularda Besi Performansı, Sindirilebilirlik, Bazı Rumen ve Kan Parametrelerine Etkisi

Özet

Bu çalışmada, kuzu bitirme karma yemlerinde artan düzeyde ham proteinin (HP) Bafra kuzularında sindirilebilirlik, rumen pH'sı, büyüme performansı, uçucu yağ asitleri (UYA), amonyak azotu (NH_3-N) ve bazı kan parametreleri (serum üre, glukoz ve total protein) üzerine etkileri araştırıldı. Araştırmada, 3-3.5 aylık yaşta, ortalama 24±0.4 kg canlı ağırlığında 30 adet Bafra erkek kuzusu kullanıldı. Araştırmada kuzular her bir grupta 10 adet olacak şekilde rastgele 3 gruba dağıtıldı. Deneme gruplarında kullanılan konsantre yemin ham protein (HP) içeriği %11, 14 ve 17 olacak şekilde düzenlendi. Her bir deneme grubu rasyonu %80 konsantre yem ve %20 fiğ samanından oluşturuldu. Kuzular günde iki kez olacak şekilde 60 gün süre ile ad libitum beslendi. Tüm deneme grupları arasında ortalama yem tüketimleri benzer bulundu. En iyi yemden yararlanma oranı (5.19) %17 HP içeren konsantre yem tüketen grupta kaydedildi. Ortalama besi sonu canlı ağırlık ve günlük canlı ağırlık artışları %17 HP içeren bitirme yemlerini tüketen grupta istatistiksel olarak önemli derecede (p<0.05) yüksek bulundu. Rasyonda HP düzeyinin artması KM ve OM sindirilebilirliğini etkilemedi fakat %17 HP içeren grupta HP sindirilebilirliği önemli derecede yüksek saptandı (P<0.05). Rasyonda artan HP düzeyinin ruminal NH3-N seviyesini yükselttiği (P<0.05), pH ve butirik asit seviyesini etkilemediği, asetik asit ve asetik asit:propionik asit oranını artırdığı (P<0.05), propionik asit oranını ise azalttığı (P<0.05) saptandı. Kan serum glikoz ve total protein düzeyi rasyonun HP düzeyinden etkilenmezken, üre düzeyi ise %14 ve 17 HP düzeylerinde %11 HP düzeyine göre yüksek bulundu (P<0.05). En yüksek ekonomik değere %187.97 ile %17 HP'li grupta saptandı. Bafra kuzularının %17 CP ve fiğ saman içeren rasyonla beslenmeleri, diğer gruplardan daha yüksek canlı ağırlık artışı, en iyi FCR ve net kâr sağlamıştır. Sonuç olarak, bu çalışmanın bulguları daha büyük ölçekli çiftlik çalışmaları ile desteklenirse, Bafra kuzusu yetiştiricileri maksimum net kar elde etmek için %17 HP içeren bitirme yemi ile besleme yapmaları yönünde teşvik edilebilir.

Anahtar sözcükler: *Bafra kuzusu, Ham protein seviyesi, Besi performansı, Sindirilebilirlik, Rumen fermentasyonu, Kan parametreleri*

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INTRODUCTION

In Turkey, which has a sheep population of approximately 31.50 million, the industry plays a crucial economic role [1]. Productivity is not only hindered by feed of low quality and quantity but also the low genetic potential of sheep. The Bafra sheep is from a Sakız x Karayaka cross and is a better than either parent in terms of yield characteristics, including fertility, milk yield and live weight [2]. This breed is widespread in the Black Sea Region of Turkey, especially in Samsun, Sinop, Ordu, Giresun, Tokat and Amasya Provinces. The majority of lambs born in spring and winter are fed under an intensive feeding system in order to meet the lamb demand in this region. The feeding of lambs on the basis of their nutritional requirements at different production stages is not a common system in Turkey. In addition, farmers tend to use feedstuffs with high energy content such as barley, corn or wheat in diets in order to obtain higher growth performance. Therefore, there is a need for supplemental protein to balance the diet and achieve optimum growth performance during the finishing period.

Balanced feeding in terms of energy and protein requirements optimizes animal growth and reproductive performance [3]. Reynal and Broderick [4] reported that a low protein diet may be unfavorable for microbial protein synthesis, ruminal digestion and the availability of nitrogen and energy. However, high levels of CP can lead to toxicity due to extreme release of ruminal ammonia [5].

The National Research Council (NRC) recommends a diet containing 14.5% CP for the maximum growth of lambs that are weaned early [6]. Several studies have been performed to determine the optimal dietary CP level for lambs. According to Andrew and Qrskov [7], the maximum nitrogen retention and live weight gain of bred lambs grown from 15 to 40 kg on a high level of nutrition was achieved at 17% dietary CP. Though feeding lambs an 18% CP diet is conventional practice, there was no difference between lambs fed 16 and 18% CP which both had a significantly higher body weight gain and DM intake than lambs fed a 10, 12 or 14% CP diet. The authors also emphasised that there were no benefits of using diets with higher than 16% CP [8-10]. Some studies have reported that DM and OM digestibility levels were not significantly different among different dietary levels of CP (10-17%), but CP digestibility levels were significantly higher in lambs feed higher CP levels [9,11,12]. Rumen NH_3-N and VFA levels increased with increasing dietary CP level [11,13-15]. Separately, it has been reported that there was no significant effect of dietary CP level on glucose and total serum protein levels, except for urea [8,16].

Many studies have been conducted on the effects of different dietary protein levels on rumen fermentation, digestibility and growth performance in weaned lambs. However, there is no data on the nutritional requirements specific to the Bafra lamb of Turkey. Therefore, the aim of this study was to investigate the effects of different dietary CP levels on rumen fermentation, digestibility and growth performance, and on some blood parameters, of weaned Bafra lambs.

MATERIAL and METHODS

The experiment was conducted at a private farm located in the Bafra district of Samsun Province, Turkey, from June 15 to July 16, 2016. The use and handling of the animals for this study was approved by the ethics council of Ondokuz Mayıs University (2015-08/71).

Animals, Diets and Experimental Procedures

Thirty male, Bafra lambs from 3 to 3.5 months of age and with mean, initial live weight of 24±0.4 kg were used in the experiment. The lambs were dewormed and vaccinated for common viral diseases before the trial which was conducted as a randomised block design. The thirty lambs were divided into three groupsof 10 lambs. The diet with 11% CP mostly consisted of maize. Protein supplements were added to the other diets to obtain 14 and 17% CP levels. The diets were formulated to be nearly isocaloric (2700 kcal/kg). Concentrates were prepared in a commercial feed manufacturing factory as a mash feed. The diets of the three groups were composed of 80% compound feed which included 11, 14 or 17% CP *(Table 1)*

Table 1. *Components and chemical composisition of the experimental diets*

Item	CP Levels (%)		
	11	**14**	**17**
Vetch straw	20.0	20.0	20.0
Maize	0	10.4	9.6
Barley	54	36.4	36.0
Wheat bran	20.8	16.8	8.8
Soybean meal	0	5.6	10.4
Canola meal	0	3.2	7.2
Molasses	3.2	5.6	6.0
Salt	0.8	0.8	0.8
Limestone	0.8	0.8	0.8
Vitamin and mineral mix[1]	0.4	0.4	0.4
Chemical composition			
CP (%)	11.3	13.7	16.0
Ruemen undegrable protein[2], (% of dietary CP)	22.5	27.5	29.4
ME[2] (kcal/kg)	2.507	2.504	2.503

[1] 50.000 mg Mn, 50.000 mg Fe, 50.000 mg Zn, 10.000 mg Cu, 800 mg I, 150 mg Co, 200 mg Se, 50.000 mg Mg, 460.000 mg $CaCO_3$, 10.000 mg Antioxidant, 10.000.000 IU vitamin A, 2.000.000 IU vitamin D_3, 30.000 IU vitamin E: [2] Calculated value

and 20% vetch straw. Fresh water was available to all animals at all times.

Lambs were fed as groups and were adapted to the feeding regime for 10 days before the experiment started. The experimental period for each group was 8 weeks. Diets were provided in equal amounts at 07:00 h and 16:00 h. The quantity of the daily diet was adjusted every day according to the previous day's feed intake by increasing at a rate 10%. Provided and refused feed were measured daily and mean feed intake was calculated as the difference between the two measurements divided by ten. The live weights of lambs were recorded before feeding in the morning on day one and on the final day (60^{th} day) of the study.

For the *in vivo* digestibility trial, five lambs in each group were placed in individual pens at the end of the final week of the experiment. Total fecal output was determined for 5 days. Individual animal's feces was weighed daily and a 10% portion taken, homogenised and frozen. The homogenised feces were then dried in a forced air oven at 60ºC for 48 h. DM, OM and CP digestibility were determined with the method of Kaya et al.[11].

On the final day of the experiment, rumen fluid samples were collected in tubes from seven lambs from each group with the aid of an oral stomach tube, 2 h after the morning feeding. The samples were collected in a manner that ensured that they were not contaminated with saliva. The pH was measured immediately with a pH meter. Samples were then filtered through four layers of cheesecloth to remove the solid, unfermented particles. For VFA analysis, a 10 mL aliquot of ruminal fluid was acidified with 2 mL of 25% metaphosphoric acid and centrifuged at 5.000 rpm for 10 min. A 1.5 mL portion of the supernatant was frozen at –20°C for subsequent analyses [17]. In addition, 10 mL subsamples of the strained rumen fluid were preserved by the addition of 0.2 mL 50% H_2SO_4 to stop bacterial activity and preserve the NH_3-N, and stored at –20°C until analysis [18].

At the end of the feeding trial, blood samples were collected immediately before the morning feeding from each lamb via jugular venipuncture into non-heparinized tubes. The samples were centrifuged at 1.000 × g for 15 min at 4°C. The serum was separated from the collected blood, then transferred into a polypropylene tube and stored at -18ºC until analysis.

Chemical Analyses

Samples of the feed rations and the feces were used to determine DM, OM, CP and ash, according to the methods described by the AOAC [19]. Ruminal NH_3-N was determined with the macro-Kjeldahl procedure [19]. For VFA analysis, a method was used as described previously [20] with some modifications, by using a gas chromatograph (GC 17A, Shimadzu, Kyoto, Japan) equipped with a Flame Ionisation Detector (FID). Just before analysis, the rumen samples were thawed and centrifuged at 4.000 *g* at 0°C for 30 min. The clear supernantant was diluted by a factor of 10 in ultrapure water, then filtered through a 0.45 µm PVDF disk filter before injection into the gas chromatograph. A TRB-FFAP capillary column (30 m×0.25 mm×0.25 µm, Teknokroma, Spain) was used. The carrier gas was nitrogen at a flow rate 5.05 ml/min; the column oven temperature range was 60-200°C (20°C increase/min), then held for 5 min at 200°C (total analysis time: 12 min); the injector temperature was 250°C; and the detector temperature was 300°C. The 1 µL samples were injected into the GC system with an autosampler (AOC 5000 plus, Shimadzu, Kyoto, Japan) at a 1:20 splitting ratio. Serum blood samples were analysed for total protein, glucose and urea concentration by using an autoanalyzer (Mindray BS120).

Economic Analysis

Economic analysis of the data was performed with the method of Mirza *et al*.[21]. The cost of an 11% CP diet was taken to be 0.845 TRY/kg, that of the 14% CP diet was 0.925 TRY/kg, that of the 17% CP diet was 1 TRY/kg, and that of of vetch straw was 0.4 TRY/kg, in 2016. 1 US Dollar was valued at about 2.97 TRY in September, 2016 and the live weight value was 14 TRY/kg.

Statistical Analyses

Date regarding various parametrs were analysed using one-way ANOVA in the SPSS 21.0 software package [22]. Differences were considered significant at 5% ($P<0.05$), and the comparison of means was carried out with the Duncan test.

RESULTS

Growth Performance and Digestibility

The effects of CP level in the diet on growth performance, digestibility, ruminal fermentation and blood parameters, and the economic analysis, are shown in *Tables 2, 3, 4,* and *5*. Increased dietary protein increased feed consumption, final live weight, average daily gain (ADG) and FCR *(Table 2)*. The lowest feed consumption was observed in the 14% CP group but it was not evaluated statistically because lambs were fed by group in the experiment. Final live weight and ADG were significantly higher for the 17% CP diet than for the 11 and 14% CP diets ($P<0.05$). At the end of the feeding trial, the average live weights for the 11, 14 and 17% CP groups were 33.66, 36.38 and 38.77 kg, respectively. ADG was highest ($P<0.05$) in lambs fed 17% CP (241.81 g), followed by 14% CP (213.58 g) and 11% CP (156.16 g). The average daily feed consumption in the 11, 14 and 17% CP groups was 1221.7 1215.2 and 1254.1 respectively. The highest and the lowest FCRs were observed in the 17 and 11% CP groups, respectively. Although there was

Table 2. *Effect of CP levels on growth performance and digestiblitiy*

Item	CP Levels (%)			P
	11	14	17	
Initial weight (kg)	24.29±0.33	24.07±0.40	24.27±0.35	NS
Final weight (kg)	33.66±0.39^{c}	36.88±0.65^{b}	38.77±0.28^{a}	*
Average daily gain (g)	156.16±2.40^{c}	213.58±4.62^{b}	241.81±4.07^{a}	*
Feed intake (g/day)[1]	1221.78±21.89	1215.25±31.39	1254.15±25.63	
Feed conversion ratio[2]	7.35	5.66	5.19	
Digestibility (%)				
DM	74.61±0.77	75.28±0.53	74.83±0.95	NS
OM	73.19±1.02	74.37±1.69	73.50±2.49	NS
CP	70.54±1.91^{c}	74.01±2.92^{b}	78.73±0.59^{a}	*

^{a,b,c} The groups in the same row labeled different letters are statistically significant (P<0.05)
[1] it was not evaluated statistically because lambs were fed by group in the experiment; [2] Feed conversion ratio= (g feed intake/g body weight gain); ***NS:*** *Non-significant, * P<0.05*

Table 3. *The effect of protein levels on ruminal pH, NH_3-N and VFA concentration*

Item	CP Levels (%)			P
	11	14	17	
pH	6.47±0.7	6.54±0.08	6.51±0.04	NS
NH_3-N (mmol L^{-1})	131.5±6,85^{c}	157.00±6.39^{b}	179.42±3.41^{a}	*
VFA (mmol L^{-1})				
Acetic acid	53.02±0.64^{c}	56.81±0.41^{b}	59.91±1.06^{a}	*
Propionic acid	27.68±0.37^{a}	25.35±0.33^{b}	22.69±1.03^{c}	*
Butyric acid	12.87±0.23	13.20±0.20	13.14±0.18	NS
Acetate:propionate	1.91±0.02^{c}	2.24±0.76^{b}	2.66±0.09^{a}	*

^{a,b,c} The groups in the same row labeled different letters are statistically significant (P<0.05)
NS: *Non-significant, *P<0.05,* ***VFA:*** *Volatile fatty acid*

Table 4. *The effects of CP levels on some blood parameters*

Item	CP Levels (%)			P
	11	14	17	
Total protein (g/dL)	6.22±0.29	6.73±0.27	6.86±0.19	NS
Glucose (mg/dL)	76.63±0.45	76.31±0.36	76.29±0.33	NS
Urea (mg/dL)	33.36±0.57^{b}	51.35±0.72^{a}	54.23±0.91^{a}	*

^{a,b} The groups in the same row labeled different letters are statistically significant (P<0.05)
NS: *Non-significant, * P<0.05*

no significant difference between the groups in terms of DM and OM digestion, the CP digestion level increased significantly with the dietary protein level (P<0.05).

Rumen Parameters

The rumen fermentation parameters are presented in *Table 3*. The NH_3-N level significantly increased with CP content (P<0.05) but ruminal pH values were not significantly affected by CP content. VFA concentrations, except for butyric acid, were significantly affected by dietary protein level (P<0.05). The molar percentage of propionic acid decreased linearly (P<0.05) with increasing protein level, while the acetic acid concentration increased (P<0.05). Increasing the CP level from 11 to 17% caused the acetate to propionate ratio to significantly increase (P<0.05) from 1.91 to 2.66.

Blood Parameters

Serum urea levels of the 14 and 17% CP groups were similar and significantly higher than for the 11% CP group

Table 5. *Economic analysis of live weight gain*

Item	CP Levels (%)		
	11	14	17
Feed intake			
Protein supplement intake (g/head/day)	990	988	1022
Straw intake (g/head/day)	231	226	232
Total feed intake (supplement+straw)(g/head/day)	1221	1215	1254
Cost of feed intake[1] (TRY/head/day)			
Protein supplement	0.83	0.91	1.022
Straw	0.09	0.09	0.09
Total cost	0.92	1.0	1.11
Average liveweight gain (g/head/day)	156	213	254
[2]Total benefit of liveweight gain at TRY/14/kg (TRY/head/day)	2.18	2.98	3.55
Net benefit (TRY/head/day)	1.26	1.98	2.44

[1] 11% CP concentrate 0.845 TRY/kg, 14% CP concentrate 0.925 TRY/kg, 17% CP concentrate 1.0 TRY/kg, straw: 0.4 TRY/kg in year 2016
[2] Price of one kilogram live body of lamb was 14 TRY in year 2016

(P<0.05) but there were no significant differences in serum TP and glucose levels for lambs fed 11, 14 or 17% CP *(Table 4)*.

Economic Analysis

The economic analysis is shown in *Table 5*. The net benefit of diets with different protein contents was calculated by giving a value of 14 TRY per kilogram of body weight gain and subtracting the expenditure on feed from that value. The net benefit was highest in lambs fed 17% CP (2.44 TRY/day), followed by 14% CP (1.98 TRY/day) and 11% CP (1.26 TRY/day).

DISCUSSION

In this study, the feed intake of the three experimental groups ranged from 1.215 to 1.254 g/day. These results support those of Bilal et al.[8] who reported that the lowest dry matter intake was observed at the 10% CP level. Also, many researchers have reported no effect of dietary CP content on DMI [23,24]. In contrast, Drouillard et al.[25] reported a 7% increase in DMI when lambs were fed a 14.5% CP diet compared with an 8.9% CP diet. Furthermore, Fluharty and McClure [26] demonstrated an increase in DMI when lambs were fed a high protein diet (18.9% CP) compared with a 14.5% CP diet. Also, Haddad et al.[9] reported an incremental increase in DMI as dietary CP content increased, being highest for 16 and 18% CP diets. In the present study, the 17% CP diet resulted in the best FCR, followed by 14% CP and 11% CP. The better FCR attained by feeding the 17% CP diet may have been due to the more efficient utilization of protein and metabolism of energy sources for growth. These results are similar to the findings of Mahmoud [23], who demonstrated a significantly higher FCR for 14% and 17% CP diets than for an 11% CP diet. Also, studies by Kebede [27] and Abebe et al.[28] reported an improvement in the feed utilization and growth performance of Arsi-bale lambs at higher protein levels.

In the current study, the average final weight was highest (P<0.05) for the 17% CP diet, followed by 14 and 11% *(Table 2)*. Improved growth performance was also reported at higher dietary CP levels (16 to 18%) than at lower CP levels (10 to 14%) by Awassi [9,29] and in Kıvırcık lambs [8]. Similarly Kaya et al.[11] and Abbasi et al.[30] reported higher growth for Morkaraman and Kooka lambs, respectively, at higher CP (16%) than at a lower level of dietary CP.

In contrast to the current study, Dabiri and Thonney [12] stated that there was no difference in live weight gain between groups fed 13, 15 or 17% CP for 42 days. Also, in a study performed on Arsi-bale lambs fed different levels of CP (9.4, 10.2, 11, 12 and 13.1%), there was no difference in final live weight [27]. In the present study, the differences in final weight are attributable to differences in the mean daily weight gain of the lambs. ADG was higher in lambs fed with 17% dietary CP diet (241.81 g) than 14% (213.58 g) (P<0.05), followed by 11% CP (156.16 g) *(Table 2)*. The highest mean daily gain and final live weight obtained with the 17% CP diet may have been due to the increased CP creating a better balanced diet with a more appropriate ratio of energy and digestible protein for growing lambs. Furhermore, the higher grow rate or live weight gain with 17% CP diet may be due to higher amount of RUP content and amino acid profile of soya bean meal and canola meal.

The DMs and OMs of the three dietary CP levels were not significantly different *(Table 2)*, but the digestibility of CP in the 11% CP group was significantly lower than that of other treatments (P<0.05). The higher CP digestibility for the lambs that were fed 17% CP supports the findings of higher digestibility of CP and rumen degradable protein

(RDP) in diets with the highest level of CP [29]. Similarly, Kaya et al.[11] reported no significant difference between groups for DM and OM digestibility but the CP digestibility level significantly increased with increasing dietary protein level. Moreover, these results are similar to the findings of Dabiri and Thonney [12] who found no differences in DM and OM digestiblity levels but higher CP digestibility in the 17% CP group than in the 13% CP group. Contrary to our results, there are other studies reported that dietary CP content increases DM and OM digestion [9,31] or that CP does not affect digestion efficency [32]. In the current study, it is possible that the lowest CP level (11%) limited the population growth and total activity of rumen microbes, whereas the higher CP levels may have induced increased microbial protein synthesis and fermentation, as suggesteded by Sharifi et al.[32].

In the current study, the ruminal pH value was not significantly affected by increasing CP level (P>0.05). pH values appear to be optimum between 6.5 and 7 for the microbial digestion of cellulose and protein [33]. This may have been due to increased rumination during the night, roughage intake in the early mornings, and nitrogen influx into the rumen when nitrogen concentrations were low [34]. The results of the present study are in agreement with several *in vitro* studies [15,34-36]. In the present study, each increase in the level of dietary CP was accompanied by a significantly higher ruminal NH_3-N concentration (P<0.05). Similiar results were reported by Sarwar et al.[13], Abadi et al.[14] and Yang et al.[15]. However, Dutta et al.[3], Vosooghi-poostindoz et al.[35] and Chanthakhoun and Wanapat [37] reported that increasing the CP level from 12 to 14.1% had no significant effect on the NH_3-N level in the rumen; they all concluded that this could have been due to a higher level of incorporation of NH_3-N in the microbial protein. Difference in the CP content among treatments is the primary reason for the observed differences in ruminal NH3-N concentration [33]. In the present study, the increase in rumen NH_3-N is probably due to an increase in proteolysis, the degradation of peptides and the deamination of amino acids in the rumen, as reported by Sharifi et al.[32].

The level of VFA production in the rumen is influenced by the carbohydrate fraction and degradability [38]. In the present study, the molar concentration of acetate and the acetate/propionate ratio in the ruminal fluid of Bafra lambs fed with a 17% CP diet were significantly higher than for those fed with 11 and 14% CP diets *(Table 3)* (P<0.05), which might be due to the availability of higher amounts of protein in the diet promoting the growth of ruminal bacteria that subsequently produce acetate by degrading cellulose [37]. In addition, heigher feed inateke of vetch straw in 17% CP group may be lead to change of VFA concentration. In contrast, the propionate concentration was higher when lambs were fed a diet with lower CP (11%). Considering that the ruminal concentration of VFAs at a particular time is the net result of their production and absorption, the lower propionate concentration in the other two groups (14 and 17% CP) in the present study may be explained by the higher proportion of barley (54%) in the 11% CP diet. It has been reported that corn is a fermentation substrate for the VFAs and therefore may increase propionate production when the lambs are fed with a lower CP content [15]. The results of our study contradicted the results of two studies in which the effects of different protein levels on rumen fermentation were investigated [11,14] but similar results were reported by Yang et al.[15] who stateded that lambs fed diets with increased protein levels had a lower propionate and higher acetate concentration.

Diseases and nutritional disorders can induce changes in blood parameters. The evaluation of the effects of dietary CP level on blood metabolites is therefore important [8]. The normal serum TP [39] and glucose [8] levels in sheep range between 5.5 and 7.5 and 44.0 and 81.2 mg/dL, respectively. In the current study, serum TP and glucose levels were in the normal range and there were no significant differences in TP and glucose levels in lambs fed with an 11, 14 or 17% CP diet *(Table 4)*. These results were compatible with those obtained by Keser et al.[8], Shalu et al.[16] and Hatfield et al.[40]. Urea is a key metabolite produced from ammonia in the liver during protein metabolism [8]. The levels of plasma urea and the dietary protein level were correlated in sheep [41]. The normal urea levels of lambs was reported as 20-30 mg/dL [42]. The urea levels in the present study were higher than the normal range *(Table 4)*. However, the increases in serum urea levels in the present study due to an increased dietary protein level are in a line with the results of previous studies [8,16,40,43].

In the present study, the cost-benefit analysis demonstrated a highest economic benefit of 2.44 TRY/day for the 17% CP diet, followed by 1.98 TRY/day for the 14% CP diet and 1.26 TRY/day for the 11% CP diet. The differences in economic benefit among the groups was due to the extra live weight gain attributable to the increase in the protein level of the diet. Similar results have been found in many studies conducted with lambs of various breeds fed different protein levels [30,44].

In the current study, Bafra lambs fed a diet containing 17% CP achieved the highest live weight and FCR and generated the highest net profit. It is therefore concluded that Bafra lamb producers should be encouraged to maximise the potential net profit by adopting the 17% CP feeding regime used in the present study, if the results of the current study are confirmed by a larger scale, on-farm study.

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Research Article

Determination of Virulence Factors of Staphylococci Isolated from Bovine Mastitis [1]

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Abstract

Staphylococcal mastitis is one of the major economic problems of cattle. The aim of this study was to identify *Staphylococcus* species that cause mastitis, to examine the virulence factors of these species and to determine the relation between these factors with the pathogenic and nonpathogenic species. In the study, 37 *S. aureus,* 13 *S. hyicus,* 9 *S. simulans,* 8 *S. chromogenes,* 5 *S. lentus,* 5 *S. epidermidis,* 2 *S. haemolyticus,* 2 *S. hominis,* 1 *S. auricularis,* 1 *S. warneri,* 1 *S. sciuri* were isolated and identified. The 41.6% of strains were determined as coagulase positive. In the coagulase positive strains, the rate of protein A, DNase, TNase, capsul, hemolyse, staphylokinase, slime (in agar), biofilm (microdilution) and hemagglutination characteristics were found 71.4%, 48.6%, 11.4%, 40%, 97.1%, 40%, 28.6%, 37.1% and 17.1%, respectively. In the coagulase negative strains, the rate of these characteristics were found 10.2%, 12.2%, 2%, 8.2%, 82%, 32.7%, 12.2%, 12.2% and 10.2%, respectively. The methicillin resistance rates in the coagulase positive and negative strains were determined as 2.9 and 16.3%. In conclusion, it was of the opinion that animals are potential carriers of staphylococcus strains that are pathogen for human.

Keywords: *Bovine, Mastitis, Staphylococci, Virulence*

Sığır İzolatı Stafilokokların Virülens Faktörlerinin Belirlenmesi

Özet

Stafilokokal mastitisler sığırlar için en önemli ekonomik problemlerden birisidir. Bu çalışmada mastitise neden olan stafilokok türlerinin sahip olduğu virülens faktörlerinin belirlenmesi ve patojenik ile apatojenik türler arasında bu faktörlerin ilişkisinin araştırılması amaçlanmıştır. Çalışma kapsamında 37 *S. aureus,* 13 *S. hyicus,* 9 *S. simulans,* 8 *S. chromogenes*, 5 *S. lentus,* 5 *S. epidermidis,* 2 *S. haemolyticus,* 2 *S. hominis,* 1 *S. auricularis,* 1 *S. warneri* ve 1 *S. sciuri* izole ve identifiye edilmiştir. Bu izolatların %41.6'sı koagülaz pozitif olarak belirlenmiştir. Koagülaz pozitif izolatların protein A, DNase, TNase, kapsül, hemoliz, stafilokinaz, slaym (agar'da), biyofilm (mikrodilüsyon) ve hemaglütinasyon özellikleri sırasıyla %71.4, %48.6, %11.4, %40, %97.1, %40, %28.6, % 37.1 ve %, 17.1 olarak tespit edilmiştir. Koagülaz negatif izolatlarda ise bu oranlar sırasıyla %10.2, %12.2, %2, %8.2, %82, %32.7, %12.2, %12.2 ve %10.2 bulunmuştur. Koagülaz pozitif ve negatif izolatlar arasında metisilin direnç oranı %2.9 ve %16.3 olarak saptanmıştır. Sonuç olarak, stafilokok taşıyıcısı hayvanların insanlar için muhtemel infeksiyon kaynağı olabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Sığır, Mastitis, Stafilokok, Virulens*

INTRODUCTION

Microbial originated mastitis is considered to be a disease of cattle that causes the vast yield loss in the whole world. Mastitis may occur as acute and chronic with regard to its clinical and subclinical character and yields loss in cattle. Mastitis infections may also end up with economic and production losses and treatment costs can pose a risk in terms of human health. In a review of the etiology of mastitis, these cases have been reported to be isolated from the more than 150 species of bacteria [1]. Clinical signs of mastitis are not specific for the etiologic agents with few exceptions, so it is important to distinguish microbial origin for the treatment of mastitis. Staphylococci are on the first

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line of mastitis agents and incidence of staphylococcal mastitis is seen in the ratio of 60-70% [2,3].

Microorganisms in staphylococcal infections by binding to the cell surface are dependent on the ability to bear the surface adhesins to colonize tissues. Adhesins of staphylococci, which are the specific receptors for extracellular matrix proteins of the host tissues, are surface proteins. These include fibronectin, fibrinogen, collagen, elastin, a plasminogen, and vitronectin. Staphylococci bare facilitating connection to the traumatized tissue and fibrin blood clots promoted by fibrinogen binding protein (Fbp). In most strains of *Staphylococcus aureus*, there are both fibronectin and fibrinogen binding protein [4]. *S. epidermidis* has solely Fb, a single fibrinogen binding protein. This protein is similar to the fibrinogen receptor of *S. aureus*.

In Turkey, although there are studies dealing with the prevalence of mastitis for the role of *Coagulase Negative Staphylococci*, the studies about virulence factors and their epidemiological aspects are insufficient. In this study, staphylococcal mastitis in cattle in province of Aydin was aimed to be examined in detail. The scopes of this study include precise identification of *Staphylococcus* species, identification of virulence factors, the relationship between nonpathogenic species and epidemiology of infection also.

MATERIAL and METHODS

In this study, 84 Staphylococci strains isolated from milks samples of 120 cows with clinical and subclinical mastitis and 80 healthy cows in different herds in the Aydın region were examined. Milk samples were evaluated by California Mastitis test. The stage of mastitis infection was determined according to the California Mastitis test procedures.

Isolation and Identification of Staphylococci

Milk samples were taken with sterile loop in the volume of 50 µL and inoculated onto Blood Agar (Bacto agar, Difco®, Detroit). The culture media were incubated at 37°C for 24-48 h. The colony morphology of the isolates were examined and staphylococci like (creamy and white or yellow pigmented) colonies were separated for further identification.

The isolated bacterial strains were identified as *Staphylococcus* sp., by the fact that catalase test positive, oxidase test negative and oxidation/fermentation (OF) test positive [5]. Species-specific identification was carried out with a commercial identification kit (Api-Staph, Bio Merieux®, Lyon, France). *Staphylococcus* species were identified with test procedure recommended by the manufacturer. *Staphylococcus* strains were evaluated by the utilization of D-glucose, D-fructose, D-mannose, D-maltose, D-lactose, D-trehalose, D-mannitol, xylitol, D-melibiose, D-raffinose, D-xylose, D-saccharose, methyl a-D-glucopyranoside, N-acetyl-glucosamine acid production, nitrate reduction, and alkaline phosphatase, acetyl-methyl-carbinol, arginine production was examined dihydrolase and urease tests.

Determination of Virulence Factors

The virulence factors of the *Staphylococcus* isolates were determined with regard to coagulase, protein A, DNase, TNase, capsule, slime and biofilm, hemolytic activity, staphylokinase, hemagglutination, methicillin resistance tests.

Coagulase test was performed via tube and slide coagulase tests. In tube coagulase test, 0.5 mLrabbit plasma (bioMerieux®) and 0.1 mL broth of bacterial culture were added together and coagulation was determined as positive reaction. Clumping factor was determined in slide coagulase test, as 1 loop of bacterial colony was homogenized with 50 µL physiological saline, and then 50 µL of rabbit plasma (bioMerieux®) was added. The developing coagulation was determined as positive reaction [5].

Protein A test was applied via commercial latex agglutination test (Staphytect Plus, Oxoid®) and performed by manufacturer's recommendation.

DNase test was performed by inoculating bacterial strains onto DNase agar. After incubation of 3 days at 37°C, one droplet of 1 N HCl was added onto agar plate, a wide pale formation on the agar was determined as positive reaction [5].

For TNase test, agar containing DNA and 1% toluidine blue was prepared. 100 µL of bacterial culture was added onto the wells on TNase agar. After incubation period, brilliant pink colour formation was determined as positive reaction [5].

Capsule formation was determined via Duguid staining method [6].

Slime formation was determined via development of black colonies on Congo Red agar [7].

Biofilm formation was determined via staining of safranine in microplates [8].

Rabbit and horse erythrocytes were used for hemolytic activity tests.

Staphylokinase activity was determined via screening of pale zone on agar that contains fibrinogen and dog serum [3].

Hemagglutination activity was determined via agglutination of erythrocytes on microplate wells [9].

Methicillin resistance was determined via disc diffusion method on Brain-Heart infusion agar [5].

Statistical Evaluation

The relationship among Coagulase positive, Coagulase

negative strains and virulence factors were statistically evaluated by Chi-square test.

RESULTS

Isolation and Identification of Staphylococci

As a result of isolation procedures in a selective and differential medium, a total of 84 *Staphylococcus* strains were isolated from 120 mastitic milk samples and 80 normal milk samples. The isolated strains were identified as in the number of 37 *S. aureus*, 13 *S. hyicus*, 9 *S. simulans*, 8 *S. chromogenes*, *S. lentus* 5, 5 *S. epidermidis*, 2 *S. haemolyticus*, 2 *S. hominis*, 1 *S. auricularis*, 1 *S. warneri* and 1 *S.* sciuri *(Table 1)*.

The virulence factors of isolates from animals with mastitis and healthy animals were shown in *Table 2* and *Table 3*.

Statistics

Coagulase positive and coagulase negative strains with Chi-square test of significance is based on their virulence factors are given in *Table 4*.

Table 1. *Staphylococcus species isolated from healthy and sick animals*

Strains	Isolation Number	Isolation from Healthy Animals		Isolation From Sick Animals	
		n	%	n	%
S. aureus	37	2	5.4	35	94.5
S. hyicus	13	0	0	13	100
S.simulans	9	0	0	9	100
S. chromogenes	8	5	62.5	3	37.5
S. lentus	5	5	100	0	0
S. epidermidis	5	1	20	4	80
S. haemolyticus	2	0	0	2	100
S. hominis	2	1	50	1	50
S. auricularis	1	1	100	0	0
S. warneri	1	1	100	0	0
S. sciuri	1	0	0	1	100

Protein, DNase and the results of the capsule test were found to be very important. TNase, hemolysis, slime and biofilm formation test results were determined to be significant when the coagulase positive and negative strains are evaluated together. Analyses of staphylokinase and hemagglutination tests were not found significant statistically.

DISCUSSION

Bovine mastitis infections cause heavy economic losses worldwide. Mastitis in Turkey is one of the most important problems of dairy cattle economically. Especially mastitis incidence can be observed up to 40% in dairy industry. Primary factors of mammary infections are often characterized in clinical or subclinical forms of Gram positive bacteria. Staphylococcal mastitis presents the first in line to 60-70%. Staphylococci are resistant bacteria commonly found in nature and natural conditions [2,3]. In this study, a detailed examination of staphylococcal mastitis in cattle is basically intended.

In this study, 84 staphylococcal strains were isolated in the ratio of 44 % *S. aureus*, 15,47%, *S. hyicus*, 10.7% *S. simulans*, 9.5% *S. chromogenes*, 5.95% *S. lentus*, 5.95% *S. epidermidis*, 2.38% *S. haemolyticus*, 2.38% *S. hominis*, 1.19% *S. auricularis*, 1.19% *S. warneri* and 1.19% *S. sciuri*, respectively. A total of 68 strains were identified from mastitis milk samples in the ratio of 51.4% *S. aureus*, 19.1% *S. hyicus*, 13.2% *S. simulans*, 4.4% *S. chromogenes*, 5.8% *S. epidermidis*, 2.9% *S. haemolyticus*, 1.4% *S. hominis*, 1.4% *S. sciuri*, respectively. The species isolated in this study and their isolation rates were found to be consistent with those of other studies in which isolation of *Staphylococcus* spp. from mastitis cases was reported [10-13].

S. aureus is regarded as pathogenic according to the conventionally coagulase pathotyping feature. However, CNS strains may also be isolated from clinical cases of mastitis [3]. In this study, 41.6% of isolates were CPS. CNS strains were identified in the ratio of 58.3% and found at a higher rate from mastitis milk samples. In this study,

Table 2. *The virulence factors of staphylococci isolated from animals with mastitis*

Strains	n	Clumping Factor n (%)	Coagulase n (%)	Protein A n (%)	DNase n (%)	TNase n (%)	Capsule n (%)	Slime n (%)	Biofilm n (%)	Hemolysis n (%)	Staphylokinase n (%)	HA n (%)
S. aureus	35	28 (80)	28 (80)	30 (85)	21 (60)	3(9)	18 (51)	5 (14)	13 (37)	34 (97)	8 (23)	5 (14)
S.hyicus	13	7 (54)	7 (54)	0	1 (8)	2(15)	0	10 (77)	2 (15)	13 (100)	12(92)	2 (15)
S.epidermidis	4	0	0	0	0	0	0	1 (25)	3 (75)	4 (100)	1(25)	3 (75)
S.chromogenes	3	0	0	0	0	0	0	0	1 (33)	3 (75)	2(67)	1 (33)
S.sciuri	1	0	0	0	1 (100)	0	0	0	0	1 (100)	0	0
S.simulans	9	0	0	0	0	0	0	0	0	9 (100)	0	0
S.hominis	1	0	0	0	0	0	0	0	0	1 (100)	0	0
S.haemolyticus	2	0	0	0	0	0	0	0	1 (50)	2 (100)	1 (50)	0
Total	68	35 (51)	35 (51)	30 (44)	23 (34)	5(7)	18 (26)	16 (24)	20 (29)	67 (99)	24 (35)	11 (16)

Table 3. *The virulence factors of staphylococci isolated from healthy animals*

Strains	n	Clumping Factor n (%)	Coagulase n (%)	Protein A n (%)	DNase n (%)	TNase n (%)	Capsule n (%)	Slime n (%)	Biofilm n (%)	Hemolysis n (%)	Staphylokinase n (%)	HA n (%)
S. aureus	2	0	0	0	0	0	0	0	0	0	0	0
S. epidermidis	1	0	0	0	0	0	0	0	0	1 (100)	1 (100)	0
S. lentus	5	0	0	0	0	0	0	0	0	1 (20)	1 (20)	0
S. chromogenes	5	0	0	0	0	0	0	0	0	4 (80)	3 (60)	0
S.warneri	1	0	0	0	0	0	0	0	0	0	0	0
S.hominis	1	0	0	0	0	0	0	0	0	0	0	0
S. auricularis	1	0	0	0	0	0	0	0	0	1 (100)	1 (100)	0
Total	16	0	0	0	0	0	0	0	0	7 (44)	6 (38)	0

Table 4. *Statistical analyses of virulence factors of staphylococci*

Virulence Factors	Results (n)				P*
	Positive		Negative		
	CoPS	CoNS	CoPS	CoNS	
Protein A	25	5	10	44	0.001
DNase	17	6	18	43	0.001
TNase	4	1	31	48	0.01
Capsule	14	4	21	45	0.001
Slime	10	6	25	43	0.05
Biofilm	13	6	22	43	0.01
Hemolysis	34	40	1	9	0.01
Staphylokinase	14	16	21	33	-
Hemagglutination	6	5	29	44	-

** Statistical analyses: 0.01, not significant; 0.05, significant; 0.001, very significant;* ***CoPS:*** *Coagulase positive strains;* ***CoNS:*** *Coagulase negative strains*

the prevalence of CNS was determined through an epidemiological aspect.

The virulence factors that express toxic, proteolytic and adhesive property were determined in strains of *S. aureus*. Staphylococcal protein A is a surface molecule found more than 95% of *S. aureus* strains [14]. In this study, the presence of protein A was determined only in *S. aureus* strains. These strains were identified as coagulase positive in the ratio of 83%.

Although the presence of DNase activity is a debated issue, Quinn et al.[15] expressed that DNase production could be regarded as a pathogenicity factor for Staphylococci from bovine clinical samples. In a previous study, it was determined that only *S. chromogenes* showed positive activity with regard to DNase production of strains isolated from bovine milk [16]. In our study, *S. aureus* strains expressed DNase and TNase activity. However, in this study, DNase production rate of *S. aureus* strains was found in the ratio of 60%. This positive result is observed to be a lesser extent in comparison with another study [17].

Capsule formation plays quite a great role in the formation of staphylococcal mastitis. *S. aureus* isolates have capsular polysaccharides in the ratio of 90% and 11 serotypes have been identified before [18]. Capsule has been proposed as an adherence factor for *S. epidermidis* [19]. In a survey research conducted in the United States, the prevalence of capsular serotypes was determined from *S. aureus* strains isolated from bovine mastitis. Compared with the predominant serotype 5 in France, the dominant group in the United States was determined as serotype 8 [20]. It has been shown that CNS *(S. haemolyticus*, *S. hyicus*, *S. simulans*, *S. warneri*, *S. lentus)* (15.5%) have capsule in less proportion [21]. In this study, capsule formation was determined in the ratio of 48.6%. This result is in line with the literature data obtained before, since the presence of capsular polysaccharide indicated mostly in *S. aureus* strains. Biofilm formation was indicated as the most important virulence factor in *S. epidermidis* and other CNS isolated from infections caused by medical instruments. In a study previously done, biofilm formation of *S. aureus* strains isolated from orthopedic implants has been detected in the ratio of 88.9%. The biofilm formation of *S. epidermidis* was also detected in the ratio of 88.9% [22]. In other studies, biofilm formation of *S. aureus* was reported in the ratios of 18-80%. Baselga et al.[23] reported slime production of staphylococci in the ratio of 12%. In this study slime production of isolates were detected in the ratio of 19%. The results of these two methods were compared and no significant differences were observed. In this study, biofilm formation was detected in higher proportion for coagulase positive strains.

Cytotoxic molecules important for *S. aureus* are classified in four types as alfa toxin, beta toxin, delta toxin and gamma toxin. In previous studies, delta toxin production of CNS was reported in the ratio of 97% [24]. In another study, beta and delta hemolysin production of *S. aureus* strains were reported in the ratio of 24%. In this study, beta and delta hemolysin production ratios of *S. aureus* strains were detected as 51.35% and 32.43% respectively. Alfa hemolysin was detected in *S. hyicus* strains at highest proportion. In previous studies, delta hemolysin was reported as the most common hemolysin in *S. epidermidis*

strains [25]. In this study, delta hemolysis was observed in the ratio of 40% in *S. epidermidis* strains. CPS produced beta hemolysin in the ratio of 51.42% while CNS strains produced in the ratio of 36.73%.

Staphylokinase, which is known as one of staphylococcal extracellular proteins, converts plasminogen to plasmin. However, there is no conclusive evidence that staphylokinase is a virulence factor. In this study, staphylokinase production was determined in the ratio of 40.47% in Staphylococcal strains. Staphylokinase production was detected in *S. hyicus* strains at highest proportion. Coagulase positive strains produced staphylokinase in the ratio of 40% while coagulase negative strains produced in the ratio of 32.65%. However, this topic is excluded from discussion since there is no proper literature for staphylokinase production. The studies for detection of haemagglutination feature in Gram positive microorganisms are insufficient and they have focused on *S. saprophyticus*. In latter studies, it was determined an association between biofilm formation and hemagglutination feature of *S. epidermidis* [9]. In this study, the haemagglutination feature was determined in the ratio of 60%. These results correlate with the data obtain in previous studies. In another study, agglutination ratio was determined as 33% for coagulase negative staphylococci except *S. epidermidis* [9]. The haemagglutination feature of *S. aureus* was found in the ratio of 23% in a study performed previously [26]. In this study, haemagglutination ratio was determined in the ratio of 14.28% for coagulase positive Staphylococci and 16.32% for coagulase negative Staphylococci. In general, this ratio was determined as low when compared with both coagulase positive and negative strains. Haemagglutination feature was found in the ratio of 13.5% only in *S. aureus* strains.

The formation of a large increase of methicillin resistance has been observed in recent years particularly in nosocomial infections [27]. Methicillin resistant *S. aureus* strains have been isolated in a study conducted in the Netherlands, albeit at a lower prevalence of staphylococci isolated from animals. Although methicillin-resistance in coagulase-negative strains was determined in the ratio of 4%, resistance in coagulase positive strains was not determined [28]. Methicillin resistance of coagulase negative strains was found in the ratio of 8% in a study conducted in United States [29]. The first MRSA strain in animals has been reported in mastitis cattle in 1972, and this number has increased in the following years [30,31]. Among the studies on methicillin resistance in mastitis milk specimens in Turkey, there is a proportional difference for MRSA. Ak et al.[10] and Güler et al.[11] reported that oxacillin resistance was absent in Staphylococcal strains causing mastitis in Konya. Kırkan et al.[12] reported that 60% of the oxacillin resistance was detected in *S. aureus* strains studied in Aydın region. Türkyılmaz et al.[13] reported that cefoxitin resistance was 17.2% in *S. aureus* strains in the same region. In this study, methicillin resistance was determined in the ratio of 2.9% for coagulase positive strains and 16.3% for coagulase negative strains. The results correlate with previous studies. *S. haemolyticus* strains exhibit a tendency to develop versatile resistance to drugs [32]. *S. haemolyticus* was determined as one of the methicillin resistant CNS [28]. In this study, two of the methicillin resistant strains were detected as *S. haemolyticus*. This result correlates with the data reported in previous studies. The study has also demonstrated that methicillin resistance should be investigated for antibiotic susceptibility testing of staphylococcal mastitis for methicillin resistant strains that are resistant to all β-lactam antibiotics. As the development of methicillin resistance in veterinary medicine may increase in the following years, methicillin resistance should be considered as an important factor in the treatment of staphylococcal mastitis. The wider and more comprehensive epidemiological studies to be carried out in this regard will clarify the situation in Turkey of the infections caused by methicillin-resistant staphylococcal strains.

In this study, the identification of *Staphylococcus* species isolated from milk samples of healthy and mastitic animals, and the virulence factors of isolates were examined. The relationship between these factors and the strains regarded as pathogen and nonpathogenic was determined also. The results indicate that isolated strains showed wide range of virulence factors. Thus, CNS were determined to have an important role in the pathogenesis of mastitis. Staphylococcal mastitis etiology, epidemiology and virulence factors were examined on the basis of the region and the prevalence of CNS were determined epidemiologically. It is considered that presence of methicillin-resistant staphylococci might create a significant problem for public health in this situation. As a result, it was concluded that coagulase-negative staphylococci also can play a role in the pathogenesis of mastitis, animals might transmit the pathogen staphylococci to human and in order to launch an effective treatment against mastitis, the exact etiology, pathogenesis and epidemiology of the infection should be determined. The large amount of staphylococcal agents found in bovine milk represents a health hazard to the animals and emphasises the need for improved hygiene practise at levels in the dairy.

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Research Article

Effect of Commercial Toxin Binder, Native Probiotic Strains, Cell Wall Yeast and Aluminosilicate in Diets Contaminated with Aflatoxin, on the Expression of *GOT2, CYP450 1A5* Genes and Serum Concentrations of Liver Enzymes in Broiler Chickens

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Abstract

This study was conducted to investigate the effect of cell wall yeast, aluminosilicate and some probiotic strains in diets contaminated with aflatoxin, on the expression of *GOT2, CYP450 1A5* genes and serum concentrations of liver enzymes including GGT, ALT, AST and LDH in broilers. 400 seven-days old chicks from strain Cobb 500 were used as a completely randomized design with 8 treatments, 5 replications. Treatments were included: 1- Negative control (NC), 2- NC + 1 mg/kg aflatoxin or positive control (PC), 3- PC + 1 kg/ton Toxeat® (a toxin binder), 4- PC + *Lactobacillus strains* (L), 5- PC + *Bacillus subtilis* JQ_{618} strain (B), 6- PC + *Saccharomyces cerevisiae*'s cell wall (Y), 7- PC + [PC+ BLY) B+ L+ Y(], 8- PC + Hydrated sodium calcium Aluminosilicate (HA). The serum concentrations of LDH, ALT, GGT and AST were increased in PC group at 42d ($P<0.05$). Tox®, L and BLY reduced serum levels of AST ($P<0.05$). Increased serum concentration of GGT was observed in PC treatment, decreased by HA, Y, B and L treatments ($P<0.05$). The results showed the upregulation of *GOT2, CYP450 1A5* in PC group. But Y, B, L and Tox® reduced the expression of *GOT2*. The groups receiving aflatoxin adsorbent compounds reduced the adverse effects of aflatoxin on increasing the expression of *CYP450 1A5*.

Keywords: *Aflatoxin, Broiler Chickens, Gene Expression, Liver Enzymes, Probiotic strains, Toxeat®*

Broiler Tavuklarda Aflatoksin ile Kontamine Diyette Ticari Toksin Bağlayıcı, Doğal Probiyotik Türleri, Maya Hücre Duvarı ve Aluminosilikatın *GOT2* ve *CYP450 1A5* Gen Ekspresyonları İle Karaciğer Enzimlerinin Serum Konsantrasyonları Üzerine Etkisi

Özet

Bu çalışma, broiler tavuklarda aflatoksin ile kontamine diyette maya hücre duvarı, aluminosilikat ve bazı probiyotik türlerinin *GOT2* ve *CYP450 1A5* genlerinin ekspresyonları ile GGT, ALT, AST ve LDH gibi karaciğer enzimlerinin serum konsantrasyonları üzerine etkisini araştırmak amacıyla gerçekleştirildi. 400 adet Cobb 500 yedi günlük civciv rastgele düzen içinde 8 uygulama ve 5 tekrar üzere kullanıldı. Uygulamalar şu şekilde gerçekleştirildi: 1- Negatif kontrol (NC), 2- NC + 1 mg/kg aflatoksin veya pozitif kontrol (PC), 3- PC + 1 kg/ton Toxeat® (toksin bağlayıcı), 4- PC + *Lactobacillus* türleri (L), 5- PC + *Bacillus subtilis* JQ618 türü (B), 6- PC + *Saccharomyces cerevisiae* hücre duvarı (Y), 7- PC + [PC+ BLY)B+ L+ Y(], 8- PC + Hidratlı sodyum kalsiyum Aluminosilikat (HA). LDH, ALT, GGT ve AST serum konsantrasyonları PC grubunda 42. günde arttı ($P<0.05$). Tox®, L ve BLY AST serum seviyesini düşürdü ($P<0.05$). PC uygulanan grupta artmış serum GGT konsantrasyonu gözlemlenirken HA, Y, B ve L uygulamaları bu seviyeyi düşürdü ($P<0.05$). Elde edilen sonuçlar, PC grubunda *GOT2* ve *CYP450 1A5* upregulasyonunu gösterdi. Ancak, Y, B, L ve Tox® GOT ekspresyonunu azalttı. Aflatoksin absorbe eden madde ilave edilen gruplarda *CYP450 1A5* ekspresyonu artarak aflatoksin tarafından oluşturulan olumsuz etkiler azaltılmıştır.

Anahtar sözcükler: *Aflatoksin, Broiler Tavuk, Gen Ekspresyonu, Karaciğer Enzimleri, Probiotik türleri, Toxeat®*

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INTRODUCTION

Mycotoxins are secondary metabolites of fungi which the possibility of their presence in foods can be provided by the conditions of production, transportation and incorrect storage. Aflatoxins are a group of mycotoxins that are produced by certain fungal species, especially *Aspergillus flavus* and *Aspergillus parasiticus* [1]. Aflatoxin B_1 has the most biological activity. Aflatoxin and Aflatoxicosis due to the consumption of contaminated diets in poultry are accompanied with symptoms such as decreased performance, liver damages and immunosuppression [2]. The investigations revealed that Cytochrome P450 enzyme[1] produced by *CYP1A5* and CYP3A37 genes is specifically responsible for the conversion of aflatoxin to the other metabolites at in *in vitro* and *in vivo* conditions [3,4]. CYP450 1A5 enzyme has high affinity for binding and metabolizing the metabolites of aflatoxin as well as the detoxification of AFM_1 [5]. Aflatoxin metabolites can be attached to the DNA and RNA and changed the level of gene expression [6]. Exposure to aflatoxin in poultry causes changes in liver enzymes gene expression levels including Xenobiotic neutralizers, cell cycle regulators, oxidative stress, DNA damages recovery, amino acid metabolizers, cell proliferation, immunity and fatty acids metabolism [7]. Aflatoxin contaminated diet leads to disturbance of the natural process of enzyme gene expression, one of these enzymes is AST that *GOT2* gene is responsible for its production. AST (*GOT2* gene expression product) is responsible for catalyzing the reversible transfer of α- amine between aspartate and glutamate [8]. The upregulation of *GOT2* affected by aflatoxin consumption causes to increase serum levels of AST, which this increment causes damages to the liver, kidneys and heart [9,10]. Researchers have been pointed out increasing the concentration of AST, ALT, LDH in the presence of aflatoxin in diets for broilers [10].

Since prevention from aflatoxin contamination is often impossible, so different methods of detoxification of mycotoxins is highly considered [6]. Among the various methods of detoxification, the impact of aluminosilicate compounds efficiency in reducing the effects of aflatoxin in *in vitro* and *in vivo* conditions has been proved [11]. Due to the limitations of aluminosilicate consumption, using biological compounds is on the agenda of nutritionists because of their numerous advantages. It has been proved that using diets based on the probiotic compounds especially *Lactobacilli* in poultry diets, have the ability to reduce aflatoxin effects on the gene expression of liver enzymes such as genes for amino acids and fat metabolizing enzymes [12]. Gao *et al*.[13] found the high ability of *Bacillus Subtilis* for reducing the effects or disable B_1, M_1 and G_1 aflatoxins. Using 0.5 to 1 gram of glucomannan *Saccharomyces cerevisiae* per kg of aflatoxin-contaminated diet reduced the histological changes in the liver, kidney, spleen and bursa fabricius [14].

[1] *Chicken Cytochrome P450 1A5*

In this study the expression of *GOT2* (Gallus gallus Aspartate transaminase), *CYP450 1A5* genes and serum concentrations of liver enzymes including Gammaglutamyl transpeptidase (GGT), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Lactate dehydrogenase (LDH), also the possible effects of various organic, mineral and biological additives were investigated under the influence of aflatoxin contaminated diets in broiler chickens because of great economic losses of aflatoxins to the poultry industry.

MATERIAL and METHODS

Aflatoxin Production

The required aflatoxin was produced by contaminating rices with *Aspergillus flavus* (PTCC 5004) (Prepared from the microbial treasures of Scientific and Industrial Research Organization of Iran). For this purpose, 1 mL of *Aspergillus flavus* suspension, containing 7×10^6 fungal spores were added to rice and were cultivated for 7 days at 28°C temperature. After fungal growth and toxin production, rice was dried by using oven at 70°C and finally its powder was obtained. Qualitative and quantitative aflatoxin content in rice powder was measured by HPLC (Waters Alliance e2695 equipped with 2475 fluorescence detector, USA) [15,16]. The content of aflatoxin in rice samples were G_2: 8 ppm, G_1: 126 ppm, B_2: 22 ppm, B_1: 289 ppm and the total concentration of aflatoxins was 445 ppm. In order to prepare the experimental diets, rice powder with a certain composition and level of aflatoxin was added and mixed to the basal diet up to a concentration of 1 mg/kg of aflatoxin B_1. According to the extent permitted of aflatoxin in poultry diets (0.02 mg/kg of feed) so contamination of the basal diet was 50 times of the extent permitted [17]. The basal diet had no aflatoxin.

Adsorbent Materials

Adsorbent compounds investigated in this study were including:

1) Toxeat®, a commercial toxin binder based on biological compounds, produced by Tak Gene Company (Tehran - Iran) contains *Lactobacilli*, *Bacilli* and Iranian native cell wall yeast based on Aluminosilicate (as a career)

2) *Lactobacilli* strains including *Lactobacillus* TD_4, *Lactobacillus* TD_{15}, *Lactobacillus* TD_3, *Lactobacillus* TD_{10} and the amount of each of the bacteria was 1×10^7 CFU/g

3) 1×10^7 CFU/g of *Bacillus Subtilis* JQ_{618}

4) *Saccharomyces cerevisiae*'s cell wall produced by Tak Gene Company (Tehran - Iran) as an organic component

The amount of mannan and glucan in the used cell wall yeast were analyzed by Tak Gene Zist Company and were respectively 430.26 mg/kg and 569.73 mg/kg.

5) Hydrated sodium calcium Aluminosilicate as a mineral component (HA)

Isolated strains of bacteria which are commercial products of Tak Gene Zist Company (Tehran - Iran) were selected among a collection of over 200 indigenous microorganisms of Iran based on their high ability of detoxification.

Experimental Treatments

In this study a total of 400 seven-day old chicks from a broiler breeder strain (Cobb 500) were used. The chicks were randomly divided into 8 treatments, 5 replications and 10 chicks in each replication (in equal proportions of male and female) and were fed with experimental diet from 7 to 42 days of age. The experimental treatments were as follows:

- Negative control group: Basal diet, (NC.)
- Positive control group: Basal diet + 1 mg/kg of feed aflatoxin, (PC.)
- Group 3: PC + 1 kg/ton of feed Toxeat®, (a commercial toxin binder) (Tox®)
- Group 4: PC + *Lactobacillus* TD_3, TD_4, TD_{10}, TD_{15} strains, (L.)
-Group 5: PC + 1 kg/ton of feed *Bacillus Subtilis* JQ_{618} strain, (B.)
- Group 6: PC + 1 kg/ton of feed *Saccharomyces cerevisiae*'s cell wall, (Y.)
- Group 7: PC + 1 kg/ton of feed the content of treatments L, B and Y, (BLY.)
- Group 8: PC + 15 kg/ton of feed Hydrated sodium calcium Aluminosilicate, (HA.)

The used feedstuffs were sent to the Tak gene laboratory for analyzing compounds by using NIR method. Diets were prepared for starter (7-14 days of age), grower (15-28 days of age) and finisher (29-42 days of age) periods. The amounts of feedstuffs and nutrient composition of the experimental diets are shown in *Table 1*. The chicks were vaccinated against infectious bronchitis, Newcastle and Gumboro but no medical program has run during the entire experimental period. In all process of the experiment the temperature and lighting control systems have been set based on the broiler husbandry instruction manuals (Cobb 500). During the experimental period, the environmental conditions were the same for all groups and given *ad libitum* access to the water and feed. The bird care and used procedures were approved by standard committee of Karaj Animal Science Research (approval date: 19/02/2016; No: 10036).

Evaluation of Liver Enzymes

At the end of the experiment (42 days of age) 3 mL of blood was taken from each chick, that way 2 randomly chicks from each pen (replicate) and 10 chicks of each treatment were selected to measure the serum levels of liver ezymes. The serum was separated for measuring the levels of GGT, ALT, AST and LDH. Analysis of serum samples was carried out with ELISA technique by using Elx 800 ELISA Reader, BioTek and commercial kits for poultry (ALT ELISA Cat. No.: MB S266858, AST ELISA Cat. No.: MB S740867, GGT ELISA Cat. No.: MB S934604, LDH ELISA Cat. No.: MB S736903) produced by MyBiosource American company [10].

Evaluation of CYP450 1A5, GOT2 Gene Expression

At the end of experiment 3 chicks from 3 replications of each treatment were selected and the birds were anesthetized by carbon dioxide gas and slaughtered through the cervical vertebra movement. Their liver samples were taken immediately after slaughter and transferred to the laboratory in vicinity of ice. A total amount of 30 mg of liver tissue were measured and all cellular RNA content of liver samples was extracted by using the instructions of GeneJetTM RNA Purification Thermo kit (Fisher Scientific, USA, Cat no. K0731) instantly [18,19]. Then the amount of extracted RNA was measured by NanoDropTM 1000 spectrophotometer (Thermo Fisher Scientific, USA) [20]. To perform RT PCR, cDNA was obtained by using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA, Cat no. K1621), according to the manufacturer instructions [18]. To carry out the quantitative RT-PCR, the volume of reaction was set on 25 uL, in which cDNA concentration and the final concentration of SYBR green were respectively set on 6.25 and 0.25 ng/µL [21]. In this study, β-actin was used as a housekeeping control that under these conditions

Table 1. *Composition and analysis of basal diets (%)*

Ingredients %	Starter (1-14 days)	Grower (15-28 days)	Finisher (29-42 days)
Corn	55	46.08	45
Soybean meal	39	29	32.6
Soybean oil	1	1.05	3.8
Wheat	-	20	15
Oyster shell-flour	1.3	1.17	1
NaCl	0.2	0.2	0.1
Premix[1]	3.5	2.5	2.5
Analysis			
AME (kcal/kg)	2995	2987	3121
Crude protein (%)	22.58	19.25	20.23
Digestible lysine (%)	1.156	0.923	0.994
Calcium (%)	1.068	0.87	0.812
Total Available phosphorus (%)	0.546	0.42	0.424
Digestible methionine (%)	0.528	0.423	0.434
Digestible methionine + cysteine (%)	0.834	0.698	0.717
Na (%)	0.212	0.187	0.145
Cl (%)	0.248	0.225	0.163

[1] Permix Vitamin and Mineral analysis: *Vitamin A: 1000 IU; vitamin D_3:3500 IU; vitamin E: 40 IU; vitamin K_3: 2 mg; vitamin B_1: 2 mg; vitamin B_2: 5 mg; vitamin B_3: 35 mg; vitamin B_5: 13 mg; vitamin B_6: 1.5 mg; vitamin B_{12}: 0.01 mg; vitamin B_9: 1.6 mg; Biotin: 1.5 mg; I: 1.25 mg; Cu: 16 mg; Zn: 100 mg; Se: 0.3 mg; Mn: 120 mg; Fe: 40 mg; Choline chloride: 350 mg; Betaine:150 mg; ME (kcal/kg) 2837; CP: 12.5%; TSAA: 6.3%; Dig Lys: 1.8%; Dig Thr: 0.85%; Ca: 21.88%; Na: 2.45%; AP: 11.5%*

no changes will occure in its expression levels [22] and PCR was designed for 80 cycles so that 15 sec at 94°C for Denaturation, 30 sec at 60°C for Annealing and 30 sec at 72°C for Elongation were intended. In the final stage, the results of fluorescence were collected and investigated by SYBR Green combined with the expanding DNA. In this research primer sequences of *GOT2* and *CYP1A5* genes were respectively designed according to the previous reports [23,24] and β-actin gene was designed based on both mentioned reports *Table 2*. Each sample was performed in 3 replications. The amount of ΔCT was obtained by subtracting the cycle threshold of sample from the amount of CT for β-actin gene. The group with the highest amount of ΔCT means it has the lowest gene expression [21]. The amount of ΔCT was calculated by Livak and Schmittgen method [25].

Statistical Analysis

The results of the experiment were analyzed as a completely randomized design with 8 treatments and 5 replications per treatment. Data were analysed by using the GLM procedures SAS version 9.2 [26] and differences between the treatments were compared by Duncan's multiple range test and the value of significance level was 0.05.

of aflatoxin on ALT serum levels with less efficiency than the treatment fed the cell wall yeast (P<0.05). Also Hydrated sodium calcium Aluminosilicate could inhibit the adverse effects of aflatoxin on increasing ALT serum levels but this effect was more limited in comparison with other investigated compounds (P<0.05). Studying the changes in serum levels of GGT showed the serum level increment of this enzyme in the positive control group (P<0.05). L., B., Y. and HA. groups were controled the incremental effects of aflatoxin on serum levels of GGT (P<0.05) whereas Tox® and BYL. groups had a lower effect on inhibiting adverse effects of aflatoxin in comparison with other groups (P<0.05).

Evaluation the Expression of GOT2 and CYP450 1A5

The results of the Tox®, L., B., Y., BYL. and HA. effects on the expression of *GOT2* and *CYP450 1A5* are presented in *Fig 1-4*. As it can be observed in *Fig. 1*, the greatest increase was for CYP450 1A5 gene expression in PC. group (P<0.05) and the other treatments showed the lowest rate of increase in the expression of this gene compared to endogenous control (P<0.05) and there were no significant differences between other treatments

Table 2. *Sequences of the investigated genes and Housekeeping gene*

Target	Accession No (GenBank)	Sequence (5'-3')	Product Length	Source
GOT2	M12105	S: ATCCTCATCCGTCCCATGTA A: GTCAGTGATGTGCTGCCAGT	201 bp	Rosebrough *et al.*[23]
CYP1A5	XM015278761	S: TCACCATCCCGCACAGCA A:AAGTCATCACCTTCTCCGCATC	201bp	Zhang *et al.*[24]
-actinβ	L08165	S: TGCGTGACATCAAGGAGAAG A: TGCCAGGGTACATTGTGGTA	300bp	Li *et al.*[22]; Rosebrough *et al.*[23]; Zhang *et al.*[24]

***S:** Sense, **A:** Anti-sense*

RESULTS

According to the the results, increasing the concentration of LDH and AST enzymes was observed in PC. group (P<0.05), but no differences were observed for serum concentrations of these two enzymes in other treatments (P>0.05) *(Table 3)*. However the serum level differences of these two enzymes was significant between PC. and other groups (P<0.05). So organic, mineral and biological compounds could well prevent the negative effects of aflatoxin on serum levels of LDH and AST. This result was obtained from the comparison of adsorbent receiving along with aflatoxin groups and NC. group that the results of this groups had no significant differences with NC. group (P>0.05). The results showed that serum levels of ALT were increased in PC. group significantly (P<0.05). Aflatoxin inhibitor compounds were able to control the increment of serum enzymes levels in all groups in comparison with positive control group. Such that there were no significant differences between NC. and Y. groups (P>0.05). Tox®, L., Y., BYL. and B. groups were inhibited the negative effects

Table 3. *Effect of Tox®, L., B., Y., BYL. and HA. on serum concentrations of liver enzymes in broiler chickens (Cobb 500) fed by diets contaminated with aflatoxin at 42 days of age*

Treatment	LDH (IU/L)	AST (IU/L)	ALT (IU/L)	GGT (IU/L)
NC	419.38±0.13^{b}	43.8±1.26^{b}	14.8± 0.78^{d}	7.42±0.41^{c}
PC	420.67±0.41^{a}	65.1±6.64^{a}	20.2±1.04^{a}	9.02±0.54^{a}
Tox®	419.78±0.15^{b}	47.8± 1.5^{b}	16.8±0.41^{c}	8.46±0.36^{ab}
L.	419.70±0.06^{b}	47.0±0.64^{b}	16.3±0.79^{c}	8.14±0.29^{b}
B.	419.72±0.08^{b}	47.2±0.83^{b}	16.2±0.25^{c}	8.02±0.51^{b}
Y.	419.47±0.07^{b}	44.7±0.71^{b}	15.7±0.35^{cd}	8.07±0.43^{b}
BLY	419.61±0.13^{b}	46.1±1.29^{b}	16.2±0.41^{c}	8.42±0.52^{ab}
HA	419.61±0.72^{b}	47.7±5.06^{b}	18.1±1.65^{b}	8.17±0.39^{b}
SEM	0.08	1.12	0.3	0.1
P-value	0.0001	0.0001	0.0001	0.0005

***GGT:** γ-glutamyltransferase, **ALT:** Alanine amino-transferase, **AST:** aspartae amino-transferase, **LDH:** Lactate dehydrogenase **NC:** Negative control; **PC:** positive control*

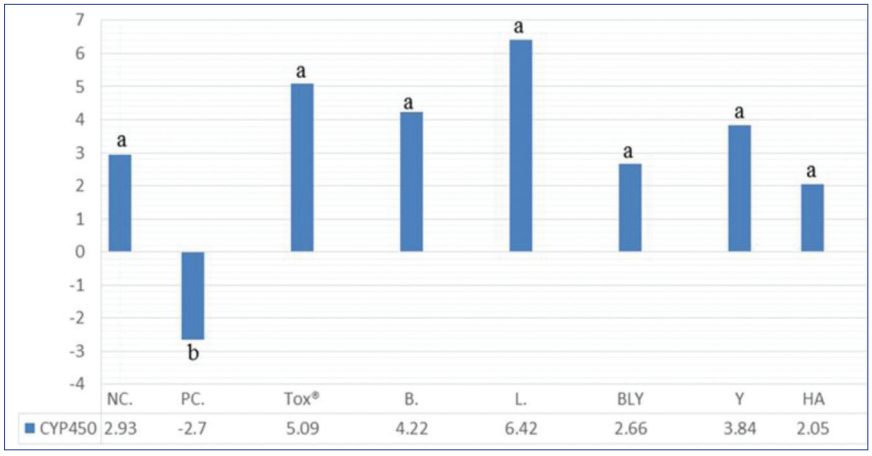
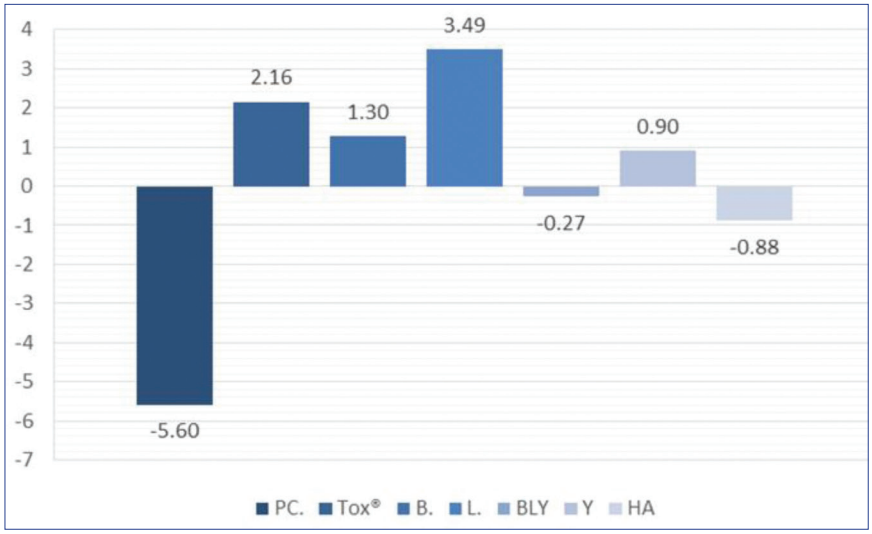


Fig 1. Effect of Tox®, L., B., Y., BYL. and HA. on *CYP450 1A5* liver enzyme gene expression in broiler chickens (Cobb 500) fed by diets treated with aflatoxin, figure has been normalized by β-actin gene (Δct), P value = 0.0152 - SEM = 0.73

Fig 2. Effect of Tox®, L., B., Y., BYL. and HA. on *CYP450 1A5* liver enzyme gene expression in broiler chickens (Cobb 500) fed by diets treated with aflatoxin, compared to control group without aflatoxin (ΔΔct)



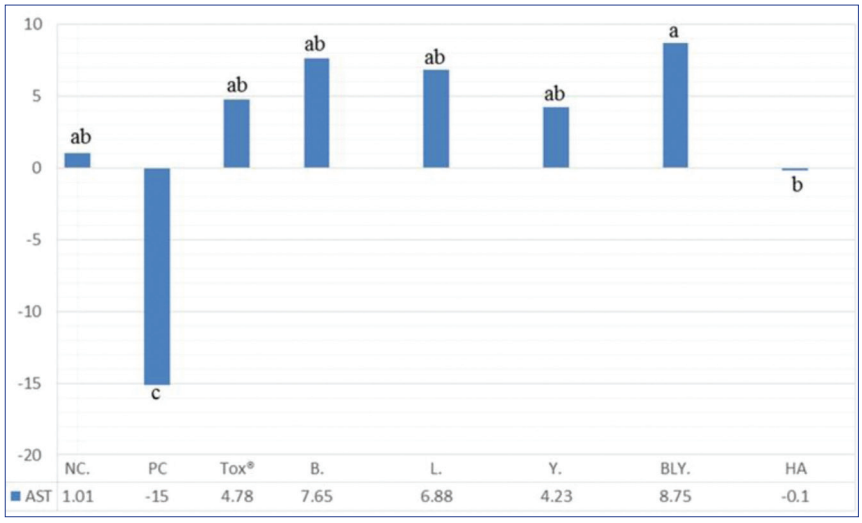


Fig 3. Effect of Tox®, L., B., Y., BYL. and HA. on *GOT2* liver enzyme gene expression in broiler chickens (Cobb 500) fed by diets treated with aflatoxin, figure has been normalized by β-actin gene (Δct), P value = 0.0008 - SEM = 1.7

received various kinds of additives compared with NC. treatment (P>0.05).

No significant treatment differences were observed between groups that received various types of additives compared with NC. group (P>0.05) but in comparison with PC. the differences were highly significant (P<0.05). Numerical comparisons of the groups received aflatoxin inhibitor indicated that L. and Tox® treatments in *CYP450 1A5* gene expression compared to endogenous control had the minimum changes. In comparing the results of *CYP450 1A5* gene expression with NC. group (ΔΔct), the highest in-

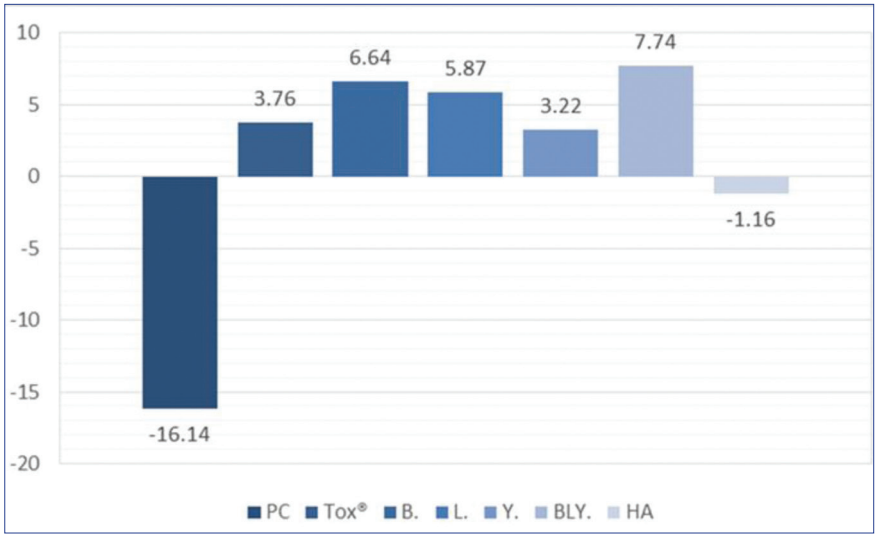


Fig 4. Effect of Tox®, L., B., Y., BYL. and HA. on *GOT2* liver enzyme gene expression in broiler chickens (Cobb 500) fed by diets treated aflatoxin, compared to control group without aflatoxin (ΔΔct)

crease of gene expression was in PC. Group and then was observed in HA. group *(Fig. 2)*. Also the results showed that the groups receiving an aflatoxin adsorbent in comparison with NC. group were able to control the effects of aflatoxin, but in comparison (ΔΔct) between all groups, L. and Tox® groups could more control the upregulation of *CYP450 1A5* gene expression.

The results of *GOT2* gene (Δct) are shown in *Fig. 3*. The PC. treatment significantly upregulated the expression of *GOT2* in comparison with endogenous control ($P<0.05$) and there were no significant differences between the other treatments ($P>0.05$), however BLY. group had the lowest gene expression and in terms of *GOT2* gene (Δct) B., L., Y., Tox® and NC. groups did not show any significant differences with each other ($p>0.05$). HA. treatment could inhibit the adverse effects of aflatoxin on upregulation of *GOT2* gene expression compared to endogenous control, but it had less ability in comparison with the other groups contain an inhibitor factor. The comparison of (ΔΔct) for *GOT2* gene showed that B., Y., L., BYL. and Tox® groups could control the adverse effects of aflatoxin on the expression of this gene, however among all groups BLY. had more effectiveness efficiency. The highest increase for *GOT2* gene expression (ΔΔct) was in PC. group. The results of (ΔΔct) are given in *Fig. 4*.

DISCUSSION

Aflatoxin has been considered as a threat to poultry nutrition from almost 50 years ago till now. This contamination makes extensive lesions in poultry and heavy economic losses to this industry by weakening the immune system and performance and also it is a threat to human health as a consumer of contaminated protein products [27]. Aflatoxins are the reason of a wide range of metabolic damages, including, liver lesions, changes in genes expression especially liver enzymes and genes involved in the metabolism of this toxin [28]. Measuring the amount of serum concentrations of liver enzymes is a good way to assess liver damages [29]. Some researchers showed that consuming aflatoxin will increase the concentration of liver enzymes especially AST, ALT and LDH [10]. In this study, an increment in serum levels of liver enzymes (ALT, AST, LDH) was observed in PC. group. Shi *et al.*[30] reported the increasment of ALT, AST and GGT enzymes due to feeding a diet contaminated with aflatoxin to broiler chickens. Researches have been proved that increased serum levels of GGT and AST, is used as an indicator for investigating liver and kidney toxicity [31]. According to what was mentioned, increased serum levels of these two enzymes (GGT, AST) in PC. group can be attributed to the liver and kidney damages in broilers fed with aflatoxin. According to the fact that many of the metabolic activities (fat and protein metabolism) and immunity (production of cytokines, chemokines, maturation of immune cells) are related to liver, therefore damage to this tissue leads to a disturbance in the immunity system function and metabolic pathways of fat and protein. All adsorbents used in this study caused to control the adverse effects of aflatoxin on increasing serum concentration of AST and GGT, but L., B., Y. and HA. groups were jointly showed better results for both mentioned enzymes. The findings of this study were in agreement with the results of Aravind *et al.*[32]. In another study, 14% increase for AST and 17% increase for ALT serum levels were observed in chickens fed aflatoxin contaminated diets [33]. One of the symptoms for hyperplasmy is a significant increase in serum levels of ALT and GGT [34]. Kasmani *et al.*[10] reported that using *Bacillus* will control aflatoxin effects on increasing liver enzyme concentrations (AST, ALT, LDH). Also in another research [32], using cell wall yeast could control the aflatoxin effects on increasing liver enzyme levels (ALT, AST, GGT) that these results are consistent with our findings for B. and Y. groups. Investigating the results of changes in liver enzymes gene expression indicated that increase in gene expression related to interleukins, liver enzymes and especially enzymes involved in the metabolism of aflatoxin occurs

under the influence of aflatoxin [28]. It seems that changes in gene expression levels in the liver occurs by using aflatoxin contaminated diets therefore acute and chronic aflatoxicosis occur due to the liver proliferation[2] [12]. Studies on the impact of diets contaminated with aflatoxin on gene expression in liver enzymes showed that the presence of toxin in the diet causes to upregulation of *CYP450 1A* gene. This cytochrome exists in chickens and turkeys and is consists of *CYP450 1A4* and *CYP450 1A5* subfamilies. Reports indicated that *CYP450 1A5* expression increases more faced with aflatoxin [35]. Yarru *et al*.[7] expressed that the expression of *CYP450* has increased by effect of aflatoxin and this causes to oxidative stress and in continue liver damage and death occur in poultry. The results of current research (PC. group) in the field of *CYP4501A5* gene expression are completely corresponded with those of previous studies mentioned. Groups L. and Tox® additives as the aflatoxin adsorbents, caused to control the upregulation of *CYP450 1A5* gene. Increasing *CYP450 1A5* expression is important because it causes to increase oxidative stress and consequently death occurs in poultry [7]. So it appears that control the expression of *CYP450 1A5* and adjusting its expression near to its level in negative control treatment under the influence of studied compounds in the diets has been protected chickens against oxidative stress caused by aflatoxin.

AFBO production was affected by *CYP450* and high affinity of this compound for binding to DNA and RNA that causes to damage to DNA and create carcinogens [36]. Control the gene expression of *CYP450 1A5* can help to reduce damages to DNA, which was observed in all treatments and of course with the higher capacity and efficiency in L. and Tox® groups. According to obtained data, chickens fed with *Lactobacillus* and Toxeat® commercial combination showed higher ability to inhibit adverse effects of aflatoxin and possible damages to DNA. More study were on serum level of liver enzyme (AST) and fewer reviews have been conducted on gene expression of this enzyme, but since investigating effects of Xenobiotics such as aflatoxin on liver enzymes, through the study of gene expression in liver enzymes or their serum concentrations or catalytic activity is possible [24], so the result of serum level of this enzyme was compared with other studies. In several experiments elevation in serum level AST was reported in broiler chickens fed diets containing aflatoxin [10]. Increasing *GOT2* gene expression (the producer of aspartate aminotransferase) was observed in PC. group of the present study in comparison with endogenous control gene and NC.

Kasmani *et al*.[10] reported that the addition of *Bacillus* to the diets contaminated by aflatoxins reduced serum level AST. Also the researchers expressed a reduction in serum level of AST by adding a commercial toxin binder containing *Saccharomyces cerevisiae* cell wall to the diets contaminated with aflatoxin in comparison with the group without additives [37]. Monson *et al*.[38] stated that probiotics in diets contaminated with aflatoxin reduces gene expression of liver enzymes and proteins. The result of *GOT2* gene expression in PC. group was consistent with the findings of Sridhar *et al*.[39] which showed the increasing of AST serum levels by consuming aflatoxin contaminated diets. Also L., B., Y. and BLY. groups in comparison with β-actin control gene (Δct), could well inhibit aflatoxin effects on *GOT2* upregulation and even there were no significant differences with NC. group. But between all groups receiving a factor as toxin adsorbent, BLY. treatment revealed more ability to inhibit aflatoxin effects on *GOT2* gene expression. The results of ΔΔct demonstrated that L., B. and BLY. groups could control *GOT2* gene expression with a better efficiency. What mentioned is in agreement with the findings of Kasmani *et al*.[10] and Yildirim *et al*.[37], in the field of AST serum levels.

According to the results of *GOT2* and *CYP450 1A5* expression and compare (Δct) and (ΔΔct) of these genes and also serum levels of liver enzymes (ALT, AST, LDH, GGT), it can be concluded that using probiotics and prebiotics in diets contaminated with aflatoxin caused to control the adverse effects of this toxin on increasing the gene expression of serum levels of liver enzymes. Compare the results of L., B., Y. groups with BLY. and Tox® groups indicated that applying several biological factors together, due to the synergistic effects of these compounds together for control negative effects of aflatoxin were efficient. Review the results of HA. group with B., L., Y. and BLY. groups determined that mineral factors have a less ability than probiotics and prebiotics to control the effects of aflatoxin on poultry, however by observing the results of Toxeat® which is a commercial biologic product based on aluminosilicate, it can be concluded that the use of HA. alongside the biological factors can help the absorption of aflatoxin in the presence of biological factors. Control the effects of aflatoxin and its absorption by Tox® and BLY. groups reduce damages to the liver and this leads to performance improvement and reduced mortality.

2 Reproduction and increment of cells

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Research Article

Bazı Kekik Türlerinin *(Thymus kotschyanus* ve *Thymus collinus)* Gastrointestinal Parazitlere Karşı Antelmentik Etkisinin Araştırılması

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Özet

Bu çalışma, Nahçıvan Özerk Cumhuriyeti arazisinde bulunan T*hymus kotschyanus* ve *Thymus collinus* adlı kekik türlerinin koyunlarda gasrointestinal parazitlere karşı antelmentik etkisinin belirlenmesi amacıyla *in-vitro* ve *in-vivo* olarak yapılmıştr. Araştırmada antelmentik preparat olarak bu bitkilerin uçucu yağları ve ekstrakları (özüt) kullanılmıştır. *In-vitro* koşullarda sindirim sisteminden toplanan helmint türlerine *(Trichostrongylus axei, Nematodirus abnormalis, Trichocephalus ovis, Haemonchus contortus)* karşı antelmentik etki belirlenmiştir. Ayrıca *in-vivo* şartlarda ise doğal enfekte koyunlarda mide-bağırsak helmintlerine karşı antelmentik etki incelenmiştir. *In-vitro* koşullarda kontrol grubuna göre deney gruplarında helmintlerin daha kısa sürede öldükleri saptanmıştır. *In-vivo* şartlarda *Th. kotschyanus* uçucu yağının antelmentik etkisi %88.1, *Th. collinus* uçucu yağının ise %82.7 olarak bulunmuştur. Bu bitkilerden hazırlanan ekstraktların antelmentik etkisi sırasıyla %69.1 ve %63.5 olmuştur. *Thymus kotschyanus* uçucu yağı preparatından alınan sonuç özüte oranla %21.6, *Th. collinus* uçucu yağı preparatından alınan sonuç ise özüte oranla %23.2 fazla olmuştur. Araştırmanın sonuçları, her iki bitkinin hem uçucu yağından, hem de özütlerinden antelmentik preparatlar hazırlanarak, koyunların gastrointestinal parazitlerinin tedavisinde kullanılabileceğini ortaya koymuştur.

Anahtar sözcükler: *Thymus kotschyanus, Thymus collinus, kekik, gasrointestinal parazit, Antelmentik*

Investigation of Anthelmintic Effects of Some Thyme Species *(Thymus kotschyanus* and *Thymus collinus)* Against Gastrointestinal Parasites

Abstract

This study was carried out *in vitro* and *in vivo* to determine the therapeutic effects of *Thymus kotschyanus* and *Thymus collinus* in the territory of Nakhchivan Autonomous Republic against the gastrointestinal parasites in sheep. Essential oils and extracts of these plants were used as antihelminthic preparation in the research. An antihelminthic effect was determined against helminth species *(Trichostrongylus axei, Nematodirus abnormalis, Trichocephalus ovis, Haemonchus contortus)* collected from the digestive system in *in vitro* conditions. Furthermore, in vivo conditions, the antihelminthic effect against gastrointestinal helminthes in naturally infected sheep was investigated. Comparing to the control group in the *in vitro* conditions, helminthes were killed in a shorter time in the experimental groups. In vivo conditions, the antihelminthic effect of *Th. kotschyanus* essential oil was 88.1% and 82.9% of *Th. collinus* essential oil. The antihelminthic effects of the extracts prepared from these plants were 69.1% and 63.5%, respectively. The result obtained from *Th. kotschyanus* volatile oil preparation was 21.6%, the result of *Th. collinus* volatile oil preparation was 23.2% which was more specific than the first one. The results of the study show that both herbal oils and extracts can be used in the treatment of gastrointestinal parasites of sheep by preparing antihelminthic preparations.

Keywords: *Thymus kotschyanus, Thymus collinus, Gastrointestinal parasite, Antihelminthics*

GİRİŞ

Bitkilerin ilaç yapımında kullanımı dünyada yaygın olarak kullanılmaktadır. Bitkilerde bulunan etken maddeler insan ve hayvanlarda fizyolojik süreçleri uyaran ve çevreye zararlı olmayan doğal kökenli maddelerdir. Bunlardan *Thymus* L. (kekik) cinsi türlerinin kimyasal içeriği, tedavi özellikleri, sanayide kullanılma üsulleri uluslararası yaygın olarak araştırılsa da, ilaçların önemli hammadde kaynağı olan bu bitkilerde antelmentik etkilerinin belirlenmesi modern dönemin güncel meselelerden biri olmuştur.

Lamiaceae Lindl (= Labiata Juss.) familyasına ait polimorfik *Thymus* L. cinsinin Kafkasya'da 44, Azerbaycan'da 28 türüne rastlanmıştır [1]. Nahçıvan Özerk Cumhuriyeti florasının

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taksonomik spektrumunda *Thymus* L. cinsi 7 türle [2], son araştırmalara göre 8 türle temsil edilmiştir. Yeni belirlenen *Thymus hyemalis* Lange türü Nahçıvan Özerk Cumhuriyeti için endemik kabul edilmiştir [3]. Bu cinsin türleri kserofit ve mezokserofit gruplara ait olup, kuru otlu, taşlı-çakıllı, subalp ve alp bozkırlarında yayılmıştır. Bunlar sıcak seven, hoş kokulu, yarı çalımlı bitkilerdir. Kimyasal bileşimi uçucu yağ, aşı maddeler, flavanoidler, katran, C vitamini, yüksek yağ asitlerinden oluşmaktadır. Tedavi etkili çoğul türlerin çayları bronşit, bronşiyal astım hastalıklarında balgam sökütürücü, iltihapi hastalıklarda antiseptik, antibakteriyel, halk tıbbında kurt salıcı olarak kullanılmıştır [4,5].

Thymus L. uçucu yağları çeşitli yönlü bakteriosit etkiye sahip olan bileşikler karışımından oluşmaktadır. Bu yağlarda bulunan timol, karvakrol ve 1,8 sineol bileşikleri özellikle antiseptik, antibakteriyel ve antelmentik etkiye sahip olan maddelerdir [6-8].

Kekik *(Thymus*L.) türlerine Nahçıvan Özerk Cumhuriyeti'nin dağ eteklerinde, aşağı, orta ve yüksek (subalp, alp, subnival) dağ kuşaklarında deniz seviyesinden 1100-3200 m yüksekliklerde rastlanmaktadır. Bu bitkilerin uçucu yağ verimi %1.45´ten %1.75'e kadar değişmektedir [9,10]. Azerbaycan florasında rastlanan kekik çeşitlerinin endüstriyel önemi ve tıpta uygulama alanları incelense de [10,11], onların antelmentik etkisi araştırılmamıştır. Nahçıvan Özerk Cumhuriyeti'nde bulunan kekik türleri bakteriosit etkili timolla daha zengin olduğundan, helmintozlarla mücadelede bu bitkilerin kullanılmasının amaca daha uygun olduğu kabul edilmektedir. Bu nedenlerden dolayı araştırma, *Thymus* L. cinsine ait olan *Th. kotschyanus* Boiss. (koçi kekik) ve *Th. collinus* Bieb. (tepelik kekik) kekik türlerinin uçucu yağ ve özütlerinin gastrointestinal parazitlere karşı antelmentik etkilerinin araştırılması amacıyla yapılmıştır.

MATERYAL ve METOT

Nahçıvan Özerk Cumhuriyeti Kengerli ve Şerur bölgelerinde deniz seviyesinden 1800-2500 m yüksekliğinde bulunan yaz otlaklarının bozkır topraklarından, Culfa bölgesinde deniz seviyesinden 2000-2800 m yüksekliğinde dağlı-çimenli fitosenozlardan *Th. kotschyanus* ve *Th. collinus* türleri tüm çiçeklenme aşamasında toplanarak kurutulmuştur.

Kurutulmuş bitkilerden hidrozilasyon yöntemi ile uçucu yağlar alınmıştır [12]. Gaz sıvı kromatografisi ile uçucu yağlar bileşimi tespit edilmiştir. Alınan koyu sarı renkli uçucu yağlardan %2´lik etil alkolde 1:5, 1:10 ve 1:20 saflaştırma oranında preparatlar ve kurutulmuş bitki materyallerinden şuruplar hazırlanmıştır. Şurup bitki kütlesi ile su 1:5 oranında karıştırılarak, 30 dakika su banyosunda tutulmuş, sürenin sonunda karışım soğutularak çift katlı bezden süzülmüştür [13].

Antelmentik etki ile ilgili araştırmalar Nahçıvan Devlet Üniversitesi Veterinerlik laboratuvarında yapılmıştır. Çeşitli amaçlarla kesilmiş koyunlardan bağırsak içeriği ve abomasum alınmıştır [14]. Seri yıkama yöntemi ile bağırsak içeriği birkaç kez yıkanarak beraklaştırılmış ve alınan sediment incelenmiştir. Koyunların abomasumları da muayene edilerek mukozada yerleşen parazitler alınmıştır. Bağırsak muhteviyatında bulunmuş parazitler *(Trichostrongylus axei, Nematodirus abnormalis, Trichocephalus ovis)* ile abomasumdan alınmış *Haemonchus contortus* fizyolojik çözeltiye (%0.9 fizyolojik tuzlu su) konulmuştur. In vitro yöntemde antelmentik etkiyi belirlemek *Th. kotschyanus* ve *Th. collinus* kekik türlerinden hazırlanan 1:5, 1:10 ve 1:20 oranda saflaştırılmış uçucu yağlı preparatların her birinden 30 ml petri kutularına dökülmüş ve sonra fizyolojik çözeltide olan canlı helmintlerden 10-15 adet bu petrilere konulmuştur. Helmintlerin hareketi izlenmiş ve hareketsizlenme durumunda onlar tekrar fizyolojik çözeltiye geçirilerek imha olmaları kontrol edilmiştir. Bu işlem birkaç kez tekrar edilerek helmintlerin tam ölüm zamanları tespit olunmuştur. Bitkilerin antelmentik etkisini belirlemek için deneyde kontrol de kullanılmıştır. Kontrol grubunda fizyolojik çözeltiye birkaç adet helmint sokularak, onların da hareketleri izlenilmiştir.

Thymus kotschyanus ve *Th. collinus* türlerinin yeşil yüzey bölümlerinden hazırlanmış bitki özütlerinin *in vitro* koşullarda antelmentik etkisini belirlemek için fizyolojik çözeltide olan helmintlerden 15-20 adet preparat olan petrilere geçirilerek, onların hareketsizlenmesi izlenilmiştir. Hareketsizlenmiş helmintleri fizyolojik çözeltiye ve yeniden antelmentik etkisi öğrenilen çözeltiye transfer edilerek ölüm süreleri kesinleştirilmiştir. Kontrol için bu tür helmitlerden aynı sayıda fizyolojik çözelti olan kasaya konulup deney kısmında olduğu gibi kontrol gerçekleştirilmiştir.

Thymus kotschyanus ve *Th.collinus* türlerinin *in vivo* koşullarında antelmentik etkisi ise doğal olarak sindirim sistemi strongylidleri ile enfekte olunmuş 30 koyun üzerinde yürütülmüştür. Araştırma için alınan hayvanlar her birinde 5 baş koyun olmak üzere 6 gruba (5'i deney, 1'i kontrol grubu) ayrılmıştır. Uygulamada ve gözaltında bulunan tüm hayvanlar aynı ortamda bulundurulmuştur. Deneyden önce her gruptaki hayvanlardan ayrı-ayrı dışkı örnekleri alınmış, flotasyon yöntemi ile incelenmiş [15] ve strongyid yumurtalar sayılarak ortalama rakam hesaplanmıştır. Birinci grupta ortalama 898.7 adet, ikinci grupta 894.5 adet, üçüncü grupta 819.3 adet, dördüncü grupta 826.9 adet, beşinci grupta 862.5 adet, altıncı grupta ise 924.6 adet helmint yumurtası tespit edilmiştir.

Thymus kotschyanus ve *Th. collinus* türlerinden alınmış eter yağları hayvanlara içirilmesi için 1:3 oranında zeytinyağı ile karıştırılmıştır. *Thymus kotschyanus* uçucu yağından hazırlanmış preparatlar birinci grup hayvanlara 3 gün sabah aç karnına 4-5 mL olmak üzere içirilmiştir. *Thymus collinus* uçucu yağı preparatı ikinci grup hayvanlara aynı olarak verilmiştir. Uçucu yağ preparatları verilen hayvanlarda toksik özellikleri oluşumunu belirlemek için klinik takip yapılmıştır. Klinik muayenelerde hayvanların kalp atımı, solunum hareketleri, bağırsak peristaltik hareketleri sayılıp,

vücut sıcaklığı ölçülmüştür. Fizyolojik süreçlerde değişikliklerin olmaması, preparatların toksik dozda olmadığını göstermiştir. *Thymus kotschyanus* ve *Th. collinus* türlerini yüzey yeşil kısmından hazırlanmış özütler üçüncü ve dördüncü grup hayvanlara uygulanmıştır. Üçüncü grupta koyunlara her başa günde 100 mL olmak üzere 3 gün *Th. kotschyanus* özütü, dördüncü grup hayvanlara ise *Th.collinus* özütü içirilmiştir. Beşinci grup koyunlara 3 gün her başa sabah aç karnına her iki bitkinin yeşil yüzey parçalarının aynı miktarda karışımından 200 g yedirilmiş, daha sonra kuvvetli yemle beslenmiştir. Deney süresince hayvanların fizyolojik süreçleri kontrol altında tutulmuş ve verilen dozun hayvanlar için toksik olmadığı kaydedilmiştir. Kontrol grubu olan altıncı grup hayvanlara ise hiçbir antelmentik preparat verilmeden doğal otlak alanlarında otlatılmıştır. Deney süresi sona erdikten sonra, hayvanlardan yeniden dışkı alınarak incelemeler yapılmıştır.

BULGULAR

In-vitro ortamda bağırsak *(Trichostrongylus axei, Nematodirus abnormalis, Trichocephalus ovis)* ve abomasum *(Haemonchus contortus)* parazitlerine karşı *Th. kotschyanus* ve *Th. collinus* kekik türlerinin antelmentik etkisi *Tablo 1*'de sunulmuştur. Deney gruplarında olan helmintlerin kontrol grubundakilere göre daha kısa sürede canlılığını kaybettikleri belirlenmiştir.

Araştırmada *Th. kotschyanus* ve *Th.collinus* türlerinden hazırlanmış ekstrakların in-vitro koşullardaki helmintosit etkisi *Şekil 1*'de gösterilmiştir.

Çalışmada *Th. kotschyanus* ve *Th. collinus* türlerinin hem uçucu yağlarının, hem de özütlerinin *in-vivo* koşularda antelmentik etkileri belirlenmiştir. Araştırma öncesi deney gruplarında strongylid helmint yumurta sayıları sırasıyla; l898.7 adet, 894.5 adet, 819.3 adet, 826.9 adet, 862.5 adet, kontrol grubunda ise 924.6 adet olarak tespit edilmiştir.

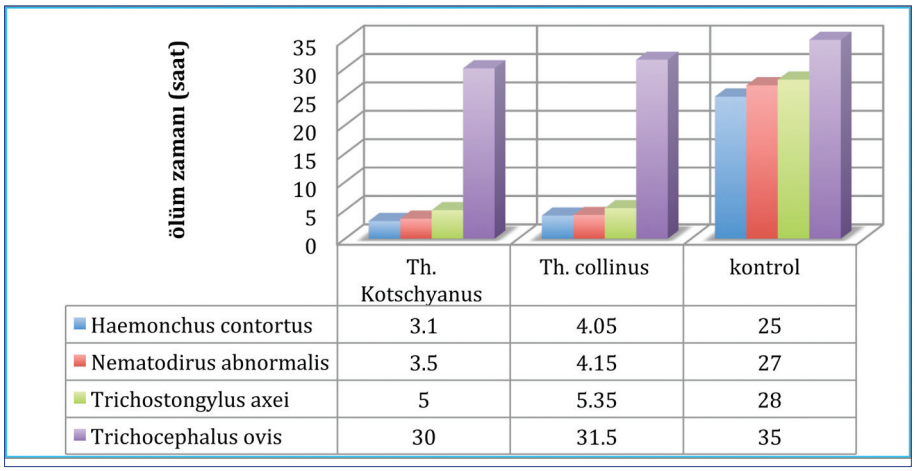
Çalışmadan sonra gruplara göre helmint yumurta sayıları deney gruplarında sırasıyla; 106.9 adet, 154.6 adet, 253.2 adet, 301.8 adet, 351.9 adet ve kontrol grubunda 922.3 adet olmuştur. Antelmentik etkinliği belirlemek için araştırmadan önce ve sonra bulunan yumurta sayıları karşılaştırılmıştır. Deney ve kontrol gruplarındaki yumurta sayısı kıyaslamsına hesaplanan entelmentik etki düzeyi *Şekil 2*'de gösterilmiştir.

TARTIŞMA ve SONUÇ

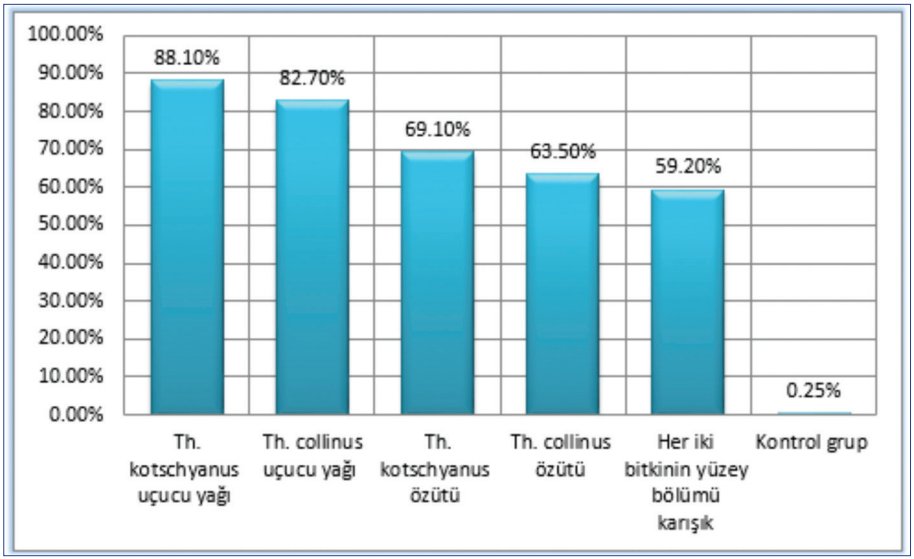
Tablo 1'de görüldüğü gibi uçucu yağ prepartlarının antelmentik etkisi saflaştırma oranına bağlı olarak farklı olmuştur. 1:20 oranında durulaşdırılmış preparatlarda *Haemonchus contortus*, *Nematodirus abnormalis* ve *Trichostrongylus axei* 2-4 saatte, 1:5 oranında durulaşmada ise 1-2 saatte tam ölüm gerçekleşmiştir. *Trichocephalus ovis*

Tablo 1. *Thymus kotschyanus ve Th.collinus türlerinden alınan uçucu yağların (%2'lik alkol çözeltisi) in-vitro ortamda gastro intestinal parazitlere karşı antelmentik etkisi*

Bitki türü	Uçucu Yağların Saflaştırma Oranı	*Haemonchus contortus*	*Nematodirus abnormalis*	*Trichostrongylus axei*	*Trichocephalus ovis*
Thymus kotschyanus	1:5	45dk.	55dk.	1s.15dk.	16s
	1:10	1s.20dk.	1s.25dk.	1s.35dk.	18s.40dk.
	1:20	2s.10dk.	2s.05dk.	2s.35dk.	20s.
Thymus collinus	1:5	2s.05dk.	2s.25dk.	2s.50dk.	20s.10dk.
	1:10	2s.35dk.	2s.55dk.	3s.20dk.	21s.40dk.
	1:20	3s.05dk.	3s.10dk.	3s.55dk.	23s.
Kontrol Grubu		25s.	27s.	28s.	33s.



Şekil 1. *Th. kotschyanus* ve *Th.collinus* türlerinden hazırlanmış ekstrakların *In vitro* koşullarda helmintosit etkileri



Şekil 2. Mide-bağırsak parazitleri ile doğal enfekte koyunlarda *Th. kotschyanus* ve *Th. collinus* türleri uçucu yağ ve özütlerinin *in-vivo* koşullarda antelmentik etkileri

1:20 oranda durulaşmış preparatda 20-23 saatte, 1:5 oranda durulaşmış preparatda ise 16-20 saatte ölüm olmuştur. Uçucu yağ miktarı yüksek karışımlarda ölüm süresinin daha kısa olması, kekik uçucu yağının esas kompanenti olan timol ve karvakrolun etkisi ile ilgilidir. Kontrol grubunda fizyolojik çözeltiye konulmuş helmintler 25-33 saat canlı kalabilmişlerdir. Deney gruplarında olan helmintlerin kısa zamanda imha olmaları, kontrol gruplarında ise uzun süre canlı kalmaları araştırılan uçucu yağların yüksek antelmentik etkiye sahip olduklarını göstermektedir.

Şekil 1'de görüldüğü gibi antelmentik preparatlara konmuş *Haemonchus contortus, Nematodirus abnormalis, Trichostrondilus axei* 3-6 saat, *Trichocephalus ovis* 30-32 saat sürede ölüme maruz kalmışlardır. Kontrol grubunda bulunan helmintler ise uzun süre (25-35 saat) canlı kalmışlardır. Bu karşılaştırma *Th. kotschyanus* ve *Th.collinus* kekik çeşitlerinin yüksek antelmentik etkili olduğunu kanıtlamaktadır.

Şekil 2'de sunulan sonuçlardan görüldüğü gibi, kontrol grubunda olan hayvanlarda araştırmadan önce ve sonra bulunan yumurta saylarında çok az yani kısmen farklılık olduğu belirlenmiştir. Deney gruplarında ise helmint yumurta saylarında belirgin farkın olması araştırılan bitkilerin antelmentik etkisi ile ilgili olmuştur. Bu da göstermektedir ki, *Th. kotschyanus* ve *Th.collinus* kekik çeşitlerinin hem uçucu yağ preparatları, hem de özütleri *in vivo* ortamında yüksek antelmentik etkiye sahiptir. Kekik uçucu yağları timol ve karvakrol maddeleriyle zengin olduğundan, bu uçucu yağ preparatlarında özütlere göre daha etkili olduğu bulunmuştur. *Th. kotschyanus* türü kekik uçucu yağı preparatından alınan sonuç özüt ile kıyaslamada %21.6, *Th.collinus* türü kekik uçucu yağı preparatından alınan sonuç ise bu bitkinin özütü ile kıyaslamada %23.2 oranında fazla olmuştur.

Son zamanlarda hayvanlarda bulunan helmintlerle mücadelede bitkilerden faydalanma oranı daha da artmıştır. Bitkilerden sentezlenen doğal etken aktif maddeler helminti imha etmekle beraber, helmintozlar sırasında hayvan vücudunda meydana gelen komplikasyonu ortadan kaldırmakta ve bağışıklık sistemini güçlendirmektedir. Ayrıca antelmentik bitkiler konak organizmasından çabuk atıldığından hayvan ürünlerinin içeriğinde kalite değişikliğine neden olmamaktadır [16,17].

İlaç olarak önemli bitkilerin yüzey bölümlerinden, köklerinden, meyve ve tohumlarından, uçucu yağlarından hazırlanmış preparatların antelmentik etkileri incelenmiştir. Çeşitli araştırmalarda nane, dağ dereotu, boymaderen bitkilerinden alınan şurublardan mide-bağırsak sisteminde parazitlik yapan *Ostertagia*'lara (Lat. *Ostertagiya Ronsom)* karşı antelmentik etkisi gösterilmiş [18], koyunlarda Moniezioza (Lat. *Monieziya Blanchard)* karşı boymaderen unu ile fenasal antihelmint preparatı karışımının yüksek antelmentik etkili olduğu belirlenmiştir [19]. Domuzların sindirim sisteminde parazitlik yapan stongillere (Lat. *Strongilida Railliet et Henry.)* ve *Trichocephalus*'lara (Lat. *Trichocephalus Schrank)* karşı kırmızı ve beyaz üç yapraklı yoncanın doğal otlatma yönteminde antelmentik etkili olduğu belirlenmiştir [20].

Nahçıvan Özerk Cumhuriyeti'nde dağlık ve düz arazilerde yaygın olan üzerlik *(PeqanumHarmala)*, devedikeni *(Alhagi pseudalhagi)* ve yoncanın *(Trifolium pratense)* antelmentik etkisi incelenmiştir. Ayrıca çaşır *(Prangos acaulis)* ile acılıkotu *(Ephedra procera)* karışımı, kalkanek *(Grammosciadum platycarpum)* ile dirçek *(Ajuga glabra)* karışımı, üzerlik *(Peqanum harmala)* ile alben (antihelmint preparat) karışımının antelmentik etkisi araştırılmıştır. Fitonsit etkiye sahip maddelerle zengin olan bu bitkilerin hem kökünden, hem de çiçeklenme fazında yüzey parçalarını kullanarak *Haemonchus*'lara (Lat. *Haemonchus Cobb)*, *Trichocephalus*'lara (Lat. *Trichocephalus Schrank)*, *Chabertia*'lara (Lat. *Chabertia Railliet et Henry)*, *Nematodirus*'lara *(Nematodirus Ransom)* karşı imha edici etkileri belirlenmiştir.

Son araştırmalarda meyer pişiknanesi *(N. meyer* Benth.) ve kedi pişiknanesi *(N. cataria* L.) türlerinden alınan uçucu yağlar ve ayrıca bitkilerin yüzey bölümlerinden hazırlanmış şurubların yüksek antelmentik etkiye sahip olduğu tespit edilmiştir [21-24]. Yapılan bilimsel araştırmaların amacı hayvanlarda önemli zararlara yol açan helmintlerin imha edilmesinde doğal ve kolay yolların bulunmasıdır. Kimyasal ilaç maddeleri parazite öldürücü etki göstermekle beraber, konak vücudunda toplanarak hayvan ürünlerinin kalite değişikliğine neden olmaktadırlar. Helmintozların tedavisinde antelmentik etkili bitkileri kullanılarak hem hayvan ürünlerini, hem de çevreyi kimyasalların zararlı etkilerinden koruyabileceğiz. Böyle bitkilerin araştırılması, otlak alanlarında doğal rezervinin korunması ve ayrıca insanlar tarafından ekilmesi helmintlerle mücadelenin doğal yollarından biri sayılır.

Paraziter hastalıklar çoğunlukla sindirim sistemine yerleşerek konaklarında ekonomik kayıplara neden olurlar. Hayvanlarda karaciğere ve mide-bağırsak parazitleri yaygın olup, bu ekonomik kayıplara sebep olan parazitlerin başında gelmektedir. Bu zararların önüne geçmek için antiparaziter ilaçlar yaygın olarak kullanılmaktadır. Antelmentik tedaviler sonucu hayvanların canlı ağırlık artışlarında ciddi yükselmeler olmaktadır [25,26]. Parazitlere karşı kullanılan ilaçların bitkisel yapıda ve doğal olması arzu edilendir.

Bu nedenle araştırmanın sonucu olarak, her iki bitki uçucu yağından ve özütünden helmintosit preparatlar hazırlanarak, bu helmintozların tedavisinde kullanılması tavsiye edilebilir.

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Research Article

Impacts of Ozone Treatment and Its Relationship with IGF-1 Levels After Injury of Soft Tissue: An Experimental Study in Rats Model

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Abstract

To investigate the effects of ozone treatment on soft tissue injury and to observe whether there is an alteration in serum IGF-1 levels after ozone treatment in an experimental rat model. Twenty-four adult Wistar albino 240-350 g male rats were randomly allocated into two groups. A standardized, experimental soft tissue injury was created on left hind limbs of animals. Group 1 underwent daily ozone treatment intraperitoneally (20 µg/mL), while Group 2 received only nutrition and routine care. All rats were evaluated regarding body weight and sensory and motor function on 5th and 15th days after experimental trauma. Blood samples were drawn from intracardiac in group 1 and group 2 serum levels of IGF-1 level were measured at the day of 15th. During follow-up period after formation of soft tissue injury, three rats in Group 2 had cutaneous infection that responded well to topical tetracycline treatment. Two groups displayed similar results regarding sensory and motor functions on 5th and 15th days. Serum IGF-1 level in Group 1 was significantly higher than that of Group 1 (P=0.03). Serum IGF-1 level was correlated with motor function on day 15 in Group 1 (P=0.04) and with motor function on day 5 in Group 2 (P=0.011). Ozone treatment may have favorable impacts on healing and regeneration process in connective and muscle tissues and these beneficial effects may be mediated by IGF-1. Further research is warranted to elucidate the role of IGF-1 in repair process and to provide additional new insights to the treatment strategies.

Keywords: *Ozone, Wound healing, Injury, Muscle, Rat, IGF-1*

Yumuşak Doku Yaralanması Sonrası Ozon Tedavisinin Etkileri ve IGF-1 Seviyesiyle Olan İlişkisi: Rat Modelinde Deneysel Bir Çalışma

Özet

Ozon tedavisinin yumuşak doku hasarı üzerindeki etkilerini araştırmak ve deneysel bir rat modelinde ozon tedavisinden sonra serum IGF-1 düzeylerinde bir değişikliğin olup olmadığını gözlemlemek amaçlandı. Yirmidört yetişkin Wistar albino 240-350 g ağırlığında erkek rat randomize olarak iki gruba ayrıldı. Her iki gruptaki ratların sol arka ekstremitelerine standartlaştırılmış deneysel yumuşak doku yaralanması oluşturuldu. Grup 1'e intraperitoneal olarak (20 µg/mL) günlük ozon tedavisi uygulandı. Grup 2'ye ise sadece beslenme ve rutin bakım yapıldı. Deneysel travmanın 5 ve 15. günlerinde tüm ratlar vücut ağırlığı, duyusal ve motor fonksiyon açısından değerlendirildi. Kan örnekleri Grup 1 ve Grup 2'de intrakardiak alındı ve serum IGF-1 seviyeleri 15. günde ölçüldü. Yumuşak doku hasarının oluşumundan sonra takip süresi boyunca Grup 2'deki üç rat topikal tetrasiklin tedavisine olumlu cevap veren kutanöz enfeksiyona sahipti. Her iki grupta, 5. ve 15. günlerde duyusal ve motor fonksiyonlarla ilgili benzer sonuçlar görüldü. Grup I'deki serum IGF-1 düzeyi grup 2'e göre anlamlı derecede yüksekti (P=0.03). Serum IGF-1 düzeyi, Grup 1'de 15. günde (P=0.04) motor fonksiyonu ile Grup 2'de 5. gündeki (P=0.011) motor fonksiyonlarıyla korele edildi. Ozon tedavisinin bağ dokusu ve kas dokusunda iyileşme ve rejenerasyon süreci üzerinde olumlu etkileri olabilir ve bu faydalı etkilere IGF-1 aracılı olabilir. IGF-1'in onarım sürecindeki rolünü aydınlatmak ve tedavi stratejilerine ek yeni anlayışlar sağlamak için daha fazla araştırma yapılması gereklidir.

Anahtar sözcükler: *Ozon, Yara iyileşmesi, Yaralanma, Kas, Rat, IGF-1*

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INTRODUCTION

Insulin-like growth factors (IGFs) have critical roles in growth, development, cellular regulation and metabolism. IGF-1 is a 70 amino acid polypeptide hormone synthesized in the liver and released into the extracellular fluids. Although the major source of IGF-1 is the liver, synthesis has been shown to take place in other organs including kidneys, ovaries, testes, placenta, pancreas, skin and lungs. Low levels of circulating IGF-1 are seen in patients with chronic liver dysfunction, renal failure, malnutrition and other diseases. The principal role of the IGF-system is to integrate growth and metabolism [1].

In-vitro administration of IGF-1 to cultured rat skeletal muscle and myoblasts resulted in activation of cellular proliferation and differentiation [2]. These properties of IGF-1 indicate its important role in the regulation of myoblast cell proliferation and differentiation. IGF-1 also induces cell proliferation and controls cell differentiation in the initial stage of muscle regeneration. Once a sufficient amount of cells has been attained, cell differentiation is initiated [2]. It has also been shown that freezing can induce regeneration of cow skeletal muscle, and culture of those cells has demonstrated strong positivity for IGF-1 in myoblasts and myotubes. These results suggest that IGF-1 stimulates the proliferation and differentiation of myoblasts during the initial and middle periods of muscle regeneration [3].

Ozone is a powerful oxidant which gained popularity recently owing to its clinically proven therapeutic effects [4]. A high-frequency current field separates the oxygen molecule into the highly reactive monatomic oxygen that combines with another oxygen molecule and forms ozone. In the oral cavity, ozone is shown to penetrate the mucus membrane and effectively destroy bacteria. Ozone displays its multi-dimensional effects using promotion of oxygenation in addition to antibacterial, antifungal and antiviral properties. Furthermore, it elicits the immune response through production of cytokines such as interleukin-2 and interferon [5]. Ozone has been used empirically as a clinical therapeutic agent for chronic wounds, ischemic ulcers and diabetic wounds [6-8]. The beneficial effects of ozone on wound healing may be linked with decreased bacterial infection, attenuation of impaired dermal wound healing and increased oxygen tension due to ozone exposure in the wound area [6-8]. Ozone has a regulatory effect on inflammation and wound healing process, and it may display these effects using growth factors [6,9-12].

Surgical interventions are associated with injury of the cutaneous, connective and muscle tissues. Healing of these injuries may be incomplete and severe sequelae may occur eventually. In this purpose, additional surgical procedures, steroids, anti-inflammatory medications and antioxidants constitute the therapeutic foci for repair of skin and connective tissue. Beyond its effects associated with protein and glucose metabolism, recent studies focussed on the roles of IGF-1 on myogenesis, cellular differentiation, trauma and metabolic diseases [13,14]. Levels of IGF-1 may increase in response to trauma to the connective tissue including soft tissue and muscle [14]. In this experimental study, we investigated the effects of ozone treatment on the soft tissue injury and to observe whether there is an alteration of serum levels of IGF-1 in response to ozone treatment.

MATERIAL and METHODS

Study Design

The study was approved from the Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK/2015-094). Twenty-four adult Wistar albino 240-350 g male rats were maintained at the same center and housed in individual cages with free access to water and animal chow. The animals were maintained in a constant 12 h light/12 h dark cycle at constant room temperature of 21°C and humidity of 60%. After induction of anesthesia by ketamine hydrochloride (80 mg/kg), animals were placed at left lateral decubitus position. The left hind limb was shaved and sterilely prepared. An incision was made at the level of left femoral neck of every rat and cutaneous, subcutaneous and muscular layers were dissected. A standardized soft tissue injury was formed using a jeweler's microforceps for 30 sec. After suturing and closure of the wound, the rats recovered from anesthesia, and they were placed back to the cages. Animals were randomly allocated into two groups (n=12 for each).

Group 1 received daily ozone treatment intraperitoneally (20 µg/mL), while Group 2 received no additional interventions other than nutrition and routine care. All rats were evaluated in terms of body weight and sensory and motor function. At the end of 2 weeks, blood samples were drawn from intracardiac of Group 1 and Group 2 animals following anesthesia with 80 mg/kg ketamine hydrochloride. Serum levels of IGF-1 were measured. Animals were sacrificed by cervical dislocation. IGF-1 protein levels in the serum were measured using commercially available ELISA kits specific for rat IGF-1 (Boster Immunoleader, Wuhan, China), with assay sensitivity < 5 pg/mL and a range of 62.5-4000 pg/mL, in compliance with the manufacturer's instructions.

Recovery of motor and sensory function were evaluated on 5th and 15th days following experimental cutaneous and muscular injury. Analysis of motor and sensory functions were assessed via free walking pattern and foot reflex withdrawal test, respectively.

Motor Function Test

For comparison, the motor activity of the rats was

assessed by the modified Tarlov system which we had used previously in the monkeys, 4 as follows: Grade 0: complete paralysis of legs; Grade 1: flicker of movement; Grade 2: good movement at all joints but without walking or weight-bearing; Grade 3: walking and weight-bearing, but not normally; Grade 4: normal [15,16].

Sensory Function Test

The sensory function was assessed by means of the foot reflex withdrawal test as described by De Koning et al.[17]. The rat was gently immobilized by hand and the sole of the foot was directed towards the examiner. An electric current was applied to the foot sole by means of two stimulation electrodes. Six current strengths ranging from 0.1 to 0.6 mA were tested. Rats with an intact innervation will instantaneously retract their paw upon skin contact with the electrodes. Rats exposed to sciatic nerve crush initially fail for this response. Failure to withdraw the foot on stimulation at 0.6 mA indicated no recovery. Rats reacting to a 0.1 mA current were considered to be completely recovered.

Outcome Parameters

This experimental study was focused on comparison of 2 groups in terms of serum IGF-1 levels on 15th day. Moreover, sensory and motor functions on 5th and 15th days were noted. Sensory function was recorded as all or none response, while motor function was graded in accordance with modified Tarlov system as described above [15,16].

Statistical Analysis

Analysis of data was made with IBM Statistical Package for Social Sciences (SPSS) software version 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Comparison of 2 independent groups in terms of quantitative variables was performed by Mann-Whitney U test. Pearson Chi-Square and Fisher's Exact tests were used for evaluation of categorical variables. Correlation between variables was tested with Spearman's rho test. Quantitative variables are demonstrated as median-interquartile range (minimum-maximum). Confidence interval was 95% and differences associated with a P value less than 0.05 were considered as statistically significant.

RESULTS

An overview of measurements in 2 groups is displayed in *Table 1*. Two groups did not differ with respect to body weights. During follow-up period after formation of soft tissue injury, 3 rats in Group 2 had cutaneous infection that responded well to administration of topical tetracycline ointment (Imex®, Assoss Pharmaceuticals, Istanbul, Turkey). In Group 1, no infections were detected. Two groups displayed similar results in terms of sensory and motor functions on 5th and 15th days. *Table 2* presents a comparative overview of these aforementioned variables in 2 groups. Serum IGF-1 level in Group 1 was significantly higher than that of group 1 (P=0.03). No remarkable differences were observed between groups in terms of motor functions on day 5 (P=0.55) and day 15 (P=0.32) or

Table 1. *An average overview of serum IGF-1 levels, motor and sensory functions on days 5 and 15 in Groups 1 and 2*

Group	Motor Function		Sensory Function		Serum IGF-1 Level (pg/mg protein)
	Day 5	Day 15	Day 5	Day 15	
1	3.33±0.654	3.92±0.29	0.92±0.291	1	11.90±2.44
2	3.08±0.792	3.67±0.493	0.67±0.490	1	10.35±0.48

Table 2. *Comparison of serum IGF-1 levels and motor and sensory functions on 5th and 15th days in 2 groups*

Variable	Day	Tarlov Score	Group 1 n (%)	Group 2 n (%)	*P* Value
Motor function	5	2	1 (8.3)	3 (25)	0.55
		3	6 (50)	5 (41.7)	
		4	5 (41.7)	4 (33.3)	
	15	3	1 (8.3)	4 (33.3)	0.32
		4	11 (91.7)	8 (66.7)	
Sensory function	5	0	1 (8.3)	4 (33.3)	0.32
		1	11 (91.7)	8 (66.7)	
	15	0	0	0	-
		1	12 (100)	12 (100)	
Serum IGF-1 level (pg/mg protein)			10.10-0.88	11.65-1.70	0.003*

** Statistically significant*

Table 3. *Correlation analysis between serum IGF-1 levels and sensory and motor functions*

Group	Variable		*P* Value	r Value
1	Serum IGF-1 level	Motor function on day 15	0.04*	0.590
	Motor function on day 5	Motor function on day 15	0.004*	0.764
2	Serum IGF-1 level	Motor function on day 5	0.011*	0.700
	Motor function on day 5	Sensory function on day 5	0.002*	0.032

** Statistically significant*

sensory functions on day 5 (P=0.32) and day 15. Correlation analysis indicated that serum IGF-1 level was correlated with motor function on day 15 in Group 1 (P=0.04) and with motor function on day 5 in Group 2 (P=0.011). Motor function on day 5 was correlated with motor function on day 15 (P=0.004) in Group 1 and with sensory function on day 5 in Group 2 (p=0.002) *(Table 3)*.

DISCUSSION

The aim of the present study was to assess the effects of ozone treatment in surgically induced soft tissue injury and to investigate whether there may be an association with IGF-1 levels. Our results demonstrated that IGF-1 levels are higher in the group receiving ozone treatment and there is a correlation between serum IGF-1 levels and motor function.

Impact of ozone treatment on wound healing has been studied in current literature [18,19]. However, the effects of ozone on a soft tissue injury model and its relationship with IGF-1 levels had not been reported in an experimental rat model. Ozone may result in increased angiogenesis and facilitate re-establishment of vascularization in the connective tissue. Thereby, improvement of oxygen and nutrient supply will enhance wound healing and repair of injury at the site of injury [18,19]. The precise mechanisms underlying these activities may be linked with anti-microbial property of ozone as well as amplification of the immune response through release of growth factors, interferons and interleukins [20]. Ozone may constitute a saf and effective therapeutic option by means of influencing oxygen metabolism, cell energy, anti-oxidant defense mechanisms, immunomodulation and vascular system [21].

In accordance with these data, our results support that ozone treatment can be a promising option for improvement of healing after soft tissue injury. We came across with 3 cases with infection at the site of traumatic injury in the experimental group that did not receive ozone treatment, whereas no infection was detected in the group that underwent ozone treatment. Even though the level of evidence is not very high, this observation reminds the anti infectious effects of ozone. Ozone can be administered through many routes depending on the site of lesion and clinical picture [22].

Adjunctive treatment alternatives must be preferred only after the failure of comprehensive wound management strategies. Further scientific evidence is necessary for supporting the use of intralesional ozone injection in treatment of chronic wounds. Notably, ozone therapy is not recommended for deep, severely infected or necrotic wounds. Moreover, since ozone treatments are frequently applied at non-academic health institutions, negative results and complications may be underestimated [23].

In addition to the inflammatory parameters, growth factors can exhibit a pivotal role in the wound-healing process. Insulin-like growth factor (IGF) not only promotes the migration of keratinocytes, but wounds with decreased levels of IGF may have less healing capacity [24]. Non-healing wounds in diabetic patients had low IGF-1 levels and exogenous application of IGF-1 promoted wound healing [25]. Remarkably, the correlation of IGF-1 with healing enhancement is a process with synergistic action with other growth factors [26].

In our study, we have focussed particularly on the injury of the connective and muscular tissue. Among the well-known factors, only IGF-1 is known to promote the hypertrophy, regeneration, proliferation and differentiation of skeletal muscles. The local production of autocrine/paracrine IGF-1 is particularly important in the skeletal muscle regeneration process. The impacts of IGF-1 on skeletal muscle cells is important for understanding the wound healing and repair mechanisms and regeneration [27].

Wound healing is a multi-dimensional and complex process that occurs with the interaction of many factors. Therefore, outcomes of the current study are prone to be influenced by multiple parameters. We could not detect a significant difference between sensory and motor functions of 2 groups. However, the occurrence of infection in the group that did not receive ozone is noteworthy. Moreover, a significant increase in serum IGF-1 levels after administration of ozone should be noted. Beneficial of effects of ozone on wound healing that may be mediated through IGF needs to be investigated in further trials. Correlation of serum IGF-1 level with a motor function on day 15 in ozone group reminds that this growth factor may be linked with late term regeneration of muscle tissue after injury.

Main limitations of the present study include experimental

design, lack of a sham group and possible impacts of metabolic, environmental and technical factors which may interfere with the outcome parameters of this study.

To conclude, the current outcomes imply that ozone treatment may have favorable impacts on healing and regeneration process in connective and muscle tissues. These beneficial effects may be mediated by IGF-1. Further experimental and clinical research are warranted to elucidate the role of IGF-1 in the repair process and to provide additional new insights to the treatment strategies.

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Research Article

Light and Scanning Electron Microscopic Structure of the Pecten Oculi in the Common Barn Owl *(Tyto alba)*

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Abstract

This study was carried out to investigate the structural properties of pecten oculi in the common barn owl *(Tyto alba)* by light and electron microscope. Fourteen eyeballs from seven owls were studied. The pecten oculi was located postero-anteriorly in the retina layer where the optic nerve enters the eye. The pecten oculi that was dark brown and pleated type consisted of 7 (n=4) or 8 (n=10) vascularised pectineal pleats. Histologically, there were numerous vessels of different size and melanocytes in the area of the pleats. Melanocytes were more frequently observed in the periphery of the pecten oculi's pleats. Scanning electron microscopy showed hyalocytes on the surface of the pecten oculi. The results of the study indicated that the pecten oculi of the common barn owl was morphologically similar to that of other nocturnal birds.

Keywords: *Common barn owl, Pecten oculi, Tyto alba, SEM*

Peçeli Baykuşlarda *(Tyto alba)* Pecten Oculi'nin Işık ve Elektron Mikroskopik Yapısı

Özet

Bu çalışma peçeli baykuşlarda *(Tyto alba)* pecten oculi'nin yapısal özelliklerini ışık ve elektron mikroskobunda incelemek amacıyla yapıldı. Çalışmada materyal olarak toplam 7 adet peçeli baykuşa ait 14 adet göz küresi kullanıldı. Yapılan incelemede pecten oculi'nin; nervus opticus'un göze girdiği bölgede, retina tabakası üzerinde bulunduğu ve postero-anterior yönlü yerleşim gösterdiği gözlendi. Koyu kahverengi renkte ve kıvrımlı tipte olan pecten oculi, 7 (n=4) veya 8 (n=10) adet damarlı pecten oculi kıvrımlardan oluşmaktaydı. Histolojik olarak; pecten oculi kıvrımları içerisinde çok sayıda orta çaplı damarlar ve melanositler bulunmaktaydı. Melanositlerin pekten oculi kıvrımlarının periferinde daha sık yer aldığı gözlendi. Elektron mikroskobik incelemede pekten yüzeyinde hyalosit varlığı tespit edildi. Peçeli baykuşlarda pecten oculi'nin morfolojik olarak diğer nocturnal türlere benzer olduğu görülmüştür.

Anahtar sözcükler: *Peçeli baykuş, Pecten oculi, Tyto alba, SEM*

INTRODUCTION

The pecten oculi is an organ found only in the eyes of birds that extends from the retinal entrance of the optical nerve up to the vitreous [1,2]. Although birds have higher metabolic rates and thicker retinas than mammals, they do not possess retinal vessels [3,4]. However, the pecten oculi is rich in vessels and pigments and is thought to play crucial roles regulating intraocular pressure and temperature by regulating blood flow, as well as in the nutrition of the retina, which is thick and avascular [5-8]. It may help regulate intraocular pH [9], stabilise the vitreous [10], reduce intraocular flashing [11], aid navigation based on the Earth's magnetic field [12], maintain the balance and integrity of the intraocular environment [13], and regulate metabolic exchange between ocular vessels and the retina [14]. It may also suppress vascular endothelial growth factor (VEGF) and inhibit vascularisation of the retina by supplying

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the eye with sufficient oxygen [15]. Among bird species three different types of pecten oculi are distinguished as conical type, vaned type and pleated type consisting of varying number of folds [16]. Morphologically, it consists of three different parts: the basal and apical parts and the pleats, which are located between the vascularised plates. Gradually decreasing in height, the folds join the basal plate, forming a bridge with the dorso-nasal end of the pecten oculi. In some bird species, there may be long extensions reaching into the vitreous over this bridge [10]. The number of folds depends on the size and shape of the pecten oculi and is associated with the behaviour and visual pattern of the bird species [17]. For example, the pecten oculi is conical in kiwis *(Apteryx)*, vaned in ostriches *(Struthio)*, and pleated in other bird species [16]. Despite such morphological variation, the pecten oculi has the same basic structure in all birds, consisting of numerous vessels surrounded by pericytes and melanocytes, with connective tissue filling the space between them [1].

Numerous studies have examined the morphological and histological structures of the pecten oculi using both light and electron microscopy in the great horned owl *(Bubo virginianus)* [18], red-tailed hawk *(Buteo jamaicensis)* [19], common buzzard *(Buteo buteo)* [20], budgerigar *(Melopsittacus undulates)* [21], black kite *(Milvus migrans)* [22], quail *(Coturnix coturnix japonica)* [23], pigeon *(Columba livia)* [10], stork *(Ciconia ciconia)* [24], and jungle crow *(Corvus macrorhynchos)* [25]. Studies on the morphological and histological structures of the pecten oculi in different owl species including nighthawk *(Chordeiles minor)* [1], great horned owl *(Bubo virginianus)* [18], barred owl *(Strix varia)* [26], spotted eagle owl *(Bubo africanus)* [27] have been published. Strobel [28] examined the pecten oculi of common barn owl *(Tyto alba)* by using ultrasonography. However to our knowledge no study on the light and electron microscopical structure of pecten oculi common barn owl *(Tyto alba)* have been published. We investigated the morphometric and histological structure of the pecten oculi in the common barn owl *(Tyto alba)*, a nocturnal bird species.

MATERIAL and METHODS

Sample Collection

The study material consisted of 14 eyeballs from seven adult common barn owls with an average body weight of 509±18 g. The owls had been brought to the Harran University Faculty of Veterinary Medicine clinic in Sanliurfa Province, Turkey, for treatment and were euthanised because of negative prognoses. The experimental procedures were approved by the General Directorate of Nature Conservation and National Parks-Turkey (Approval no. 70525) and Harran University Animal Experimentation Local Ethics Committee (Approval no. 2017/003/01). The eyeballs were harvested immediately after the death of the animals, which had no detectable visual problems clinically or pathologically. To facilitate diffusion of the fixative solution, 10% formaldehyde was injected into each eyeball. Then the specimens were kept in 10% formaldehyde until the morphological and histological examinations were performed.

Morphometric Analysis

The eyeballs were cut equatorially and the pecten oculi, which is located in the posterior eyeball, was examined morphometrically using a stereomicroscope (Olympus-SZX7, Olympus Optical, Japan) with an attached camera (Olympus Cam-SC50). Nomina Anatomica Avium [29] was used for the nomenclature. Descriptions of the morphometric measurements on bulbus oculi including axial globe length, equatorial diameter and corneal diameter as well as on pecten oculi including height of pecten, length of apical border and length of basal border were shown in *Fig. 1*. Weight of the bulbus oculi was measured by using a balance of 0.0001 g sensitivity.

Histological Examination

Four eyeballs from two owls were used for the histological examination. The specimens were washed in flowing water and then fixed in 10% formaldehyde for 24 h. After dehydration through a series of graded alcohols, the tissue samples were cleared in xylene and embedded in paraffin. The paraffin blocks were cut into serial sections of 5 µm thickness. After deparaffinisation and rehydration, the sections were stained using Crossmann's modification of Mallory's trichrome method [30]. Six serial cross-sections in 5 µm intervals for each sample were examined using the Bs200Pro image analysis program (BAB software) for measuring the diameters of the vessels. The average diameters of vessels belonging to each group were determined with standard errors.

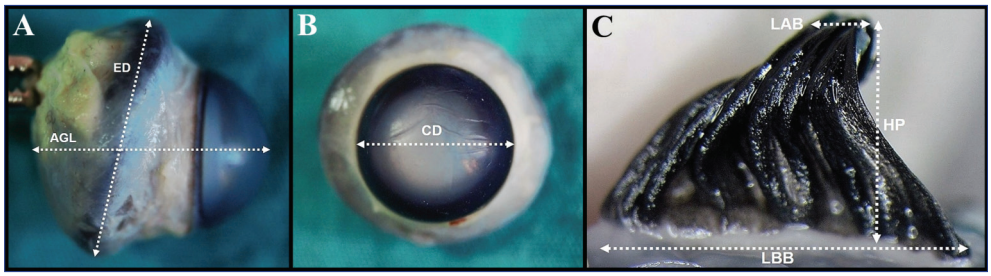

Fig 1. Measurements taken for morphometric analysis of the bulbus oculi (A, B) and pecten oculi (C). AGL: axial globe length, ED: equatorial diameter, CD: corneal diameter, LBB: length of basal border, LAB: length of apical border, HP: height of pecten

Scanning Electron Microscopical (SEM) Examination

For scanning electron microscopy (SEM) analyses, four eyeballs from two owls were used. After dissecting out the eyeballs, they were washed twice with phosphate-buffered saline (0.1 M, pH 7.4) and fixed in 2.5% glutaraldehyde for 48 h. Then the samples were treated with 1% osmium tetroxide for 1 h and dehydrated through a series of increasing concentrations of acetone (25%, 50%, 75%, and 100%, three repetitions each) and dried in a critical point drier. The samples were coated with gold-palladium using a Polaron SC7620 sputter coater and examined with SEM (Leica, LEO 440, UK) at different magnifications.

RESULTS

The dark brown, pleated type pecten oculi was located in the retina layer postero-anteriorly where the optic nerve enters the eye. The pecten oculi consisted of three distinguishable parts: the base, apical (or bridge), and pleats. The basal part was near the optic nerve and was wider than the apical part. Four samples carried pecten oculi of 7 and 10 samples had pecten oculi of 8 vascularised pleats *(Fig. 2-A,B)*.

Minimum-maximum and mean and standard error values of the macroscopic measurements were given in *Table 1*. In stereomicroscopic examinations of the eyeballs, the axial length and equatorial and corneal diameters were 17.56±0.19, 18.01±0.27, and 11.95±0.17 mm, respectively *(Table 1)*. The mean width of the basal part of the pecten oculi was 4.431±0.09 mm, while that of the apical part was 1.447±0.06 mm. The height of the pecten oculi, defined as the distance between the base and the highest point of the apical, was 2.741±0.08 mm. The ratios of the mean height of the pecten oculi to the axial, equatorial and corneal diameters of the eyeball were 0.16, 0.15, and 0.23, respectively.

Histologically, there were numerous vessels of moderate size and melanocytes in areas where pleats extended from the base to the apical *(Fig. 3* and *Fig. 4-A)*. Three different types of vessel were distinguished based on

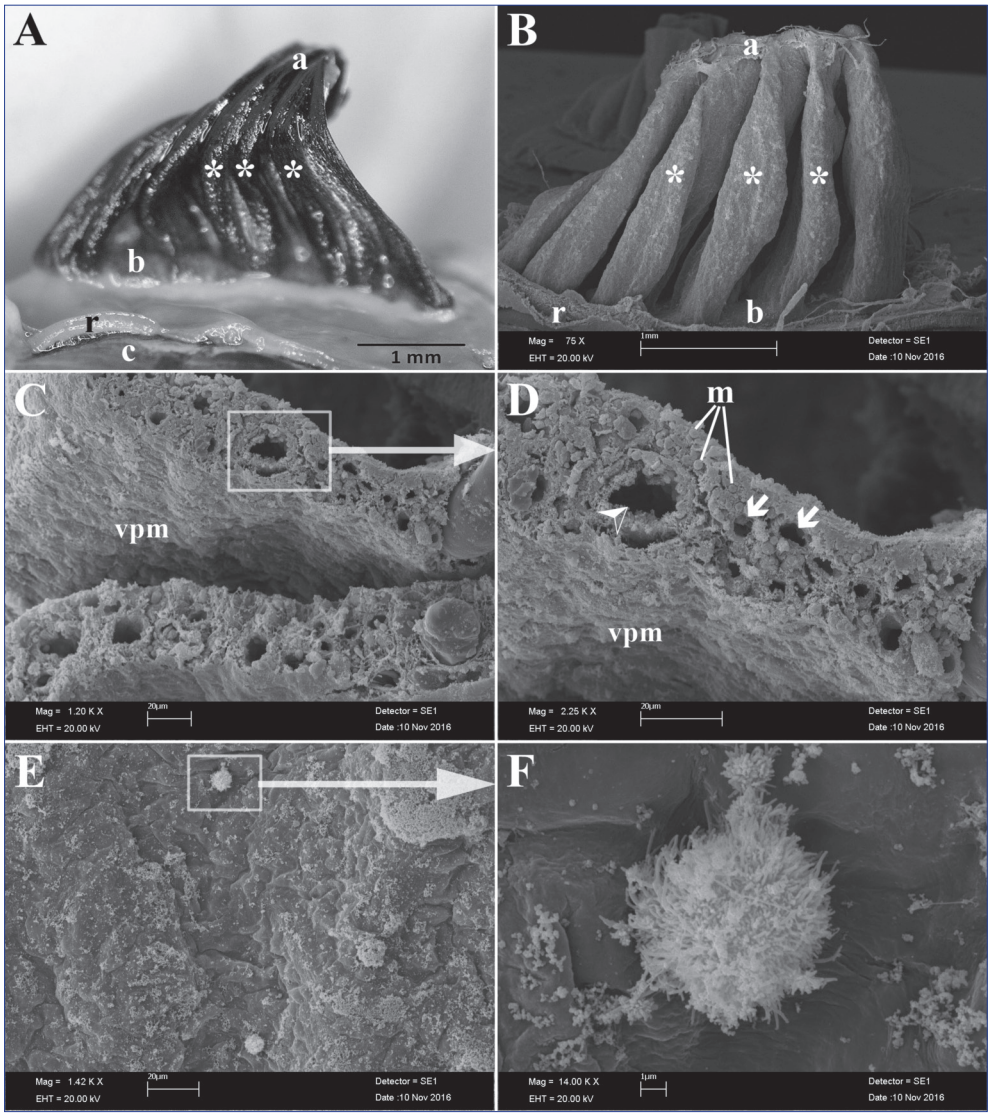

Fig 2. Stereomicroscopic (A) and scanning electron microscopic (B) view of the pecten oculi *in situ*. Scanning electron micrography of the cross-sectional (C, D) and outer (E, F) surface of the pecten oculi. * pectineal folds, a: apical border, b: basal border, r: retina, c: choroidea, m: melanocytes, *arrow:* afferent blood vessels, *arrow head:* efferent blood vessels, vpm: vitreo-pectineal limiting membrane

Table 1. *Body, ocular and pecten oculi measurements of the common barn owls*

Parameters	Min - Max	Mean ± SE
Body and ocular measurements		
Body weight (g)	466 - 550	509±18
Bulbus oculi weight (g)	1.96 - 2.28	2.13±0.05
Axial globe length (mm)	16.93 - 18.79	17.56±0.19
Equatorial diameter (mm)	17.04 - 19.70	18.01±0.27
Corneal diameter (mm)	11.09 - 12.58	11.95±0.17
Pecten oculi measurements		
Height of pecten (mm)	2.481 - 3.036	2.741±0.08
Length of apical border (mm)	1.276 - 1.791	1.447±0.06
Length of basal border (mm)	4.158 - 4.811	4.431±0.09

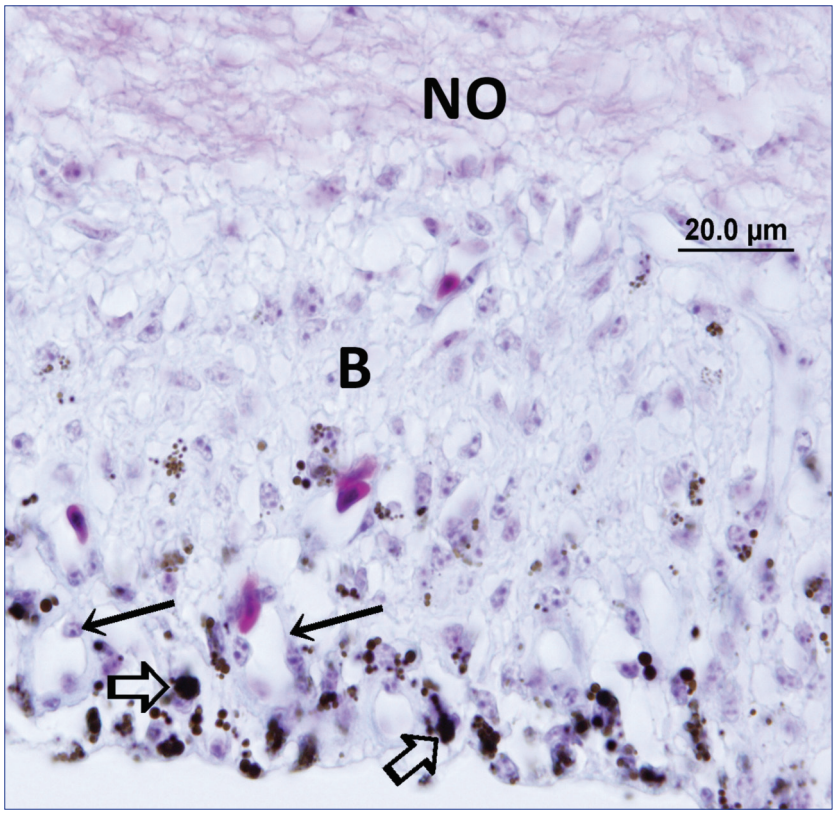

Fig 3. Light microscopic appearance of the basal border of the pecten oculi. B: basal border, NO: optic nerve, *thin arrow:* vessels, *white arrow:* melanocytes

diameter, and defined as primary (largest diameter), secondary (moderate size), and tertiary (small) vessels *(Fig. 4-B,C)*. The primary vessels were central with respect to the pleats, while the secondary vessels were peripheral. Tertiary vessels (capillaries) were observed among the primary and secondary vessels. There were more primary vessels at the basal part, while the number of capillaries increased towards the apical. The average diameters of the primary, secondary, and tertiary vessels were 83.59±19.44, 47.20±12.02, and 23.57±6.59 µm, respectively. Melanocytes were first observed in the area at which the pleats arose from the base and more were found among vessels at the periphery of the pleats *(Fig. 4-C,D)*. No hyalocytes were observed in light microscopy examinations.

SEM showed that the pleats started from the basal part, formed rib-like segments, and merged at the apical part extending into the vitreous *(Fig. 2B)*. The distance between the pleats was greater basally than apically. A vitreo pecteneal limiting membrane separated the pecten oculi from the corpus vitreous. In transverse sections, the pleats contained numerous afferent and efferent vessels along with capillaries *(Fig. 2-C,D)*. Melanin was observed among the capillary net and at the periphery of the pleats *(Fig. 2-D)*. The surface of the pleats appeared rough due to the presence of capillaries. Several star-like hyalocytes with numerous thin, irregular extensions were observed on the outer surface of the pleats *(Fig. 2-E,F)*.

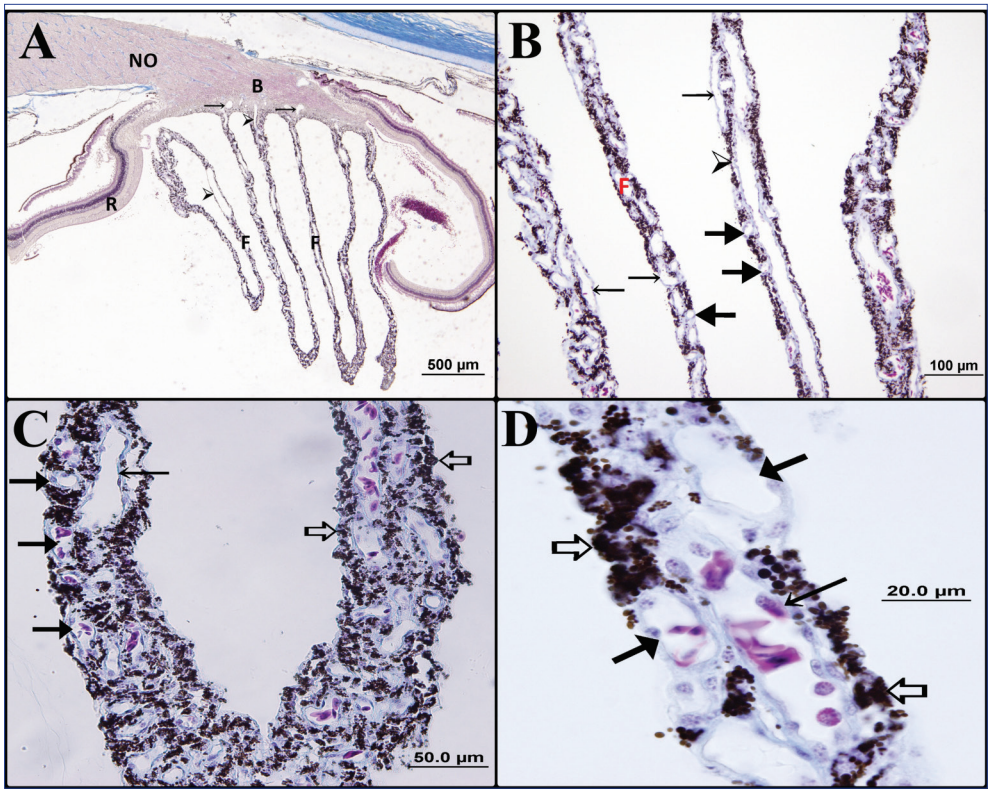

Fig 4. Light microscopic appearance of the pecten oculi (A) and pectineal folds (B-D). R: retina, B: basal border, NO: optic nerve, F: Pectineal folds, *arrow head:* primary vessels, *thin arrow:* secondary vessels, *thick arrow:* tertiary vessels, *white arrow:* melanocytes

DISCUSSION

Similar to other bird species pecten oculi of common barn owl was a pigmented, vascular structure extending into the vitreous located below the postero-temporal region over the optic disc [18,19,22,31]. The pecten oculi of the common barn owl had three different parts, in accordance with the literature: a basal part located near to where the optic nerve enters the eye, pleats arising from the basal part, and an apical part where the pleats merge [21].

Although numerous variants have been described about the morphological structure of the pecten oculi [32], there are generally three different types of the pecten oculi, being conical in kiwis, vaned in ostriches, and pleated in other bird species [16,18,33]. We found that the pecten oculi in common barn owls was of the pleated type, similar to that reported for the great horned and barred owls [18,26]. The physiological significance of the pleats has been explained by different authors so that the presence of the pleats increases the surface area of the pecten oculi without increasing its volume and provides mechanical stability to pecten oculi [27,34].

The size and number of pleats varies among bird species and numerous studies have associated the number of pleats with the function of the pecten oculi. Several studies have indicated that visually more active species have larger pecten oculi consisting of more pleats compared to bird species with less visual activity [21,26,27]. Accordingly, diurnal species, which require visual acuity have larger pecten oculi with more pleats than nocturnal bird species requiring visual sensitivity [17,27]. Among owl species, long-eared, tawny, and eagle owls have short and compact pecten oculi while barn and little owls have longer and narrower pecten oculi [28]. The barred owl [26], great horned owl [18], and emu [35] have a larger pecten oculi relative to the size of their eyeballs. We found that the number of pleats in common barn owl (7-8 pleats) lower than that in barred owl (8-10 pleats) [26] while it was higher than that reported in spotted eagle owl (5-6 pleats) [27] and nighthawk (4-5 pleats) [1]. Pecten oculi of common barn owl was found to be similar to that of great horned owl [18] with respect to number of the pleats. However length of pecten at basal border in barn owl (4.431±0.09) was lower than that in great horned owl (5-6 mm) [18] and higher than that in spotted eagle owl (2.77±0.09) [27] while the pecten hight in barn owl (2.741±0.08) was lower than that great horned owl (5-6 mm) [18] and spotted eagle owl (6.02±0.16) [27]. These results were in accordance with those of Kiama et al.[27] who reported that the number of pleats may not reveal the true size of pecten oculi.

By using eye ultrasonography, Strobel [28] has reported that the length of the pecten oculi is 4.03 mm in the common barn owl, 4.04 mm in the long-eared owl, 5.10 mm in the tawny owl, 4.29 mm in the little owl, and 7.52 mm in the eagle owl. Ravelhofer [36] has reported that the length and height of the pecten oculi in vultures are 11.25 and 6.17 mm, respectively. Braekevelt [18] has reported that the basal length, bridge length, and distance between the basal and apical parts are 5-6, 3, and 5-6 mm, respectively,

in the great horned owl *(Bubo virginianus)*. These values were more higher in the common barn owl. The differences might be attributed to the body sizes of the species studied.

Onuk *et al*.[24] determined that the ratio between the height of the pecten oculi and the diameter of the eyeball in storks was 0.4, while we found that the ratio between the height of the pecten oculi and the equatorial diameter was 0.15. This might be attributable to the differences in the habits of the species under study as storks are diurnal species in contrast to common barn owls.

Numerous capillaries along with afferent and efferent vessels are found in pecten pleats [1,7]. We also observed capillaries and vessels of different sizes in the common barn owl. However, in contrast to other bird species, vessels and melanocytes were observed beginning from the basal part of the pecten oculi. Capillaries were more abundant and were located among the primary and secondary vessels. We did not find any lymph vessels in the pecten oculi, unlike in the mallard *(Anas platyrhynchos)* [13]; this is in accordance with studies on other species [21,24].

Melanocytes are frequently observed in the pecten oculi [1,19,24,37]. We also observed melanocytes at the periphery of the pleats beginning from the basal part and becoming more intensive at apical part. Melanocytes have been reported to be more abundant in the apical and peripheral regions of the pleats than at the basal region [1,18,24]. Because no other cell types function as supporting components in the pecten oculi, melanocytes are thought to play a structural role [17,19]. However it has been also suggested that melanocytes regulate the temperature of pecten oculi by absorbing light and contribute to the metabolic function [9,17] or protect the eye from harmful effects of sunlight [33].

Although the presence of a vitreo-pecteneal limiting membrane covering the pecten oculi has been reported in all bird species [9,26,38], macrophage-like hyalocytes around this connective tissue have been observed only in some species including the chicken [38,39], budgerigar [21], quail [23,40], mallard [41] and emu [35] and not in the pigeon [42], red-tailed hawk [19], or nighthawk [1]. We also found a vitreo-pecteneal limiting membrane in the common barn owl. While macrophage-like hyalocytes were not observed by light microscopy, they were detected on the surface of the pleats via SEM. We suggest that there might be two reasons for this observation. Firstly, hyalocytes might be freely circulating within vitreous in birds as in mammals [43]. These hyalocytes can be lost during the tissue following procedures for light microscopic examinations while they can be seen in 3D SEM using different tissue preparation methods. Secondly, the number of hyalocytes in common barn owl might be low so as to be seen by using SEM which is capable of providing a more detailed information compared to light microscope.

In summary, we investigated the anatomical, morphometric, and histological structure of the pecten oculi of the common barn owl in detail and compared it to that of other bird species. The results suggest that the morphological, morphometric and histological properties of pecten oculi in common barn owl was similar to other nocturnal bird species.

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Research Article

Determining the Priority Selection Emphasis on Characteristics in Terms of Optimized and Non-Optimized Conditions of Production System in Dairy Cows

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Abstract

The aim of this study is assessment of the priority selection emphasis on characteristics in terms of optimized and non-optimized conditions of production system in dairy cows. Optimization is the approach of finding the best accessible value of a predefined objective function within a determined range of effective variables. In optimized conditions for cow future profitability, the decision to cull cattle from herd is based on future predicted profitability of cows. Dynamic planning is an efficient mathematical method which is used to study and optimization of multi-stage decision problems. One of the main challenges in estimating economic value of characteristics is inefficiency of production system. In this study, in order to estimating the economic values and relative importance of characteristics in efficient and inefficient conditions of production system, the average data of six Holstein herds was used which is recorded under supervision of breeding center. After optimizing production system for culling and replacement policies in the herd, a unit is added to the considered characteristic average in order to calculate the economic value of a characteristic after this changes, the production system optimized. The current value of the system calculated in optimized condition before and after changing characteristic and economic value of characteristics obtained from their difference. Relative importance of characteristics such as calving interval, milk production, milk fat, birth weight, mature live weight, increasing daily weight pre-weaning, increasing daily weight after weaning and the first calving age in non-optimized condition of production system are estimated respectively 2.06, 95.62, -4.34, -0.64, 1.90, 1.25, 1.93 and 2.22 percent and In optimized condition of production system are 0.42, 92.55, -0.642, 0.32, 2.00, 1.15, 1.81 and 2.37 percent respectively.

Keywords: *Dairy cows, Economic values, Iran, Non-Optimized, Optimized, Selection emphasis*

Süt İneklerinde Üretim Sisteminin Optimize Edilmiş ve Optimize Edilmemiş Koşulları Açısından Öncelik Seçiminin Belirlenmesi

Özet

Bu çalışmanın amacı, süt ineklerinde üretim sisteminin optimize edilmiş ve optimize edilmemiş koşulları açısından öncelik seçiminin değerlendirilmesidir. Optimizasyon, önceden belirlenmiş bir objektif fonksiyonun erişilebilir en iyi değerini belirlenmiş etkili değişkenler aralığında bulma yaklaşımıdır. Optimize koşullarda ineklerin gelecekteki verimliliği için sürüden bir sığırın çıkarılması kararı ineklerin gelecekteki tahmin edilen verimliliğine bağlıdır. Dinamik planlama, çok aşamalı karar problemlerinin incelenmesi ve optimizasyonu için kullanılan etkili bir matematiksel yöntemdir. Özelliklerin ekonomik değerini tahmin etmede başlıca zorluklardan biri, üretim sisteminin verimsizliğidir. Bu çalışmada, üretim sisteminin uygun ve uygun olmayan koşullarında ekonomik değerlerin ve özelliklerin göreceli öneminin tahmin edilmesi amacıyla, ıslah merkezi gözetiminde kaydedilen altı Holstein sürüsünün ortalama verileri kullanılmıştır. Sürülerde atılma ve değiştirme politikaları için üretim sistemini optimize ettikten sonra, bu özelliklerin ekonomik değerini hesaplamak için dikkate alınan karakteristik ortalamaya bir birim eklenir, bu değişikliklerden sonra üretim sistemi optimize edilir. Sistemin mevcut değeri, karakterler ve bu karakterlerin farklılıklarından elde edilen ekonomik değerleri değiştirmeden önce ve sonra optimize edilmiş koşulda hesaplanmıştır. Buzağılama aralığı, süt üretimi, süt yağı, doğum ağırlığı, ergin canlı ağırlık, sütten kesme öncesi günlük ağırlık kazanımı, sütten kesme sonrası günlük ağırlık kazanımı ve ilk buzağılama yaşı gibi karakterlerin oransal önemi üretim sisteminin optimize olmamış koşulunda sırasıyla 2.06, 95.62, -4.34, -0.64, 1.90, 1.25, 1.93 ve 2.22, optimize koşulda ise 0.42, 92.55, -0.642, 0.32, 2.00, 1.15, 1.81 ve 2.37 olarak belirlendi.

Anahtar sözcükler: *Süt ineği, Ekonomik değerler, İran, Optimize olmayan, Optimize, Seleksiyon vurgusu*

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INTRODUCTION

In livestock breeding, making decision in terms of effective characteristics on profitability (correction objectives) and the share of each characteristic in profitability (economic value or relative importance) are the first steps to development of breeding programs [1]. Economic value of a characteristic indicates the share of genetic growth of which to develop economic efficiency of production system [2,3]. Wrong estimation of this value leads to lack of conformity of breeding program with economic conditions of production system [4]. Bio-economic models are one of the important tools for estimating economic value. In this method, the economic value of the characteristic will be assessed by simulating one unit change in one of the elements of characteristic operation (production and operation) while others remain without change so that its impacts on economic output of the production unit can be calculated [5,6]. Simulation is a process of modeling a real system and doing examinations on the model in order to determine system behavior or assessment of different strategies in a way that simulation model introduces the best production condition according to existing facilities and system capacity. Optimization was found the best accessible value of a predefined objective function within a determined domain of effective variables. In optimized condition for future profitability of cow, livestock culling decision is based on future predicted revenue of cow and by this method; the decision of elimination or maintenance of livestock will be carried out [7]. Difference of production models, characteristic definition and assumptions related to impact of management systems on genetic improvement of a particular one, makes direct comparison more difficult among different countries. In order to compare the suggested selection indicator of a country with others, relative emphasize of the characteristics is calculated [7].

One of the main challenges in estimating economic value of characteristics is inefficiency of production system. As the impact of breeding is long-term, it is necessary to perform it for optimized system and not for non-optimized systems. On-optimized systems lead to skewed estimation of economic values and relative importance of characteristics [8]. Optimized replacement decisions, directly affected by milk price fluctuations, replacement expenses and price of additional heifers, is one of the most important factors for dairy farms [9]. In order to determine optimized replacement decision under different production conditions, dynamic planning is used [10]. Dynamic planning includes stage, condition or state and optimized policy. Every planning problem converts into trivial problems which called a stage. Every stage includes decision making and one or more condition or state. Decision in each stage is made based on the determined condition of the system in that stage. Optimized policy in each stage represents the best decision from that stage to final stage. In this method, production system is divided into limited or unlimited periods and stages along time horizon. In each stage the situation of the system is observed and a decision related to the system is made. This decision affects the system situation in the next stage certainly or occasionally [10]. Each stage depends on some states. Based on the state and decision, intermediate revenue is obtained. In probable dynamic planning method, situation occurrence includes uncertainty therefore; the decisions depend on the conditions occurrence possibility and decision which is made in the previous period. In order to consider uncertainty in prediction, Markov chain model will be used [11]. To select several characteristics simultaneously, the relative importance should be considered. In total genetic-economic indicator, economic value determines the amount of selection emphasize in order to obtain optimized genetic progress with the maximum profitability [12]. So, the aim of this study is to determine relative emphasize of different characteristics in optimized and non-optimized production system.

MATERIAL and METHODS

In this study, in order to estimate relative emphasize of characteristic in optimized and non-optimized condition of production system, the average data of six Holstein herd is used which recorded under consideration of country breeding center. Herd data and economic and bio parameters used for modeling for base scenario has been represented in *Table 1*.

Profit obtained from the difference of revenues and costs.

Table 1. *The data of studied herd and economic and bio parameters used for modeling for base scenario*

Parameters	Amount	Symbol
Birth weight (kg)	43.28	BW
Mature live weight (kg)	680	LW
Preweaning daily gain (kg)	0.65	DG
Postweaning daily gain (kg)	0.506	PDG
Preweaning survival rate (%)	0.95	SR
Postweaning survival rate (%)	0.98	PSR
Survival rate to 24 h of birth (%)	0.98	S24
Age at first calving (days)	720	AFC
Milk price per kg milk (Riyal)	16000	Pm
Natural pasture silage cost per kg DM (Riyal)	2020	Psil
Concentrate cost per kg DM (Riyal)	13200	Pconc
Price per kg LW (Riyal)	70000	PLw
Productive lifetime (days)	1800	PLT
Milk yield per cow per year (kg)	13280	MY
Amount of DM consumed from silage per cow per day (kg)	20	Sil

Revenue and costs of every cow in a year is as follow:

P = R-C

In the above equation, P is annual profit, R is annual revenue and C is annual cost per each cow. Annual revenue per every cow through milk sale (R_{milk}), male calf ($R_{male\ calves}$), eliminated cow ($R_{cows\text{-}age}$), culled heifers ($R_{culled\ heifer}$) and manure (R_{manure}) is calculated as follow:

$R = R_{milk} + R_{male\ calves} + R_{cows\text{-}age} + R_{culled\ heifer} + R_{manure}$

Annual cost for each cow is calculated as follow:

C = CFeed - birth - w + CFeed - w - ma + CFeed - ma - afc + CFeed - cows + CHealth - birth - w + CHealth - w - ma + CHealthh - maafc + CHealth - cows + CLabor - birth - w + CLabor - w - ma + CLabor - ma - afc + CLabor - cows + CReproduction - heifers + CReproduction - cows + CFix

The parameters which are used in above equations are defined as follow:

C Feed-birth-w: nutrition cost of heifer from birth to weaning, CFeed-w-ma: nutrition cost of heifer from weaning to 18 month age, CFeed-ma-afc: nutrition cost of heifer from 18 month age to first calving, CFeed-cows: nutrition cost of dairy cow, CHealth-birth-w: health and hygiene cost of heifer from birth to weaning, CHealth-w-ma: health and hygiene cost of heifer from weaning to 18 months age, CHealthh-ma-afc: health and hygiene cost of heifer from 18 months age to first calving, CHealth-cows: health cost of a cow, CLabor h- birth-w:labor cost from birth to weaning, CLaborh- w-ma: labor cost from weaning to 18 months age, CLaborh- ma-afc: labor cost from 18 months age to first calving, CLabor-cows: labor cost for each cow, CReproduction-heifers: reproduction cost of the heifer, CReproductin-cows: reproductive cost of cow, CFix: fix costs.

The relation between costs and revenues determined by a mathematical method and economic coefficient of characteristics estimated using system analyze method:

$$V_I = \frac{P_{m_{i+\Delta}} - P_{m_i}}{\Delta}$$

In which V_i is economic coefficient, Pmi+Δ is average profit per each cow after adding a genetic unit in I characteristic, P_{mi} is average profit per each cow before genetic progress and Δ is I increase of character which is used to determine economic coefficients. Optimization of dynamic planning depends on replacement decisions in each state and stage which depends on operation in current state and optimized decision in next stage. In order to optimize production system, Markov chain simulation in the probable dynamic planning method is used. A set of Markov processes is considered as follow and every moment is placed in a particular state of $S_1,\ldots, S_n$ and the state of system changes in discrete times and regular intervals according to a set of probabilities. Accordingly, if the state is indicated for times t = 1.2…. by q_t, in order to show the operation of this process in format of Markov processes, the current state should be determined based on previous states which is shown in the following equation:

$$P(q_t = S_j | q_{t-1} = S_i, q_{t-2} = S_k, \ldots) = P(q_t = S_j | q_{t-1} = S_i)$$

In this equation, $P(q_t)$ shows system state and $P(q_t = S_j/q_{t-1})$ is a transient probability which defines the movement from one state to another along a determined period. Accordingly, future behavior of the system depends on its current state. In this study, planning horizon is 10 lactation period and each period is a stage to decision making. It should be noted that by considering 10 calving in the model, in fact we do not have the 11th calving. So, system value does not affect decision making after planning horizon and is considered as zero and since in the last lactation period the cow is replaced intentionally, system value in the last stage equals to slaughter or scrap value in management concepts. State variables used in dynamic planning to describe cows situations includes power generated in 3 levels with a production less than 5000 kg, 5000-7000 kg and more than 7000 kg which include 0.02, 0.35 and 0.63 percent of cows respectively and reproduction operation classified in 4 levels by calving intervals of 410, 450, 490 and 530 days. To indicate state parameters, condition vector of the cow at Tth stage is defined as follow:

$$S_T = [S_t^{parity}, S_t^{prod}, S_t^{reprod}]$$

Which S_t^{parity} is the number of lactation period of dairy cow, $S_t^{parity} = 1,2,\ldots,10$, S_t^{prod} is production capacity ($S_t^{prod} = 1,2,3$),1 for low production dairy cow, 2 for medium and 3 for high production. and S_t^{reprod} is the condition in pregnancy time $S_t^{reprod} = 1,2,3,4$, $S_t^{reprod} = 1$ is is an ideal state (there is no delay in pregnancy), 2 is a state with 40 days delay in pregnancy and calving in next year, and respectively, 3 and 4 are 80 and 120 days delay in pregnancy. A decision made at the end of T stage is (maintenance = 0 and replacement = 1). Decide to maintain means that the cow will remain another calving period in the herd. Replacement decision relates to results of cow sale and replacement with a new cow in the first lactation period.

Function of lactation period efficiency in each stage is as follow:

If we decide to maintain the dairy cow:

$R(s^{parity,prod,reprod}, x = 0) = MR(s^{parity,prod,reprod}) - FC(s^{parity,prod}) - TL(s^{parity,prod,reprod})$

And if decide to replace livestock with heifer:

$R(s^{parity,prod,reprod}, x = 1) = MR(s^{parity,prod,reprod}) - FC(s^{parity,prod}) - TL(s^{parity,prod,reprod}) - HC + SR(s^{parity}) + GP(s^{parity})$

Which $MR(s^{parity,prod,reprod})$ is milk production efficiency and is a function of lactation period, production capacity and pregnancy condition, $FC(s^{parity,prod})$ is nutrition cost which is function of lactation period and production capacity, $TL(s^{parity,prod,reprod})$ is losses value and is a function of lactation, production capacity and pregnancy condition. HC Is the cost of replaced heifer $SR(s^{parity})$ is efficiency of cow sale to slaughterhouse and is a function of lactation and $GP(s^{parity})$ is the value of genetic progress and is a function of lactation. Efficiency and costs which are not dependent on replacement rate, removed from the model. Optimized decision calculated numerically with a continuous repeat using CompEcon toolbox in MATLAB software [13]. Continuous repeat can be used to optimization under an infinitive planning horizon and when some the state are relatively small. Also in this study, annual profit of the cattle designed by the MATLAB programming language to simulate bio-economic system of cattle and then the amount of revenues and costs estimated in the system and finally relative emphasize calculated as follow:

$$RE = \frac{(EV_i \times GSD_i)}{\sum_{i=1}^{t}(EV_i \times GSD_i)} \times 100$$

Which RE EV_i, GSD_i are relative emphasize, economic value and genetic standard deviation of the i^{th} characteristic and t is the number of characteristics in the correction objective. It should be noted that different genetic standard deviation must be derived from valid scientific sources [13,14]. By comparing relative emphasize between countries, it is possible to make essential decisions in order to sperm imports and improving progeny test programs. After optimizing production system for replacement and culling decisions for the cattle, one unit is added to average of the considered character and then production system can be optimized again according to this change. Current system value calculated in optimized condition before and after changing the characteristic and economic value and relative emphasize obtained from their difference.

RESULTS

In this study, revenues and costs are represented separately. The results show that the most of revenues and costs of dairy cows breeding unit is obtained from milk sale (89% of the total revenues) and costs related to health and nutrition (55% of the total costs) respectively. Economic value of a characteristic calculated for production and operation characteristics which is defined as change in profit for each dairy cow in year for one unit genetic merit of considered characteristic when other characteristic remain stable. Economic value of characteristics in non-optimized conditions of the system has been shown in *Table 2*. Results showed that economic value of a characteristic

Table 2. *Economic value of characteristics in non-optimized conditions of the production system*

Trait	Economic Value (Riyal)
Calving interval	38764.84
Calving rate	41966.26
Survival rate 24 h after calving	31123.38
Milk production	52806.22
Milk fat	-90500.69
Lifetime of production	-1354.72
Mature live weight	44448.54
Birth weight	-9377.8
Preweaning daily gain	14809.23
Postweaning daily gain	23952.72
First calving age	45951.32

follow different factors such as production, economic and nutrition parameters of production system. In this study, optimizing livestock replacement and culling policies carried out in the format of productive and reproductive characteristics. *Table 3* shows the current expected value using replacement strategies and optimized elimination for three capacitive livestock group. It can be seen as increases of culling rates as the cow get older. By considering 10 calving in the model, we do not have the 11^{th} calving. In 10^{th} calving, there is no future condition assumption for cow and system value in this stage is equivalent to scrap value. Such that, system value after the end of planning horizon do not impact the decision making and would be considered as zero. For 9^{th} calving, since its future condition is 10^{th} calving, the amount of elimination is much greater than other calving. One of the main criteria in estimating current expected value is organizing cows in the cattle based on future revenue and cost and according to this amount, decides to maintain or cull the cow in the herd. Therefore, without considering these values, the cows are culled earlier than optimized time which leads to cattle profitability reduction. Probability of transition from one lactation period to next period using data related to reproductive condition (4 states of calving interval) in every lactation period has been represented using logistic regression in *Table 4*.

Assume that 0.34 indicates the ideal probability of calving interval in the first lactation. Coefficients related to different levels of milk production for low, medium and high productive levels are 0.02, 0.35 and 0.63 respectively. To calculate economic values of characteristics in optimized condition, first, the coefficients related to different productive and reproductive conditions of different lactation periods obtained. After estimating multiplication of current expected values to coefficients related to different productive and reproductive conditions of different lactation periods, the weighted average of total current expected values in frequency of herd composition

Table 3. *Current expected value of different productive and reproductive conditions related to different lactation periods (in million Riyals)*

Lactation	Production Capacity											
	Low Production				Average Production				High Production			
	Pregnancy Status				Pregnancy Status				Pregnancy Status			
	a	b	c	d	a	b	c	d	a	b	c	d
1	9.540371059 (K)	1.537438682 (K)	3.534198504 (K)	5.531073751 (K)	5.505448073 (K)	7.502515695 (K)	0.499275518 (K)	496150765.2 (K)	5.467387450 (K)	7.464455072 (K)	9.461214894 (K)	1.458090142 (K)
2	4.545097240 (K)	6.542164862 (K)	8.538924684 (K)	0.535799932 (K)	8.511106888 (K)	0.508174511 (K)	2.504934333 (K)	501809580.5 (K)	..474346944 (K)	2.471414566 (K)	5.468174388 (K)	7.465049635 (K)
3	6.543259387 (K)	8.540327009 (K)	0.537086832 (K)	2.533962079 (K)	9.510980426 (K)	2.508048049 (K)	4.504807871 (K)	6.501683118 (K)	476448765.8 (K)	0.473516388 (K)	2.470276210 (K)	5.467151457 (K)
4	6.535657786 (K)	9.532725408 (K)	1.529485231 (K)	3.526360478 (K)	5.505855977 (K)	7.502923599 (K)	9.499683421 (K)	1.496558669 (K)	474476134.4 (K)	7.471543756 (K)	9.468303578 (K)	1.465178826 (K)
5	0.523315284 (K)	2.520382906 (K)	4.517142728 (K)	7.514017975 (K)	5.496740729 (K)	7.493808351 (K)	0.490568174 (K)	4.487443421 (K)	469431334.4 (K)	6.466498956 (K)	8.463258778 (K)	1.460134026 (K)
6	1.507869673 (K)	3.504937295 (K)	6.501697117 (K)	8.498572364 (K)	3.485253612 (K)	5.482321234 (K)	8.479081056 (K)	0.475956304 (K)	462927385.6 (K)	9.459995007 (K)	1.456754830 (K)	3.453630077 (K)
7	5.490797701 (K)	7.487865323 (K)	9.484625145 (K)	1.481500393 (K)	3.472848645 (K)	5.469916267 (K)	7.466676089 (K)	0.463551337 (K)	456411188.1 (K)	3.453478810 (K)	5.450238632 (K)	7.447113879 (K)
8	4.473874735 (K)	6.470942357 (K)	8.467702179 (K)	1.464577427 (K)	6.461273811 (K)	8.458341433 (K)	1.455101256 (K)	3.451976503 (K)	451622147.0 (R)	3.448689769 (R)	5.445449591 (R)	7.442324838 (R)
9	7.459805688 (R)	9.456873310 (R)	2.453633133 (R)	4.450508380 (R)	4.453201033 (R)	6.450268655 (R)	8.447028477 (R)	0.443903725 (R)	445770796.1 (R)	3.442838418 (R)	5.439598240 (R)	7.436473487 (R)
10	4.450928367 (R)	6.447995989 (R)	8.444755811 (R)	0.441631059 (R)	4.444981291 (R)	6.442048913 (R)	8.438808735 (R)	0.435683983 (R)	438290830.9 (R)	1.435358453 (R)	3.432118275 (R)	5.428993522 (R)

a: ideal pregnancy status, b: 40 days delay in pregnancy, c: 80 days delay in pregnancy, d: 120 days delay in pregnancy

Table 4. *Results related to probability of reproductive conditions in different lactation periods with logistic regression*

Calving Interval	Lactation								
	1	2	3	4	5	6	7	8	9
410 Days (ideal pregnancy status)	0.34	0.33	0.31	0.30	0.29	0.28	0.26	0.23	0.22
450 Days	0.23	0.22	0.21	0.20	0.20	0.19	0.18	0.18	0.17
490 Days	0.15	0.15	0.14	0.13	0.13	0.12	0.14	0.15	0.15
530 Days	0.28	0.30	0.34	0.37	0.38	0.41	0.42	0.44	0.44

Table 5. *Economic value of characteristics in optimized and non-optimized conditions of production system*

Trait	Economic Value in Non-optimized Conditions (Riyal)	Economic Value in Optimized Conditions (Riyal)
Calving interval	38764.84	16772.5
Calving rate	41966.26	90808.8
Survival rate 24 h after calving	31123.38	67311.3
Milk production	52806.22	108721
Milk fat	-90500.69	-28462
Lifetime of production	-1354.79	4433.5
Mature live weight	44448.54	98778.1
Birth weight	-9377.8	10019.2
Pre-weaning daily gain (kg)	14809.23	28849.4
Post-weaning daily gain (kg)	23952.72	47863.9
First calving age	45951.32	104447

Table 6. *Relative importance of characteristics in optimized and non-optimized condition of production system*

Trait		Genetic Standard Deviation	Relative Importance (%) in Optimized Condition	Relative Importance (%) in Non-optimized Condition
Productive	Milk production	561.7	92.55	95.62
	Milk fat	14.9	-0.642	-4.34
Reproductive	Age at first calving	15	2.37	2.22
	Calving interval	16.52	0.42	2.06
Growth	Postweaning daily gain	24.95	1.81	1.93
	Preweaning daily gain	26.15	1.15	1.25
	Mature live weight	13.32	2.00	1.9
Survival	Productive Life Time	0.29	0.0195	-0.001
	Birth weight	21.44	0.32	-0.64

extracted to determining the amount of optimized state profit in base scenario. After optimizing production system for replacement and culling policies within the cattle to calculate economic value of a characteristic, one unit added to the average of the considered characteristic and based on this change, production system optimized again. Current value of the system calculated in optimized conditions before and after characteristic change and economic value obtained from their difference. In this study, absolute economic coefficients in optimized condition of production system and economic value of characteristics in optimized and non-optimized conditions of production system are represented in the *Table 5*. According to this table, economic value of milk production, age of the first calving and calving intervals in non-optimized condition of production system are 52805.22, 45951.32 and 38764.84 Riyals per kg respectively and after optimizing economic value system these values obtained as 108721.4, 104447

and 16772.5 Riyals per kg for a cow per year. According to *Table 6* relative importance of characteristics in optimized and non-optimized condition of production system milk production in Optimized Condition and non-optimized condition were 92.55, 95.62 percent respectively.

DISCUSSION

According to the results, milk production has the highest economic value. Positive economic value for milk production indicates that genetic improvement of milk production has positive effect on the system. It should be noted that any factor which leads to reduce costs of milk production or increase the revenue obtained from milk sale, leads to increase economic value of milk production. The differences between economic values of characteristics in optimized and non-optimized conditions are due to using optimized livestock replacement method which was used as farmer policy in replacement and impacts the profit. In optimized condition in order to improve future profitability of cow, elimination decisions are based on predicted future revenues. In non-optimized condition, cows are removed earlier or later than optimized time and therefore profitability is reduced. The role of optimization in total profitability has been reported in different studies. Accordingly, in order to maximize cattle profit, elimination decisions should be optimized. Mir Mahdavi et al.[15]; Sadeghi et al.[16] and Sahragard et al.[17] estimated economic value of milk production 232, -353.6 and 983.3 Riyals respectively. Also, in this study, economic values of milk fat, production lifetime and birth weight in non-optimized conditions are -90500.69, -1354.72 and -9377.8 Riyals respectively and in optimized condition are -28461.8, 4433.5 and 10019.2 respectively. Sayed Sharifi et al.[14] reported economic values of milk production, age of the first calving and calving intervals in non-optimized condition of production system -7297.84, -80752.14 and -197416.81 respectively and economic values of these characteristics in optimized condition of production system 20833.51, -34277.73 and -6450.21 Riyals respectively.

Negative coefficients indicate profit reduction after increasing one genetic unit because of higher cost than revenue [18]. According to the various economic values in different reports, it seems that the reason is significant variety of production systems, management, production level and market conditions and local economy which means input price change and increasing price of milk sale. Studying reported sources indicate that in most of the studies economic coefficients of production characteristics and one or two characteristics of durability and reproduction has been estimated and relative importance of different characteristic in reported studies for the country and other provinces are differ from each other. Accordingly, the comparison of relative importance of characteristics obtained from this study has been represented in *Table 6*. Using dynamic planning as one of the optimization methods to realize future condition from the point of management variables of production system such as elimination and replacement policies, leads to unbiased estimation of economic values and relative importance. From *Table 6* it can be seen that in optimized condition of production system, the characteristics of milk production, age of first calving and mature live weight have the highest relative importance therefore, in order to increase production system efficiency, these characteristics should be selected in priority. According to using strategies of optimized replacement and culling dairy cows with ages higher than optimized age, observed that there is difference in estimating relative importance in comparison to non-optimized condition of production system in terms of characteristics ranking. Non-optimized production system leads to skewed estimation of economic values and relative importance and selection improper orientation in selection indicator [19]. Therefore, it is better to perform economic values and relative importance of characteristics for optimized systems and not for non-optimized systems.

The simultaneous change in the price of inputs and outputs in the system balances the effects of each other in determining the economic values of the traits, but a large increase in output prices than inputs, and vice versa causes a lot of changes in the economic value of the traits. Therefore, in these cases, it is suggested that the economic values of the traits should be recalculated again.With regard to the process of changes in economic values, increases in food prices have high influence on the profitability of breeding systems. Therefore, the pricing policy should be more precise and more sensitive. Using dynamic planning as one of the optimization methods for identifying future conditions in terms of management variables of the manufacturing system, such as culling and replacement policies, will result in an unbiased estimation of the economic values of the traits. The non-optimal production system is a fundamental challenge in estimating the economic value of the traits. Due to the long-term effects of breeding, it is necessary to determine the economic values for optimal systems. The reason for the differences between the economic values of the traits in optimal conditions and non-optimal conditions is the use of an optimal replacement strategy of the livestock so that it influences the stockbreeder's policy in the replacement of the profit. In order to improve the future profitability of cows, deciding to remove livestock should be based on the anticipated future incomes of the cows.

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Research Article

Development of PPA-ELISA for Diagnosing *Streptococcus suis* Infection Using Recombinant Sao-M Protein As Diagnostic Antigen

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Abstract

Streptococcus suis, an important zoonotic agent, is responsible for outbreaks of human infections. The accurate and rapid detection of *S. suis* may help control the potential outbreak and ameliorate patient outcomes. In the present study, Sao-M was used to establish a horseradish peroxidase enzyme-linked staphylococcal protein A immunosorbent assay (PPA-ELISA) for the diagnosis of *S. suis* infection. Results of chessboard titration test showed that the optimal concentration of coating antigen and dilution of serum were 8 μg/ml and 1:80, respectively. The cut-off was confirmed as OD450≥0.351 for positive response. The specificity of test indicated that rSao-M had no cross-reaction with antisera against the other 6 species of pathogens. The variation coefficient of intra-batch and inter-batch in the repeating tests was less than 9.5%. Comparative analysis by using conventional ELISA kit and established GDH-based ELISA showed that the present PPA-ELISA has higher specificity and sensitivity than GDH-based ELISA. A total seroprevalence of 6.6% in 500 pig serum samples indicated the method's applicability to detect *S. suis* infection. Cumulatively, the results suggested that the PPA-ELISA is a rapid, sensitive and specific diagnostic method and could be used as a new tool for large-scale epidemiological surveys and serological diagnosis of *S. suis* infection.

Keywords: *Streptococcus suis, PPA-ELISA, Sao-M, Diagnosis*

Diagnostik Antijen Olarak Rekombinant Sao-M Protein Kullanılarak *Streptococcus suis*'in Tanısı Amacıyla PPA-ELİSA Geliştirilmesi

Özet

Streptococcus suis, insanlarda enfeksiyona neden olan salgınlardan sorumlu önemli bir zoonotik ajandır. *S. suis*'in doğru ve hızlı bir şekilde tespit edilmesi enfeksiyona bağlı salgınları kontrol edebilir ve hastalığa bağlı etkileri azaltabilir. Bu çalışmada, Sao-M, *S suis* enfeksiyonunun teşhisi amacıyla peroksidaz enzim-bağlı stafilokokal protein A immün testi (PPA-ELİSA) için kullanılmıştır. Satranç tahtası titrasyon testinin sonuçları, kaplama antijeninin optimal konsantrasyonu ve serum sulandırılmasını sırasıyla 8 μg/mL ve 1:80 olarak göstermiştir. Pozitif cevap için eşik değeri OD450≥0.351 olarak belirlendi. Testin spesifitesi rSao-M'nin diğer 6 patojen türe karşı kullanılan antiserum ile çapraz reaksiyon vermediğini gösterdi. Tekrar testlerinde yığın içi ve yığınlar arası varyasyon katsayısı %9.5'ten az idi. Geleneksel ELİSA kiti ve kurulan GDH tabanlı ELİSA'nın karşılaştırmalı analizinde, mevcut PPA-ELİSA testinin GDH tabanlı ELİSA testinden daha yüksek spesifite ve sensitiviteye sahip olduğunu gösterdi. 500 domuz serumu örneğinde toplam seroprevalansın %6.6 olduğu ve bunun yöntemin *S. suis* enfeksiyonunun saptanmasında uygunluğunu gösterdi. Sonuçlar, PPA-ELİSA'nın hızlı, duyarlı ve spesifik bir tanı yöntemi olduğunu ve *S. suis* enfeksiyonunun serolojik tanısı için büyük kapsamlı epidemiyolojik araştırmalarda yeni bir araç olarak kullanılabilirliğini göstermektedir.

Anahtar sözcükler: *Streptococcus suis, PPA-ELISA, Sao-M, Tanı*

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INTRODUCTION

Streptococcus suis (S. suis), a Gram-positive bacteria, can cause pig meningitis and pneumonia, sepsis, sudden death and other diseases [1], also infect humans via impaired skin or mucosa, is an important zoonotic infectious disease pathogen [2]. Before 2005, *S. suis* was classified into 35 serotypes based on differences in capsular polysaccharide antigens [1,3,4]. In 2005, serotypes 32 and 34 were suggested to class into *Streptococcus orisratti* [4], and subsequently serotypes 20, 22, 26 and 33 were also removed from the *S. suis* taxon [5,6]. Hence, there are currently 29 remaining true *S. suis* serotypes [7]. Serotypes 1/2, 2, 3, 4, 5, 7, 8, 9, 21 and 31 have been determined to cause aggressive disease both in humans and/or pigs. Among these, serotype 2 is the most commonly associated with disease [8]. *S. suis* was initially reported as an etiological agent in 1954, and subsequently frequently-occurring bacterial infection [1,8]. Since originally reported in 1968, over 700 human cases of *S. suis* infection have been described in 2009 and further reached a staggering 1642 in 2013 [1,3,9]. *S. suis* is the most frequent cause of adult meningitis in Vietnam and the third most common cause of community-acquired bacterial meningitis in Hong Kong [10-12]. Of note, two large-scale outbreaks of lethal SS2 infection with a hallmark of streptococcal toxic shock-like syndrome (STSLS) occurred in China in 1998 and 2005, respectively, raising grave concerns in public health [13-15]. Unfortunately, there is no effective/specific human vaccine or therapeutics against *S. suis* infections is available so far [16]. Considering the high pathogenicity of *S. suis* infection in humans and pigs, it is crucial to develop a method for rapid and convenient diagnosis [16].

Standard bacteriological and biochemical analysis are routinely used to isolate and identify *S. suis* from clinical samples such as cerebrospinal fluid and blood samples, which are laborious and time consuming, and of low sensitivity [2,17,18]. At present, molecular biology technique based on PCR is the most commonly technique which is used for *S. suis* detection. Okwumabua et al.[17] developed a PCR method that targeted gdh encoding glutamate dehydrogenase, designated gdh PCR. Ishida et al.[19] also developed a PCR method targeting the recN gene, designated recN PCR. In addition, colloidal gold-based immunochromatographic strip test, and amperometric immunosensor techniques were also used for the detection of *S. suis* infection [2,20]. These methods could be used for the rapid and sensitive detection of *S. suis*; however, most of these methods are mainly used by professional technicians and require expensive instruments [2]. Therefore, establishing a simple, rapid, and cost-effective assay for the rapid detection of *S. suis* is an urgent problem to be solved in livestock production and scientific study.

Over the past few decades, the ELISA technique is the most universally used immunoassay in the laboratories to detect both bacteria and viruses. This method has the advantages of low cost, fast application, high sensitivity, ease of use and high reliability, and is possible to detect antibodies or antigens while screening large numbers of samples in a single experiment [7,21]. Surface antigen one (Sao) was identified by screening of a phage display library using convalescent swine sera [22]. Sao is a common surface protein, containing a C-terminal membrane anchoring Leu-Pro-X-Thr-Gly (LPXTG) motif and therefore is anchored to the cell wall peptidoglycan by the housekeeping sortase A, which mediates numerous virulence factors during *S. suis* infection [23]. Sao is highly conserved protein among most *S. suis* strains and has become an important candidate for the subunit *S. suis* vaccines [22]. Immunization with recombinant Sao protein was capable of provoking strong humoral antibody responses, diminish clinical signs and bacterial dissemination, improve survival rates and provide cross-serotype protection in pig and mouse vaccination protocols [24], indicating rSao is a suitable antigen for subunit *S. suis* vaccine development [25]. Sao protein is encoded by three allelic variants of gene of difference lengths, Sao-S (1.5 kb), Sao-M (1.7 kb) and Sao-L (2.0 kb), and Sao-M is the most prevalent variant comprising about 80% [26]. We suspect that Sao-M has great potential to become a diagnostic antigen for detection of *S. suis* infection.

Therefore, the objective of the present study was to develop an indirect ELISA test for the serodiagnosis of *S. suis* infection and to optimize the conditions for its use in basic clinical laboratories or in the field with basic levels of equipment. Furthermore, the performance of this test was evaluated and compared with the standard ELISA test.

MATERIAL and METHODS

Bacterial Strains and Serum (Samples)

In this experimental study, the strain *S. suis* SC22, an MRP^+ EF^+ SLY^+ strain, was provided by Shandong Binzhou Animal Science & Veterinary Medicine Academy (Binzhou, China). It was isolated from a diseased pig in Sichuan Province. Strain SC22 was cultured in Todd-Hewitt broth supplemented with 0.6% yeast at 37°C. The serum against *S. suis* was collected from pigs experimentally infected with the SC22 strain and saved by the aforementioned institute. Antisera against *S. enterica*, *E. coli*, *A. pleuropneumoniae*, *S. aureus*, *S. zooepidemicus*, and *S. equisimilis* were stored by the aforementioned institute. Clinical serum samples were collected from growing pigs from various geographical locations (Shandong, Henan, Anhui, Jiangsu, Zhejiang and Hubei).

Reagents

Horseradish peroxidase-labeled Staphylococcal protein A (HRP-PPA) was purchased from Boster (Wuhan, China). Porcine *S. suis* Antibody ELISA Test Kit, which uses the whole-bacteria lysis as antigen to for detect porcine *S.*

suis all sub-types, was purchased from SenBeiJia Bioloical Technology Co., Ltd (Nanjing, China). Twain-20, bovine serum albumin (BSA) were purchased from Solarbio LIFE SCIENCES Co. Ltd (Beijing, China). Skim milk was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Other reagents, imported or domestically produced, were of analytical grade. Preparation of coated solution, sealing solution, diluent and washing solution were refer to reference Xia [7].

Preparation of Recombinant Sao-M Protein

Specific primer was designed based on sequence of Sao-M coding gene from GenBank accession no. JF 810176. The primer of the Sao-M gene was: Sao-F: GCGGGATCC ATGAATACTAAGAAATGGAG and Sao-R: CAGAAGCTTGAACTA ATTTACGTTTACGTG [25]. The forward and reverse primers contained *Bam*H I and Hind III recognition sequences, respectively. The PCR product was purified by SanPrep PCR Cleanup kit (Sangon, Shanghai, China) and cloned into the prokaryotic expression vector pET-30a according to the manufacturer's instructions (Novagen, Darmstadt, Germany). The identity of the insert in pET30a was verified by DNA sequence analysis (Sangon, Shanghai, China). The recombinant plasmid was transformed into the *Escherichia coli* (E. coli) strain BL21 (DE3) and cultured in LB at 37°C until the absorbance was in the range of 0.6-1.0 at 600 nm. IPTG was added at a final concentration of 0.8 mmol/L and the culture was further incubated at 37°C for 5 h with shaking at 200 r/min. The recombinant Sao-M protein domain (rSao-M) was efficiently expressed in *E. coli* BL21 (DE3), and nickel ion-agarose affinity chromatography was subsequently used to obtain highly pure recombinant protein as described in a previous study [7]. The concentration of total bacterial protein and purified protein were measured using NanoDrop 2000/2000C spectrophotometer (Thermo Scientific; Boston, USA) to determine the concentration of target protein in total bacterial protein.

Procedure of Indirect PPA-ELISA

The purified rSao-M was appropriately diluted in carbonate buffer solution (CBS) and coated onto a polystyrene 96-well microtiter plates at 4°C, overnight. After the plates were washed four times with Phosphate buffered saline (PBS) -Tween-20 (PBST), 5% (w/v) skim milk-PBST was used to block non-specific binding sites at 37°C for 2 h. After washing three times with PBST, 100 µl of serum sample with a titer of 1:80 was then added to the plates and incubated at 37°C for 1 h. 96-well plates were washed three times and further incubated for 1 h with horseradish peroxidase-labeled PPA diluted in PBST-2.5% skim milk. After further washing, the reaction was visualized by the addition of a TMB substrate for 10 min at room temperature (RT). The reaction was terminated with Stop Solution and the results were revealed by Enzyme-linked immune apparatus. The preimmunization serum of New Zealand rabbits and the sera of immunized rabbits were served as negative and positive controls, respectively. Simultaneously, sterile carbonate buffer solution was served as a non-antigen-coated control and PBST-2.5% skim milk was used for the blank control (no serum or no conjugate added).

Optimization for PPA-ELISA

The primary antibody (serum came from a diseased pig in Sichuan Province as described above) and rSao-M were used at twofold dilutions from 1:20 to 1:320 and from 0.5 µg/ml to 64 µg/mL, respectively. The serum and coating antigen dilution corresponding to the largest value of P/N at the value of OD_{450} of positive serum was about 1 and the negative serum with a lower value of OD_{450} were considered as optimal serum dilution and optimal antigen concentration. PBS and CBS were selected as washing and coating buffers, respectively. Horseradish peroxidase-labeled Staphylococcal protein A (PPA) was used as second antibody at a 1:2000 dilution according to the instruction of the manufacturer (BOSTER, China). Serial dilutions of bovine serum albumin, skim milk, horse serum and fetal calf serum were used to screen the best blocking buffer condition. Dilutions were made in PBST. Effects of temperature and time on the performance of the PPA-ELISA were also optimized. The optimal conditions were used for all subsequent PPA-ELISA tests.

Determination of Positive and Negative Thresholds of PPA-ELISA

Indirect ELISA was carried out with 40 pig serum samples in triplicate (all samples were negative in the commercial ELISA kit analysis). The end results of OD_{450}nm were computed as mean (x) and standard deviation (S). According to statistical principles, the thresholds were defined depending on the criteria as follows: a sample OD_{450}nm value $\geq x + 3S$ was regarded as positive; a sample OD_{450}nm value $\leq x + 2S$ was regarded as negative; and the values between $x + 3S$ and $x + 2S$ were regarded as doubtful. The doubtful samples were double tested and regarded as positive if the value still suspicious. In this situation, clinical symptoms were considered for diagnosis if applicable, and further monitoring was performed.

Evaluation of PPA-ELISA

Cross-reaction was assessed by testing of sera from positive controls and antisera against *S. enterica*, *E. coli*, *A. pleuropneumoniae*, *S. aureus*, *S. zooepidemicus*, and *S. equisimilis* [17]. In blocking test, first, the rSao-M protein and *S. suis* positive serum were diluted with PBS at the optimal dilution. Second, the diluted serum was divided into two groups: A and B. Third, 50 µl of diluted rSao-M protein was mixed well with 50 µL A (P) and then the mixture was incubated at 37°C for 1 h. A mixture of 50 µL of B and 50 µL of CBS (N) served as the control. The percentage inhibit (PI, PI = (OD_{450}nm value of N - OD_{450}nm value of P)/OD_{450}nm value of N) was calculated.

At the identical experimental conditions, five negative serum samples and five positive serum samples were chosen randomly and each sample was examined 5 times. For inter-batch experiment, five batches of the purified rSao-M protein were performed at different plate on different occasions at different time. The coefficient of variation was computed (C.V %).

The storage conditions (-20°C, 4°C, 25°C, 37°C) and the duration (1-6 months) of studies were calculated to reflect the analytical stability of the assay.

The analytical sensitivity of the system was evaluated by measuring the reactivity between rSao-M antigen with twofold serial diluted (ranging from 1:20 to 1:1280) specific anti-*S. suis* serum and negative control serum with a 40-fold dilution.

To validate the indirect PPA-ELISA as a clinical diagnostic tool, 160 clinical samples from cases of suspected of *S. suis* infection were tested using our indirect PPA-ELISA and commercial ELISA. Confirmed *S. suis*-positive and negative sera served as controls, and the results obtained from PPA-ELISA and commercial ELISA were compared to computed their detection coincidence.

Clinical Application of the Indirect PPA-ELISA Test

500 sera from growing pigs from various geographical locations (Shandong, Henan, Anhui, Jiangsu, Zhejiang and Hubei) were tested to evaluate the positive rate of *S. suis* infection and further to understand the relationship between the *S. suis* infectivity and Sao-M.

Statistical Analysis

The data were presented as mean ± SD. All graphical illustrations were constructed either by GraphPad Prism5 software or in Microsoft Excel sheet. Student's t-test was used for all statistical comparisons. Significance (P) value summary: $P \leq 0.05$; $P \leq 0.01$.

RESULTS

Expression and Purification of Recombinant Protein rSao-M

E. coli BL21 (DE3) harboring the pET30a-Sao-M plasmid displayed efficient expression after induced by IPTG, an approximately 110 kDa protein band appeared as evidenced by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) electrophoresis. The protein bands were analyzed using Alpha VIEW software, and the results indicated that the soluble Sao-M fusion protein accounted for 44.8% of the total bacterial protein in supernatant. The expressed Sao-M fusion protein was purified using nickel ion-agarose affinity chromatography according to the instructions *(Fig. 1*, line 5-9). As shown by SDS-PAGE, the purified protein was manifested as a single band, about 110 kDa *(Fig. 1*, line 5-9). The protein concentration was 1.16 mg/mL as measured using NanoDrop 2000/2000C spectrophotometer.

Standardization of PPA-ELISA

The Indirect PPA-ELISA was standarded with the conditions of an 80-fold dilution of swine serum and 8 μg/mL of antigen by the square titration experiments. The optimal conditions for the orthogonal experiments were as follows: reaction temperature was 37°C, blocking solution was 5% (v/v) BSA, blocking time was 2 h, primary/second antibody incubation time was 1 h, and chromogenic time was 10 min.

Establishment of Positive and Negative Thresholds

The range of OD_{450}nm values was between 0.138~0.338 with an average of 0.216 and a standard deviation of 0.045, as measured from 30 negative serum samples. The threshold between negative and positive samples was 0.306 which indicates that, a serum sample can be regarded as positive at OD_{450}nm ≥0.351, or negative at OD_{450}nm ≤0.308, or as suspicious with a value between 0.308-0.363. The suspicious sample was measured again and considered as positive with a questionable value.

Specificity and Sensitivity of Indirect PPA-ELISA

According to established PPA-ELISA conditions, all the antibodies against *S. enterica, E. coli, A. pleuropneumoniae, S. aureus, S. zooepidemicus,* and *S. equisimilis* showed negative OD_{450}nm values, indicating a negative cross-reactivity of this antigen with above sera (data not shown). Our blocking test also showed that the OD_{450}nm value of the positive samples sharply reduced after blocking with rSao-M protein *(Table 1)*, thereby indicating that the rSao-M may be specific.

Evaluation of Assay Repeatability

The variation coefficient is less than 8.5% within intra-batch experiments, whereas less than 9.5% with inter-batch experiments using different batches of purified recombinant antigens *(Table 2)*. These results suggested a high reproducibility of this study.

Evaluation of Assay Stability

The stability test was conducted by comparing results from coated microtiter plates tested at four different temperatures for six different times. The results showed that the sensitivity of diagnostic test antigen remained unchanged for 6 months after storing at -20°C and 4°C. However, after storage for 6 months at RT and 37°C the assay showed a slight decrease in the development of the discs, the latter decrease by even more; the results showed that the assay has a good "shelf life" *(Table 3)*.

The Coincidence Rate Between PPA-ELISA and Two Established ELISA

The commercial ELISA Kit for detection of all subtypes

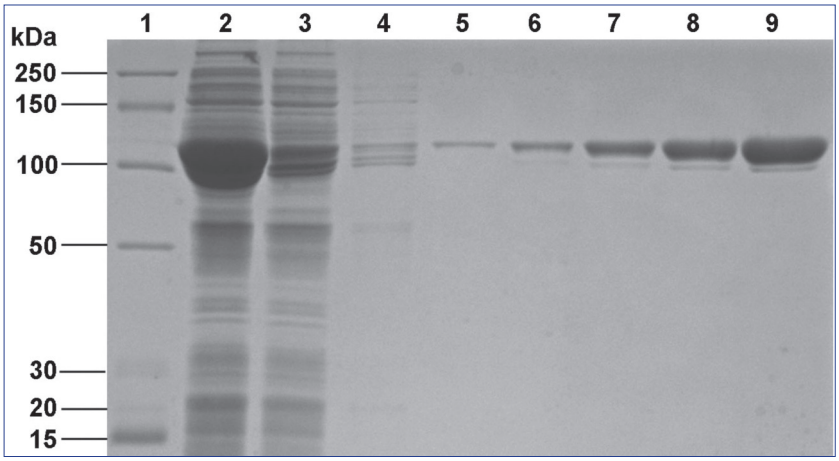

Fig 1. SDS-PAGE of BL21/pET-30a-Sao-M cell lysate. Line 1. Protein ruler; Line 2. All fraction of BL21/pET-30a-Sao-M cell lysate; Line 3. Insoluble fraction of BL21/pET-30a-Sao-M cell lysate; Line 4. Precipitation fraction of BL21/pET-30a-Sao-M cell lysate; Line 5-9. Purified His recombinant proteins with different concentration gradient imidazole

Table 1. *Special-blocking test of PPA-ELISA*

Positive Sera and Treatment		Dilution of Serum Sample			
		1:40	1:800	1:160	1:320
Sample 1	(N-P)/N	61.8%	65.1%	68.1%	71.5%
Sample 2	(N-P)/N	63.3%	67.2%	69.5%	76.1%
Sample 3	(N-P)/N	64.5%	67.6%	71.3%	77.1%

Table 2. *The different values among wells in plate and among plates for PPA-ELISA*

Sample NO.	Repeat Detections					Average X	Standard Deviation	CV Value (%)
	1	2	3	4	5			
Intra-batch experiments								
1	0.802	0.899	0.845	0.861	0.950	0.871	0.056	6.43
2	1.245	1.006	1.135	1.122	1.049	1.111	0.092	8.28
3	1.081	1.066	1.069	1.071	1.125	1.078	0.016	4.12
Inter-batch experiments								
4	0.756	0.689	0.613	0.777	0.748	0.717	0.066	9.20
5	1.033	1.061	1.038	1.145	1.046	1.085	0.063	5.80
6	1.250	1.155	1.239	1.151	1.140	1.187	0.053	4.47

Table 3. *Stability of being coated ELISA plate*

Preserving Time/month	Serum /Batch	Titer Changes Detected by ELISA Plate Stored in Different Preserving Conditions			
		-20°C	4°C	Room Temperature	37°C
1	1	2^8	2^8	2^8	2^8
	2	2^9	2^9	2^9	2^9
3	1	2^8	2^8	2^8	2^8
	2	2^9	2^9	2^9	2^8
6	1	2^8	2^8	2^8	2^8
	2	2^9	2^9	2^9	2^7
9	1	2^8	2^8	2^7	2^7
	2	2^9	2^9	2^8	2^6

Table 4. *Comparison among three kinds of testing methods for detecting sera samples*

Total Number of Samples	No. of Samples Positive by Commercial ELISA Kit	No. of Samples Positive by Indirect Dot-ELISA	No. of Samples Positive by Indirect PPA-ELISA
True- positive	119	115	117
False-positive	0	3	2
False-negative	0	4	2
True-negative	41	38	43
Specificity (%)	100	92.68	95.56
Sensitivity (%)	100	96.64	98.32

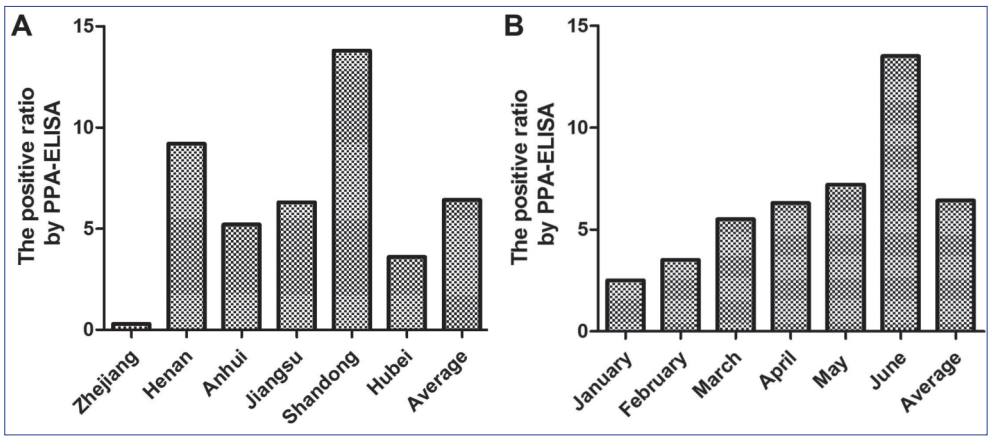

Fig 2. The positive detecting of *S. suis* by PPA-ELISA in 2015. A. The positive detecting of *S. suis* by PPA-ELISA in various geographical locations in 2015; B. The positive detecting of *S. suis* by PPA-ELISA in various months in 2015

of porcine *S. suis* was used as the standard method for confirmation to establish the authenticity of the PPA-ELISA. A total of 160 clinical samples from cases of suspected of *S. suis* infection were examined simultaneously using the indirect PPA-ELISA developed in this study, Dot-ELISA developed in our laboratory and commercialized ELISA diagnostic kit. Among the 160 immune sera samples in the study, positive reactions for *S. suis* were detected in 117 (73.13%) by PPA-ELISA. Two (1.25%) of the samples were negative in the PPA-ELISA but were positive when tested with the commercial kit. Apart from 41 negative samples, two (1.25%) showed a false-positive reaction, these samples were negative in the commercial ELISA Kit. The PPA-ELISA was positive with 73.13% of the samples while commercial Indirect ELISA Kit was positive in 74.38%. Thus, the PPA-ELISA developed in our laboratory showed a specificity of 95.56% and a sensitivity of 98.32% (Table 4), which higher than that of the Dot-ELISA developed in our laboratory [7].

Clinical Application of the Indirect PPA-ELISA Test

A total of 500 serum samples from growing pigs from various geographical locations (Shandong, Henan, Anhui, Jiangsu, Zhejiang and Hubei) were analyzed for the occurrence of *S. suis* infection by the PPA-ELISA method developed in the present study. The result showed that the total seroprevalence was 6.6% (33/500) *(Fig. 2A)*. The positive rate of sera in Shandong Province was 13.8%, only 0.3% in Zhejang Province and less than 10% in the other provinces *(Fig. 2A)*. As shown in *Fig. 2B*, from January to June in 2015, PPA-ELISA positive rate of SS infection displayed an upward trend, and the positive rate in January and February was low and significantly increased in June. These results indicated that the prevalence of *S. suis* is closely related to that of climate, the positive rate of *S. suis* will generally increase in high fever and high humidity environment.

DISCUSSION

Due to its fast spread and high mortality, *S. suis* infection has become a huge threaten for economic and healthy [27]. Therefore, serological surveillance is useful in determining the infection status and could play an important role in the control of *S. suis* in both pigs and humans [28]. ELISA has been confirmed to be a rapid and sensitive method for serological surveillance and detection of bacterial pathogens [28]. In this study, the Sao-M was applied as a diagnostic antigen to develop an accurate and rapid PPA-ELISA detection method, which can be conveniently applied in serological surveillance, with high sensitivity, specificity, and feasibility.

The diagnosis of *S. suis* infection is essentially based on the results of traditional microbiological and biochemical analysis. However, these methods are routine and complex and are unable to accurately distinguish *S. suis* from other related bacteria [17]. Moreover, numerous advanced molecular techniques, including PCR, colloidal gold-based immunochromatographic strip test, and amperometric

immunosensor techniques, could be used for the rapid and sensitive detection of *S. suis* yet require expensive equipment and highly skilled personnel [2,18,20]. ELISA becomes a widely used serological diagnostic technique, as also recommended by OIE International Trade, in animals quarantine for infectious diseases and epidemical investigation due to its strong specificity, sensitivity, objective criteria, and so on [29]. Based on the above, we turned our attention to ELISA which is an essential immunological technology for diagnosis of the diseases.

In addition to serotypes 2, serotypes 1/2, 3, 4, 5, 7, 8, 9, 21 and 31 possess a certain degree of virulence to both in humans and pigs and cause highly hazardous to porcine industry worldwide. Rapid detection of all subtypes of *S. suis* is of great significance for epidemiological survey of *S. suis* and widely monitoring the prevalence of *S. suis* in swine. The Sao protein is highly conserved among *S. suis* strains and Sao-specific antibodies have been shown to react with cell lysates of 28 of 33 *S. suis* serotypes and 25 of 26 serotype 2 isolates in immunoblots [22]. Sao protein is encoded by three allelic variants of gene of difference lengths, Sao-S (1.5 kb), Sao-M (1.7 kb) and Sao-L (2.0 kb) and Sao-M is the most common type of Sao [24]. Moreover, Sao-M in *S. suis* is well conserved and the protein exhibits strong immunogenicity [24]. Based on these observations, Sao-M was chosen as a diagnostic antigen for the development of a serodiagnostic test to detect *S. suis* in this study. Comparing with the GDH-based ELISA measuring system, which previously developed in our laboratory [7], the present PPA-ELISA has higher specificity and sensitivity.

The accuracy of the Indirect ELISA test was compared to the commercial ELISA kit to test the accuracy, our results showed the present Indirect ELISA showed 95.56% sensitivity and 98.32% specificity. Only 1.25% false negativities were observed. Cross-reactivity was not observed between *S. suis* antibody and other bacterial genera. Moreover, a total of 500 swine serum samples from various geographical locations were measured by Indirect PPA-ELISA to determine the seroprevalence of *S. suis*. The results showed that the total seroprevalence was 6.6% (33/500). Furthermore, our results indicated that the prevalence of *S. suis* is closely related to that of climate, the positive rate of *S. suis* generally increases in high fever and high humidity environment, which is in agreement with previous studies [30-32].

In 2007, Li et al. established Sao-based ELISA for detecting titers of Sao-specific total IgG and IgG subclasses in mouse and swine sera [24]. In 2012, Hsueh et al. [25] established ELISA using Sao-L and Sao-M for detecting titers of Sao-L-specific total IgG and Sao-M-specific total IgG. In the present study, Sao-M was used to establish PPA-ELISA for the diagnosis of *S. suis* infection. Compared with the former two groups the present PPA-ELISA displayed less operation time, since the optimized parameters. In addition, Staphylococcal protein A (SPA) could bind to Fc segments of serum IgG in human and various mammals (pigs, dogs, rabbits, monkeys, mice, mice and bovine), and the enzyme-labeled SPA (HRP-SPA) could replace the second antibody for ELISA, can be achieved at the same time on guinea pigs, rabbits, pigs and other animal serum detection. Moreover, the present PPA-ELISA was used in clinical test. Enolase, an immunodominant antigen involved in the virulence of Streptococcus species [33,34] was also used as diagnostic antigen for ELISA and used in clinical test [35]. However, they just tested small amount of samples in Jiangsu province. In our study, 500 sera from growing pigs from six geographical locations (Shandong, Henan, Anhui, Jiangsu, Zhejiang and Hubei) were tested and further to probe whether the prevalence of *S. suis* is associated with climate.

Some limitations of this study should be noted. Firstly, the present PPA-ELISA failed to distinguish wild-type from vaccine strains of the *S. suis*. Secondly, sample sizes was still small though 500 samples were tested in this study. In the future, we will increase the sample sizes from more geographical locations to enhance the practicability of the PPA-ELISA.

In conclusion, our study establishes an indirect ELISA method for detection of *S. suis* antibody, which provides a technical support for quarantine, diagnosis, antibody surveillance, which may prevent the further spread of this emerging pathogen.

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Conflicts of Interest

All authors declared that there are no conflicts of interest.

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Short Communication

Identification, Characterization and Expression Analysis of *Biglycan* in Asian Elephant *(Elephas maximus)* [1]

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Abstract

The aims of this study were to investigate the coding sequence and the deduced amino acid sequence of Asian elephant's biglycan gene as well as its expression in different tissues and conditions using wound healing as a model. The results showed that Asian elephant biglycan coding sequence was 1,110 base pair (bp) long (accession number: JQ753329), encoding 369 amino acids. The coding and amino acid sequences between Asian and African elephants revealed 99% and 98% similarity, respectively. The conserved domains of biglycan protein were also observed. In addition, its expression was found in 15 tissues with a predominant expression in cartilage and spleen. For expression analysis in the wound healing process, it was found that the level of biglycan mRNA was influenced by many factors, including age, type of wound and stage of wound healing.

Keywords: *Asian elephants, Biglycan, Gene expression, Sequencing, Wound healing*

Asya Filinde *(Elephas maximus)* Biglikanın Tanımlanması, Karakterizasyonu ve Ekspresyon Analizi

Özet

Bu çalışmanın amacı Asya filinde biglikan kodlayan sekans ve sonuçlanan amino asit sekansı ile farklı dokulardaki ekspresyonunu araştırmak ve yara iyileşmesindeki durumunu incelemektir. Elde edilen sonuçlar Asya fili biglikan kodlayan sekansın 1110 baz çifti (Ulaşım Numarası: JQ753329) olduğunu ve 369 amino asit kodladığını göstermiştir. Asya ve Afrika filleri arasında kodlama ve amino asit sekansları birbirleriyle sırasıyla %99 ve %98 oranında benzerlik gösterdi. Biglikan proteinde korunmuş domainin varlığı gözlemlendi. Ayrıca biglikan ekspresyonu 15 farklı dokuda tespit edilirken kıkırdak ve dalakta baskın olarak eksprese edildiği belirlendi. Yara iyileşmesindeki ekspresyon analizinde biglikan mRNA seviyesinin yaş, yara tipi ve devresi gibi birçok faktör tarafından etkilendiği tespit edildi.

Anahtar sözcükler: *Asya fili, Biglikan, Gen ekspresyonu, Sekanslama, Yara iyileşmesi*

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INTRODUCTION

Asian elephants (*Elephas maximus*) have a close relationship with Thais and their culture for more than 700 years. However, Asian elephants became an endangered species in the International Union for Conservation of Nature (IUCN) Red List and the first account of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Health problem is an important factor influencing their life quality [1], especially diseases or disorders in musculoskeletal system and wounds [2]. Although many clinical studies have been done on musculoskeletal system and wound healing of Asian elephants, little is known about the musculoskeletal genes of this species.

Many genes involving musculoskeletal system have been identified and characterized. Biglycan, also known as BGN, PG-1, DS-PG1, and PG-S1, is a member of the small leucine-rich proteoglycan (SLRP) family. This proteoglycan is associated with extracellular matrix (ECM) formation as an important structural component and signaling molecule [3], which can be found in many organs [4]. Therefore, the alteration of biglycan at DNA, RNA and protein can influence the progression and the recovery of many diseases and disorders, such as degenerative joint disease, chronic heart failure and other inflammatory diseases [5-7]. The *biglycan* gene has been identified in many organisms, but, for Asian elephant, only the intron region of the gene was studied. In addition, there was no previous report on its gene expression profile in various tissues of the Asian elephant, which is generally studied together to indicate the gene function and its expression pattern on pathological conditions. Hence, this study was conducted to investigate the coding sequence of the *biglycan* gene, the mRNA expression of this gene in the various tissues of the Asian elephant including its expression pattern in some pathological conditions.

MATERIALS and METHODS

This research consists of two main studies. The first is the identification and characterization of coding sequence of the *biglycan* gene and its deduced amino acid sequence in Asian elephant. The other study is to provide the expression levels of *biglycan* gene in various tissues of Asian elephant, including tissues undergoing wound healing at different conditions as an example model.

Animals

Different tissues (skin, pancreas, heart, cartilage, large intestine, kidney, lung, muscle, spleen, liver, cecum, lymph node, small intestine, placenta, thymus) were collected from six Asian elephants (age of 1 day to >60 years) immediately after death. Wounded skin samples were taken from three living elephants with different wound conditions from the Thai Elephant Conservation Center (TECC) (Lampang, Thailand) and the Friends of the Asian Elephant Organization. The information regarding the Asian elephants is provided in *Table 1*. These tissues were stored at -80°C until use.

RNA Isolation and Reverse Transcription

Total RNA was extracted from 20 mg of tissue samples using InviTrap® Spin Universal RNA Mini Kit (Invitek, Germany), according to the manufacturer's protocols. The contaminated genomic DNA was eliminated using DnaseI (Fermentas, USA), following the manufacturer's instructions. The RNA quantity, purity, and integrity were verified using both native RNA electrophoresis on 1% agarose gel and UV absorbance ratio at 260 nm and 280 nm. cDNA was synthesized from 100 ng of total RNA using M-MuLV® Revertid reverse transcriptase (Fermentas, USA) at 65°C for 5 min, 37°C for 5 min, and 42°C for 90 min in Thermal Cycler (Biorad, USA).

Experiment 1: Identification and Characterization of *Biglycan* Coding Sequence

Cloning and Sequencing of Coding Sequence of Asian Elephant Biglycan cDNA

The first partial coding sequence of *biglycan* was amplified using primer pair (F1/R1). This primer pair was firstly designed from the conserved regions of various mammals including cattle (NM_178318), pigs (XM_003135475), mice (NM_007542), rats (XM_001057996), orangutans (NM_001132116), dogs (NM_001003229), rabbits (NM_001195691), and sheep (NM_001009201). The partial coding

Table 1. *Name and description of Asian elephants used in this study*

No.	Name	Age (years)	Sex	Sampling Tissues
				Gene Expression Analysis in Various Tissues
1	Bua-ngern	50	F	skin, pancreas, heart, cartilage, large intestine, kidney, lung, muscle, spleen, liver, cecum
2	Kod	>65	M	skin, heart, large intestine, kidney, lung, spleen, liver, small intestine
3	Somjai	60	F	cartilage, kidney, spleen
4	Thongtae	3	M	heart, lung, spleen, liver, lymph node, small intestine
5	Baby	1 day	M	heart, kidney, spleen, liver, lymph node, thymus, placenta
6	Lomsak	2	M	heart, kidney, small intestine
No.	**Name**	**Age (years)**	**Sex**	**Gene Expression Analysis in Wound Healing**
7	Momae	18	F	nearly closed, acute wound on left foot
8	Mogradee	19	F	new, acute wound at belly
9	Saithong	35	F	chronic wound on forehead

sequence of *biglycan* was then cloned into the TA cloning vector (RBC Bioscience, Taiwan), and sequenced by 1st BASE, Malaysia. The obtained partial sequence was then used together with African elephant *biglycan* gene (XM_003421701.2) to design the F2/R2 and F3/R3 primer pairs, to amplify the whole coding sequence. Next, the *biglycan* fragments amplified from primer F2/R2 and F3/R3 were also cloned and sequenced. The coding sequences were acquired from three individual Asian elephants.

Nucleotide and Amino Acid Sequence Analysis

Three partial coding sequences were analyzed using the BLAST program [8] and combined into a complete *biglycan* coding sequence by Clustal X program [9]. The amino acid sequences were deduced using the six-frame translation program (www.bioline.com/media/calculator/01_13.html) and analyzed by the BLAST program.

Experiment 2: Gene Expression Analysis of Biglycan in Asian Elephant

Expression Analysis of Asian Elephant Biglycan mRNA Expression in Different Tissues and Wound Healing Using Real-time PCR

The mRNA expression of *biglycan* in different samples was performed using Real-time Thermal Cycler, MyiQ5 (Biorad, USA) with 2X SYBR Green qPCR Master Mix (RBC Bioscience, Taiwan), following the manufacturers' instructions. The primer pair F1 and R4 (5'-CAGGTTCAA AGCCACTGTTCTCC-3'), which was designed based on the newly discovered *biglycan* coding sequence, was utilized in this experiment. The housekeeping gene, *glyceraldehydes 3-phosphate dehydrogenase* (*GAPDH*), was used as the internal normalization with the GAPDH primer (F: 5'-ATC ACTGCCACCCAGAAGA-3', R: 5'-TTTCTCCAGGCGGCAGGT CAG-3') designed from the accession number, FJ423089.1. PCR reaction was performed at 95°C for 5 min; 45 cycles of 95°C for 15 s, 62°C for 30 s, 72°C for 30 s, and, 72°C for 7 min. The expression of *biglycan* was calculated by the $2^{-\Delta CT}$ method and normalized by the *GAPDH* expression [10].

Statistical Analysis

Statistical analysis using student's t-test and analysis of variance (ANOVA) was conducted to determine the difference in the mean values of the expression level between groups of the different tissues and mRNA levels of individual wounded-skin samples, respectively. The *P* value for significance was set at $P \leq 0.05$.

RESULTS

Identification and Characterization of the Coding Region of Asian Elephant Biglycan Gene

The full length of *biglycan* cDNA of the Asian elephant was amplified, found to be 1,110-bp long *(Fig. 1)* and deposited in the GenBank database as accession number, JQ753329. The sequence shared 99% homology with that of African elephants. Among the three Asian elephant *biglycan* sequences derived in this study, a single nucleotide polymorphism (SNP) at position 303 was exhibited as thymine (T) or cytosine (C), resulting in a silent mutation which was translated to asparagine *(Fig. 1)*. The 369-amino-acid sequence of Asian elephant *biglycan* in the primary structure was presented in *Fig. 1*. The putative molecular weight was 3.7 kDa with an isoelectric point of 8.39. This amino acid sequence also shared 98% homology with that of the African elephant.

Expression Analysis of Asian Elephant Biglycan in Different Tissues Using Real-time PCR

It was found that the Asian elephant *biglycan* mRNA was ubiquitously expressed in different levels in various tissues *(Table 2)*. High levels of expression in cartilage and spleen were observed, while the *biglycan* mRNA levels in skin, pancreas, muscle, cecum, and placenta were relatively low (expression level <0.1). Significantly higher expression level of *biglycan* mRNA was found in young elephant group (1 day - 3 years), compared to those of the old group (≥50 years), in spleen, liver and small intestine. But in kidney, lung and heart, there was no significant difference between the two groups. For gene expression in wounded skin at different pathologic conditions, the result was showed in *Table 3*.

DISCUSSION

Due to the close relationship in evolution between the Asian and African elephants as they belong to the same order, Proboscidae, we hypothesized that both coding sequence and amino acid sequence of biglycan of the Asian elephant might be similar to those of the African elephant. The 99% and 98% homology of the biglycan coding and amino acid sequence, respectively, between the Asian and African elephants agreed with our hypothesis. Six conserved structures including signal peptide sequence, pro-peptide region, LRR regions, Cysteine loop (CX_3CXCX_6C) at N-terminal that defines it as a member of SLRP classI, Cystein loop at C-termnial and Ser-Gly dipeptides, the GAG attachment site, were also observed and similar to the structure of biglycan in other species [4,11]. These similar structures may suggest the function of biglycan as an important component for connective tissue formation [5,6,11].

This study was the first report of *biglycan* gene expression profile in various tissues of the Asian elephant. From the result, *biglycan* mRNA was predominately expressed in cartilage and spleen. This was consistent with the pre-vious study in other species [12,13]. Moreover, the decreased expression level of *biglycan* in spleen, liver and small intestine in the old elephant group was found.

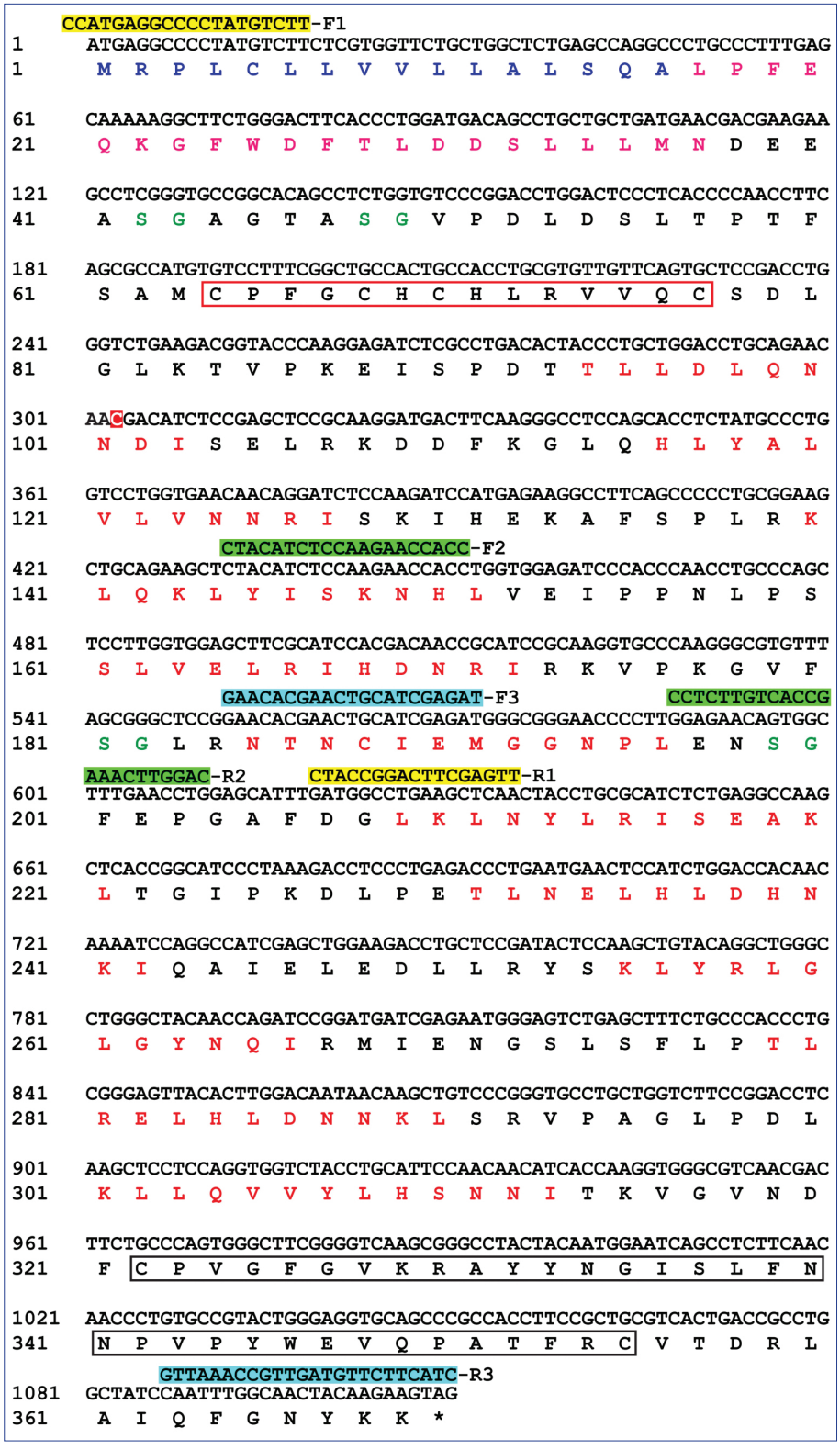

Fig. 1. The nucleotide and deduced amino acid sequence of Asian elephant biglycan including the primers, and their binding sites, used to clone this coding sequence. The primer sequence with its name were shown above the nucleotide sequence with highlight. Forward primer sequences are given in 5′ to 3′ direction. Reverse primer sequences are shown as 3′ to 5′ direction. The red highlight with white letters indicates a nucleotide with polymorphism (T or C). The asterisk shows a stop codon as TAG. The blue and pink letters indicate the putative signal peptide (amino acid 1-16) and pro-peptide region (amino acid 17-37), respectively. Four Ser-Gly dipeptides are indicated by green letters. The Cystein loop at N-terminal (C^{64} to C^{77}) is given in red box. Ten LRR regions (x-L-x-x-L-x-L/I-x-x-N-x-L/I) are indicated by red letters. The letters in black box represent the Cystein loop at C-terminal (C^{322} to C^{355})

This was similar to the decline of *biglycan* mRNA in human articular cartilage which correlates to the increasing age [14]. Although the difficulty in acquiring normal sample was a critical factor that affected the limited expression profile of the gene as shown in *Table 2*, these data could support the function of biglycan and indicated the preliminary trend of its gene expression pattern in various tissues of the Asian elephants.

Skin wound was chosen as a model in this study due to its simplicity of sample collection and observation of the pathophysiology of the samples. Because age and gender are the important factors that affect the physiological changes of wound healing [15]. The significant difference of *biglycan* expression between Momae and Mogradee (similar age and same sex) might be due to the different stage of wound healing process, as Momae had the nearly closed wound (maturation stage) but Mogradee had the open wound with pus (inflammation and proliferation stages). The higher expression of *biglycan* gene in Mogradee, compared to that of Momae, might be a result of its function as an early response gene in inflammatory condition and a participating gene in ECM formation during the proliferation stage [3,7]. For Saitong, the lowest expression level might be due to the older age of Saitong than those of the other elephants. Besides, loss of growth factors in chronic wound [16] especially transforming growth factor-β (TGF-β) might be a critical factor for the low expression of *biglycan* that is usually stimulated by TGF-β in ECM formation [14]. Thus, the expression pattern of *biglycan* mRNA in wound healing in Asian elephant in this study was influenced by age, stage of wound healing and wound type, which was similar to the previous studies [5]. This was also in agreement with

Table 2. *The relative expression of Asian elephant biglycan gene between young and old groups in various tissues*

Tissues	Relative expression	
	Young Group	Old Group
Skin	NS	0.007±0.002
Pancreas	NS	0.038±0.018
Heart	0.134±0.062	0.361±0.282
Cartilage	NS	2.085±0.437
Large intestine	NS	0.103±0.034
Kidney	0.038±0.012	0.142±0.181
Lung	0.260±0.004	0.168±0.115
Muscle	NS	0.003±0.002
Spleen *	3.072±1.138	0.313±0.206
Liver *	0.474±0.377	0.024±0.011
Cecum	NS	0.042±0.007
Lymph node	0.294±0.251	NS
Small intestine *	0.964±0.538	0.101±0.014
Placenta	0.016±0.002	NS
Thymus	0.267±0.048	NS

The data are represented as mean ± standard deviation. NS indicates no tissue samples. The asterisks show the significant difference of gene expression between the young and old groups (P<0.05)

Table 3. *The relative expression of Asian elephant biglycan gene in the wounded samples*

Name	Relative Expression
Momae	0.048±0.009 [a]
Mogradee	0.500±0.154 [b]
Saitong	0.010±0.001 [a]

The data are represented as mean ± standard deviation. [a,b] indicate the significant difference of relative expression among three elephants (P<0.05)

the alteration of expression pattern of *biglycan* by growth factors and pathologic conditions [4].

In this study, the first coding sequence of Asian elephant *biglycan* (1,110 bp) with a deduced amino acid sequence (369 amino acid) and some conserved structures were presented. High expression levels of *biglycan* were found in cartilage and spleen. The expression levels of *biglycan* mRNA that was related to the physiology of wound healing were also revealed. These study provided the preliminary information for further study on developing biomarker for the detection and treatment of disorders related to biglycan in Asian elephant.

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Short Communication

Effects of Chitosan Oligosaccharides Supplementation on the Cell Cycle of Immune Organs in Broilers

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Abstract

The objective of this study was to investigate effects of COS on cell cycle and relative weights of thymus, bursa of fabricius (BF) and spleen in broilers. Three hundred and sixty 1-day-old healthy Arbor Acres male broilers were randomly divided into control, COS I, COS II and COS III groups, which were respectively fed with diets containing 0, 200, 350 and 500 mg/kg COS for six weeks. The results showed that the relative weights of immune organs were higher in the COS group II than those in the control group at 42 d. When compared with the control group, increased percentages of G_2/M phase thymocytes in the COS groups II and III at 21 d, and decreased percentage of G_0/G_1 phase cells, increased percentage of G_2/M phase cells and PI (proliferation index) of thymus were observed at 42 d. The percentage of G_0/G_1 phase BF cells was lower, and the percentage of S phase cells and PI of BF were higher in the COS group II at 21 d. The increase of G_2/M phase splenocytes and PI, decrease of G_0/G_1 phase splenocytes in the COS group II could be seen at 42 d.

Keywords: *Chitosan oligosaccharides, Cell cycle phase, Immune organs, Flow cytometer*

Broilerlerde Kitosan Olisakkarit İlavesinin İmmun Organlarda Hücre Siklusu Üzerine Etkileri

Özet

Bu çalışmanın amacı Kitosan Oligosakkaritlerin (COS) broiler tavuklarda hücre siklusu ile timus, bursa Fabrisius (BF) ve dalak organ ağırlıklarına etkisini araştırmaktır. Üç yüz altmış adet bir günlük sağlıklı Arbor Acres erkek broiler rastgele olarak kontrol, COS I, COS II ve COS III olmak üzere gruplara ayrıldı ve sırasıyla gruplara 0, 200, 350 ve 500 mg/kg COS altı hafta süresince verildi. İmmun organların orantısal ağırlıkları 42. günde COS II grubunda kontrol grubuna göre daha yüksekti. Kontrol grubu ile karşılaştırıldığında 21. günde COS II ve COS III gruplarında yüzde G_2/M faz timositler artmıştı. 42. günde COS II grubunda kontrol grubuna göre daha yüksekti. Kontrol grubu ile karşılaştırıldığında 21. günde COS II ve COS III gruplarında G_0/G_1 faz hücrelerin yüzdesi azalmış, G_2/M faz hücrelerin yüzdesi ile timusun proliferasypn indeksi (PI) ise artmıştı. 21. günde COS II grubunda G_0/G_1 faz BF hücrelerin yüzdesi daha düşük ve S faz hücrelerin yüzdesi ile BF'nin PI'sı daha yüksekti. 42. günde COS II grubunda G_2/M faz splenositlerde ve PI'da artma ile G_0/G_1 faz splenositlerde azalma görülebilir.

Anahtar sözcükler: *Kitosan oligosakkaritler, Hücre siklusu fazı, İmmun organlar, Flow sitometre*

INTRODUCTION

Chitosan oligosaccharides (COS) are a type of oligosaccharides, which are obtained by chemical and enzymatic hydrolysis of chitosan [1,2]. COS have several biological functions, including anti-bacterial, anti-tumor, anti-oxidant, anti-inflammatory and immuno-enhancing functions [3]. COS could inhibit the proliferation of human lung cancer cells and gastric cancer cells by inducing cell cycle blockage in S or/and G_2/M phase [4-6]. There was a report about the

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effect of COS on the normal Schwann cells, which showed that COS could promote cell proliferation [7].

Previous researches have shown that dietary COS could improve immune functions by stimulating antibody production, but related mechanism has not been explored. Since COS could accelerate proliferation of normal Schwann cells *in vitro*, it is noteworthy that whether COS could promote cell proliferation of immune organs in broilers. Hence, this study was conducted to explore the effects of dietary COS on immune organs of broilers by detecting relative weight, and the cell cycle distribution of thymus, bursa of Fabricius (BF) and spleen.

MATERIAL and METHODS

Chickens and Diets

Three hundred and sixty 1-day-old healthy Arbor Acres (AA) male broiler chicks (bought from Sichuan Yuguan agriculture Co., ltd, Sichuan province, China) were randomly divided into four equal groups of 90 each. They were fed on diets as follows: control group (basal diet), COS group I (200 mg/kg COS), COS group II (350 mg/kg COS), and COS group III (500 mg/kg COS) for 42 days. Nutritional requirements were adequate according to the National Research Council (NRC, 1994) and Chinese Feeding Standard of Chicken (NY/T33-2004). The use of animals and all experimental procedures were approved by Sichuan Agricultural University Animal Care and Use Committee.

Relative Weights of Immune Organs

At 21 and 42 days of age, after the body weight was measured, six birds in each group were euthanized and necropsied. Thymus, BF and spleen were dissected from each chick and weighed after dissecting connective tissue around the organ. Relative weight was calculated by following formula:

Relative weight = Organ weight (g) / Body weight (kg)

Cell Cycle by Flow Cytometry Method

Six broilers in each group were euthanized at 21 and 42 days of age, the thymus, BF and spleen were removed from each broiler. Single-cell suspension was prepared by dissecting each sample into homogenate and filtering through 300-mesh nylon gauze. The cells were washed and suspended in cold phosphate buffer solution (PBS, pH 7.2-7.4) at a concentration of 1×10^6 cells/mL. 500 µL cell suspension was transferred into culture tube. And then, 1 mL of 0.25% Triton X-100 was added for perforation. The mixture was incubated for 20 min at 4°C, washed and resuspended with 100 µL PBS. Five µL propidium iodide (PI) (BD Pharmingen, USA, 51-66211E) was added into the cell suspension and incubated for 30 min at 4°C in the dark room. Finally, 400 µL PBS was added and the cell cycle distribution was assayed by FACSCalibur flow cytometer (BD Co. Ltd., San Diego, CA, USA) within 45 min. The results were analyzed by ModFit software [8].

Proliferating index value (PI) = $(S+G_2M) / (G_0G_1+S+G_2M) \times 100\%$

Statistical Analyses

SPSS 20.0 for Windows was used for statistical analyses. The results were presented as means ± standard deviation (M ± SD). Statistical analyses were performed using one-way analysis of variance or t-test, and LSD was employed for multiple comparisons. A value of $P<0.05$ was considered significant, a value of $P<0.01$ was considered extremely significant.

RESULTS

Relative Weights of Immune Organs

The relative weight of thymus in the COS group II was significantly higher ($P<0.05$) than that in the control group at 21 days of age, and significantly higher ($P<0.05$ or $P<0.01$) than those in the COS group I and control group at 42 days of age. No significant difference was observed among the experimental groups at 21 days of age, while the relative weight of BF in the COS group II was higher ($P<0.05$) than that in the control group at 42 days of age. The relative weight of spleen in the COS group I was higher ($P<0.05$) than those of the control group and COS group I at 42 days of age, but there was no significance between the four experimental groups at 21 days of age. The results were shown in *Table 1*.

Cell Cycle Determination

Compared with the control group, the percentage of G_2/M

Table 1. *The relative weight of immune organs in broilers (g/kg)*

Time	Group	21 Days of Age	42 Days of Age
Thymus	control	3.095±0.653^a	3.169±0.591^A
	COS group I	3.764±1.000	3.849±0.727^a
	COS group II	4.358±0.876^b	5.094±1.581^{Bb}
	COS group III	3.528±0.976	4.113±0.870
Bursa of Fabricius	control	2.306±0.276	1.610±0.430^a
	COS group I	2.397±0.329	1.739±0.318
	COS group II	2.722±0.430	2.051±0.349^b
	COS group III	2.670±0.391	1.839±0.325
Spleen	control	1.018±0.132	1.127±0.092^a
	COS group I	1.079±0.197	1.142±0.121^a
	COS group II	1.112±0.121	1.397±0.219^b
	COS group III	1.151±0.284	1.236±0.205

Data are presented with the means ± standard deviation (n = 6). Values within a column followed by different capital letters were significantly different (P<0.01) between two groups (A-B). Values within a column followed by different small letters were different (P<0.05) between two groups (a-b)

phase thymocytes was increased (P<0.05) in the COS group II and III at 21 days of age. The percentage of thymocytes in G_0G_1 phase was markedly lower (P<0.05) in the COS group II at 42 days of age, and the percentage of G_2/M phase cells and PI of thymus in the COS group II were evidently higher (P<0.05) than those in the control group at 42 days of age. An arised tendency of thymocytes in S phase was observed at 21 and 42 days of age, although there was no significant difference. The results are shown in *Fig. 1*.

Compared with the control group, the percentage of BF cells in G_0G_1 phase was markedly decreased (P<0.05) in the COS group II at 21 days of age, while the BF cell percentage in S phase and PI value were significantly

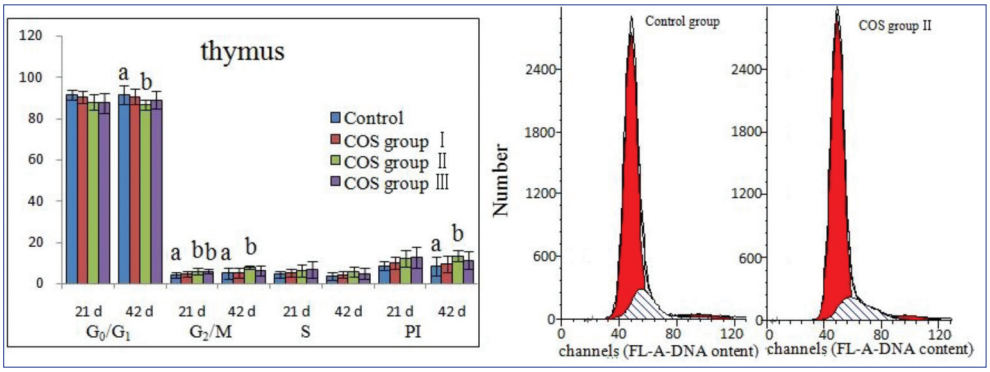

Fig 1. Cell cycle distribution of thymocytes in the broilers fed on the control and COS supplemention diets. Bar graph indicates the percentages of thymocytes in G_0G_1, G_2/M and S phases and PI value (a-b: P<0.05, n=6). The DNA histogram by FCM shows that the first red peak is in G_0G_1 phase, the second red peak is in G_2/M phase, and the white peak with blue stripe is in S phase. At 42 days of age, the percentage of thymocytes in G_2/M phase is higher in the COS group II than those in the control group

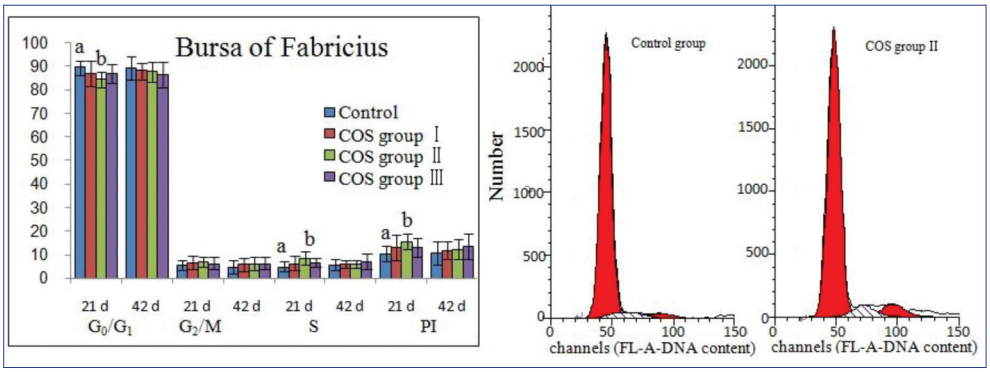

Fig 2. Cell cycle distribution of BF cell in the broilers fed on the control and COS supplemention diets. Bar graph indicates the percentages of BF cells in G_0G_1, G_2/M and S phases and PI value. (a-b: P<0.05, n=6). The DNA histogram by FCM shows the percentage of BF cells in S phase is higher in the COS group II than those in the control group at 21 days of age

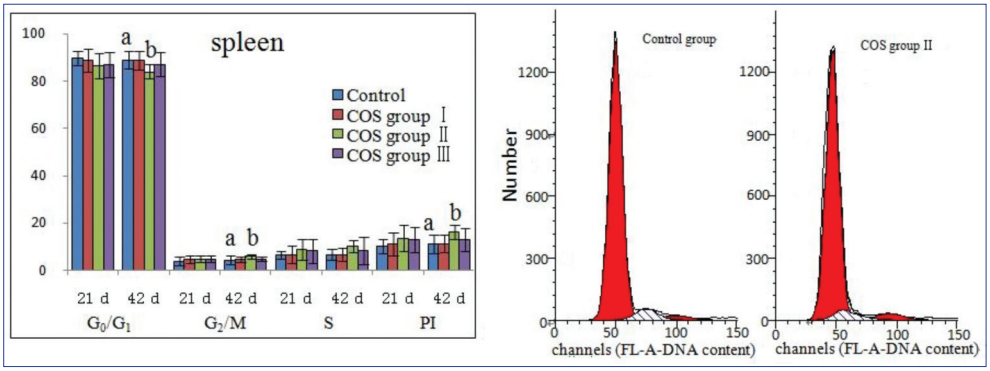

Fig 3. Cell cycle distribution of splenocytes in the broilers fed on the control and COS supplemention diets. Bar graph indicates the percentages of splenocytes in G_0G_1, G_2/M and S phases and PI value (a-b: P<0.05, n=6). The DNA histogram by FCM shows the percentage of splenocytes in G_2/M phase is higher in the COS group II than those in the control group at 42 days of age

increased (P<0.05). An arised tendency of BF cells in G_2/M phase was observed at 21 and 42 days of age, although there was no significant difference. The results are shown in *Fig. 2*.

No significant differences were observed among the four groups at 21 days of age (P>0.05). The percentage of splenocytes in G_0G_1 phase was decreased (P<0.05) in the COS group II compared with that in the control group at 42 days of age, and the percentage of G_2/M phase splenocytes and PI of spleen in the COS group II were significantly higher (P<0.05) than that in the control group at 42 days of age. An arised tendency of splenocytes in S phase was observed at 21 and 42 days of age, although there was no significant difference. The results are shown in *Fig. 3*.

DISCUSSION

Thymus and BF of avian species are known as the central immune organs for diversification and maintenance of T cell and B cell respectively. The spleen, a crucial non-specific peripheral lymphoid organ, has a dominant role in the generation of immune responses [9]. In the present study, it was observed that the relative weights of thymus, BF and spleen in the COS group II were increased when compared with those in the control group. These results suggested that dietary COS supplements with 350 mg/kg could accelerate development of immune organs in broilers. Previous studies [10,11] showed that 50 mg/kg and 100 mg/kg dietary COS could increase the relative weights of thymus, BF and spleen in chicks. However, another research suggested that the relative weight of thymus and BF was increased and that of spleen was decreased in the chickens fed with 50 mg/kg dietary COS [12]. According to previous studies and this research, there were no consistent results about optimal COS level existing enhancing-effects on the relative weight of immune organs, and opposite results were found about the relative weight of spleen in chickens fed on the same dosage of COS. The possible reasons could be related to several factors, including the species and, age of bird, the dosage and duration of feeding COS.

In this study, the percentages of thymocytes, BF cells and splenocytes in G_2/M phase or/and S phase were increased, and those in G_0G_1 phase were decreased in some degree, when compared with those in the control group. At the same time, the increased proliferation index of thymus, BF, and spleen was seen at 42 days of age. As we know, G_0G_1 phase is a resting phase before it enters the process of synthesizing new DNA (S phase) in preparation for another round of mitosis, and G_2/M phase is a division stage [13]. This results suggested that when 350 mg/kg COS were supplemented into the broiler's diets, more cells in the three immune organs entered into the division stage, which might result in the increase of relative weights of these immune organs, and might finally induce the improvement of immune function. As far the effects of COS on cell cycle of normal cells, there was only one in vitro research, which showed that the proliferation index and the expression of cyclin D_1 of normal Schwann cells treated with 0.25, 0.5 and 1.0 mg/mL COS were increased when compared with those of control [7]. The increased proliferation index observed in our study was in agreement with the result of this research, which hints at the possibility that appropriate levels of COS could improve the proliferation of normal cells *in vivo* and *in vitro*. Its mechanisms probably based on the activation of signaling pathways related to DNA and protein syntheses, which need to be furtherly studied.

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Short Communication

Effect of LED Lighting during Incubation of Fayoumi Eggs on Hatchability and Chick Performance

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Abstract

This study aims to evaluate the effect of LED lighting during incubation of Fayoumi eggs on hatchability and hatch chick performance. The experiment was carried out in three groups with total number of 2100 eggs. Eggs were incubated 24 h of complete darkness (G1); 24 h of complete LED lighting (G2); and 12 h of LED lighting then 12 h of darkness (G3). From the obtained results, there were no effects of LED lighting on hatchability percent and dead embryos. There were significant differences observed in chick performance among the three groups, chick weight at hatch was the heaviest in group of eggs exposed to complete LED lighting during incubation (G2) with high vitality percent.

Keywords: *Chicken's eggs, LED lighting, Hatchability, Chick performance*

Fayoumi Yumurtalarda İnkubasyon Süresince LED Işıklandırmanın Yumurtadan Çıkma ve Civciv Performansına Etkisi

Özet

Bu çalışmanın amacı Fayoumi yumurtalarda inkubasyon süresince LED ışıklandırmanın yumurtadan çıkma ve civciv performansı üzerine etkisini araştırmaktır. Toplam 2100 yumurta kullanılarak ve üç grup olacak şekilde çalışma yürütüldü. Yumurtalar 24 saat tümüyle karanlık (G1), 24 saat tümüyle LED ışıklandırma (G2) ve 12 saat LED ışıklandırma ve sonrasında 12 saat karanlık (G3) olacak şekilde inkube edildiler. Sonuçlar LED ışıklandırmanın yumurtadan çıkma yüzdesi ve embriyo ölümlerine etkisinin olmadığını gösterdi. Her üç grup arasında civciv performansında anlamlı bir fark gözlenmedi. Yumurtadan çıkma zamanında civciv ağırlığı inkubasyon sırasında tümüyle LED ışığa maruz kalanlarda (G2) en fazla olup yüksek hayatta kalma yüzdesine sahipti.

Anahtar sözcükler: *Tavuk yumurtası, LED ışıklandırma, Yumurtadan çıkma, Civciv performansı*

INTRODUCTION

To meet the high demand on poultry products, producers are adopting new technologies that will enable them to increase production at a reduced cost. Most of these production technologies focus on enhancing the environmental conditions surrounding the avian population. These environmental conditions are critical elements during embryogenesis of avian well-being. By using light-emitting diode (LED), breeders can increase production and reduce mortality with lower energy consumption and longer life use than fluorescent or conventional incandescent lighting [1].

Fayoumi chickens have a good hatchability percent as a local strain [2]. Generally, chicken's eggs are often incubated commercially in semi or complete darkness, but under natural conditions, avian embryos would certainly receive some light stimulation during development [3]. Avian embryos have a pineal gland sensitive for light that affects their growth [4]. During incubation, exposing eggs to light can increase the embryo's growth [5] and decrease time of incubation [6]. However, numerous studies have shown the importance of exposing embryos to light [7,8], but few of them [9] have shown the LED lighting effects on hatchability and chick performance. Huth and Archer [9] indicated that providing LED light during incubation can improve chick quality.

Therefore, the current study was conducted to investigate the effect of complete/complement LED lighting during

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chicken's eggs incubation on hatchability and hatch chick performance.

MATERIAL and METHODS

A total number of 2100 Fayoumi chicken *(Gallus Gallus domesticus)* eggs were randomly distributed into three groups (700 eggs for each group and each one was further divided into 7 replicates) and incubated in a commercial hatchery under the recommended conditions (37.5°C and 60% RH) with/without LED lighting as follows: the first group (control) was incubated 24 h of complete darkness (G1); while the second group was incubated 24 h of complete LED lighting (yellow, 6 Watt, 540 Leumans) (G2); and the third group was incubated 12 h of LED lighting (yellow, 6 Watt, 540 Leumans) then 12 h of darkness (G3). The study was approved by Alexandria University Animal Ethics Committee (2016-2/18). All chicks were removed at 21-day of incubation.

Data of hatchability percent, embryo mortality [at 7th (early dead) and 18th (late dead) day of incubation] and chick performance (weight, vitality) at hatch were recorded. All the hatched chicks were weighted and examined in order to determine their vitality which included their activity, appearance, eyes, walking, wing flapping, unhealed navel and weakness to stand. The chick vitality levels were using a scale from 1 (poor quality) to 5 (high quality) according to Farghly et al.[10].

One-way ANOVA was used to investigate treatment effects on hatchability and hatch chick performance by SPSS statistical software [11]. Duncan's multiple range test was used to detect differences among means of the three groups.

RESULTS

The effect of LED lighting during eggs incubation on hatchability and chick performance was presented in *Table 1*. There was no significant effect (P<0.05) on hatchability percent of eggs. Also, there was no significant effect (P<0.05) of LED lighting on early and late dead embryos. Differences were observed among groups in incubation period, eggs of G2 (LED) had the lowest (P<0.05) incubation time (hours). Also, there was a difference observed in chick performance among the three groups, chick weight at hatch was the heaviest in group of eggs exposed to complete LED lighting (G2) with high vitality level.

DISCUSSION

To reach high economical efficiency in hatchability, optimal incubation conditions must be providing. Light is one of these conditions that improves embryonic growth and hatchability performance of avian eggs [7]. Also, the type and amount of light could affect hatchability and chick performance [5]. LED lighting provides an approximation of daylight than the spectral gaps of other lightings. Therefore, we used LED lamps in this study by exposing the Fayoumi eggs to complete/complement LED lighting during the incubation to test its impact. In agreement with previous study of Archer et al.[8], there was no effect of complete/complement LED lighting on hatchability percent of eggs *(Table 1)*. Also, we observed that early and late dead embryos were not change among groups. This result corresponds to findings by Huth and Archer [9]. The eggs exposed to complete/complement LED lighting were hatched before the control eggs; thereby, increasing productivity. It means that LED lighting affect the incubation period. It can also affect the hatch window of eggs. The earliness in the hatching time due to light providing may can be refer to accelerate embryonic development rate [12]. Eggs exposed to complete LED light (G2) showed an increase in hatch chick weight compared to G1 and G3 eggs. Light regime helps to increase the embryonic plasma T3 levels which have a positive correlation with metabolic rate and development of chicken embryos [13]. Moreover, lighting treatment regulated melatonin production at post hatch period, which in turn affects some physiological functions of chicks [14]. However, this result is in agreement with Farghly and Mahrose [15] who reported that the eggs incubated under continuous lighting produced heavier

Table 1. *Means ± SE of hatching and chick performance of Fayoumi eggs as affected by LED treatment during incubation period*

Traits	Groups		
	G1 (control)	G2 (LED)	G3 (LED + darkness)
Hatchability (%)	59.01±0.33	60.21±0.40	60.02±0.38
Early dead embryo (n)	27.52±0.20	25.83±0.27	26.30±0.25
Late dead embryo (n)	23.83±0.13	23.11±0.10	21.90±0.11
Incubation period (hrs)	518±0.98^{a}	507±0.81^{c}	512±0.80^{b}
Egg weight at the start of incubation (g)	38.47±2.20	39.98±2.00	39.87±2.03
Chick weight at hatch (g)	34.01±0.18^{b}	37.10±0.17^{a}	36.85±0.17^{a}
Chick performance (%)	90.04±0.50^{b}	95.60±0.55^{a}	93.80±0.50^{a}

^{a,b,c} *Means in the same row with different superscripts are significantly different (P<0.05);* ***G1:*** *24 h of complete darkness,* ***G2:*** *24 h of complete LED lighting,* ***G3:*** *12 h of LED lighting then 12 h of darkness*

chicks than those incubated in the dark. With similar trend, Farghly et al.[10] reported that the highest value of embryo weight was observed under incubated light flashes.

Furthermore, the G2 chicks showed high vitality level than G1 and G3. These results indicate that providing LED light during incubation can improve hatch chick performance. This result is in accordance with that observed by Khalil [16], who revealed that chicks hatched under light regime incubation appeared to be more active when compared with those hatched under dark incubation. Huth and Archer [9] indicated that providing LED light during incubation can improve chick quality. These improvements mainly related to the physiological and metabolic responses to light during embryonic development.

According to the results obtained in this study, there is no statistical difference between the groups in terms of hatchability, early dead embryo and late dead embryo. There is a significant association between the providing of LED lighting during chicken eggs incubation and the hatch chick performance. Light induced from the LED lamps during incubation improved chick's performance. Data of G2 and G3 were closely, therefore, it recommends that complete or complement LED lighting could be applied during eggs incubation to have high hatch chick's performance. Further studies with more details especially on chick behavior and physiology are needed to provide obvious explanation.

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Case Report

Pathological and Parasitological Investigations in an Adult Bottlenose Dolphin *(Tursiops truncatus)*

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Abstract

This case aimed to perform pathological and parasitological examinations on an adult male bottlenose dolphin *(Tursiops truncatus),* which was found dead on the coast of Aegean Sea (Kusadasi/Davutlar/Aydin-Turkey). At necropsy, intestinal volvulus and fibrinopurulent peritonitis were observed. In the histopathologic examination, severe vascular changes including oedema and hemorrhages were observed in the intestines formed by volvulus and peritoneum. In the parasitological examination revealed some ectoparasites (Copepoda: Pennellidae) on the skin. Also, *Stenurus minor (S. minor)* was detected in the lungs. In conclusion, severe ectoparasite infestation, intestinal volvulus, *S. minor* in the lungs, and severe renal calcinosis were defined in the adult bottlenose dolphin.

Keywords: *Dolphin, intestinal volvulus, Pennellidae, S. minor*

Şişe Burunlu Bir Yunusta *(Tursiops truncatus)* Patolojik ve Parazitolojik İncelemeler

Özet

Bu raporda, Ege Denizi sahilinde (Kusadasi/Davutlar/Aydin-Turkey) ölü olarak bulunan yetişkin şişe burunlu erkek bir yunusta *(Tursiops truncatus)* patolojik ve parazitolojik bulgular değerlendirildi. Nekropside, intestinal volvulus ve fibrinopurulent peritonitis gözlendi. Histopatolojik incelemede, volvulus oluşan bağırsak bölümleri ile peritonda ödem ve hemorajileri içeren şiddetli vasküler değişiklikler görüldü. Parazitolojik incelemede, deri üzerinde bazı ektoparazitler (Copepoda: Pennellidae) görüldü. Ayrıca, akciğerlerde *Stenurus minor (S. minor)* tespit edildi. Sonuç olarak, yetişkin şişe burunlu bir yunusta şiddetli ektoparazit enfestasyonu, intestinal volvulus, akciğerlerde *S. minor* ve şiddetli böbrek kalsinozu tanımlandı.

Anahtar sözcükler: *Yunus, intestinal volvulus, Pennellidae, S. minor*

INTRODUCTION

Parasitic infections represent a potential threat to endangered populations of marine mammals. However, little information is known about the role parasites in the deaths and strandings of dolphins [1]. Lungworms (Metastrongyloidea: Pseudaliidae) have been implicated as the main factor of natural mortality of dolphins [2].

The parasites belonging to the Pennellidae family are the common ectoparasites found in large pelagic fishes. A series of planktonic free swimming larval phases exist in their life cycles after metamorphosis, males are free-living; however, females attach to the body surface of host organisms [3]. The copepods display a permanent parasitism, and they feed on mucus, epithelial scrapings, and tissue fluids. No literature shows their direct parasitic effect; however, the wounds caused by parasite may serve as points of entrance for pathogenic microorganisms [4].

In this case, severe ectoparasite infestation, intestinal volvulus, *S. minor* in the lungs, and renal calcinosis were defined in an adult bottlenose dolphin.

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CASE HISTORY

Necropsy was performed on an adult male bottlenose dolphin *(T. truncatus)* found dead on the coast of Aegean Sea (Kusadasi/Davutlar/Aydin-Turkey, 370 46'N 270 15'W). Following necropsy, tissue samples (skin, lung, liver, intestines, kidneys, heart and testicles) were fixed in 10% buffer formalin solution, processed routinely, 5 µm sectioned and stained with hematoxylin and eosin (H.E). Furthermore, the sections were stained by melanin removal method II for melanin and periodic acid-Schiff reaction (PAS) for lipofuscin [5]. Skin samples with parasites and tissue samples from the lungs were preserved in 70% alcohol for further parasitological examination.

In the external view, 125 lesions 3.0–5.0 mm in diameter (52 of them had parasites) were counted, especially on the cauda-lateral and abdominal parts of the skin *(Fig. 1A)*. The parasites were gray-black in color, filament-like structures and 4.5-6.0 cm in length. Some teeth of the dolphin were extracted or rasped.

At necropsy, serosanguinous fluid with fibrin flecks was found in the abdominal cavity. There was an 180º volvulus of 35 cm segment of the jejunum. The involved intestinal segment was dark red in color with fibrinonecrotic exudate on the serosal surfaces *(Fig. 1B)*. Some postmortem changes (autolysis, putrefaction, and pseudo-melanosis) were also observed in all organs.

The histopathological examinations revealed parasitic dermatitis through the parasitic tunnels, surrounded by inflammatory reaction characterized by macrophages, neutrophil leucocytes with areas of hemorrhages *(Fig. 2A)*. These lesions progressed through the muscle tissue, leading to muscle necrosis. There was a thick fibrinopurulent exudate with severe neutrophil leucocytosis, oedema and

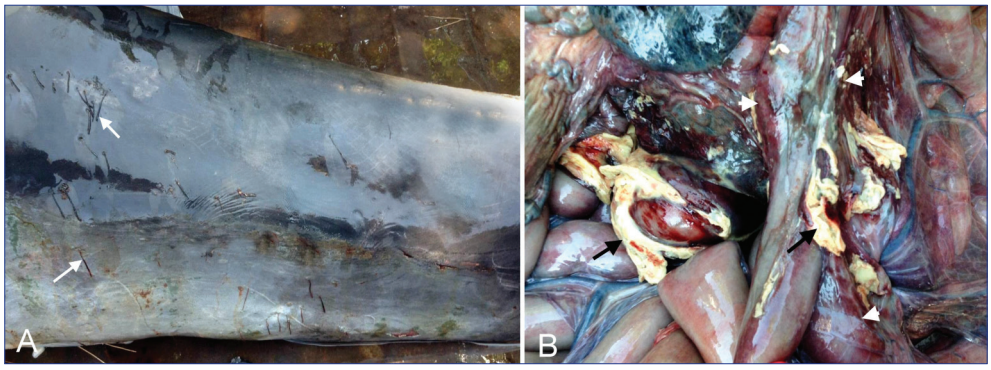

Fig 1. A. Gray-black-colored ectoparasites (Copepoda: Pennellidae) on the skin *(arrows)*. B. The intestinal segment was dark red in color *(white arrowheads)*. Fibrinopurulent exudate *(dark arrows)* in the abdominal cavity

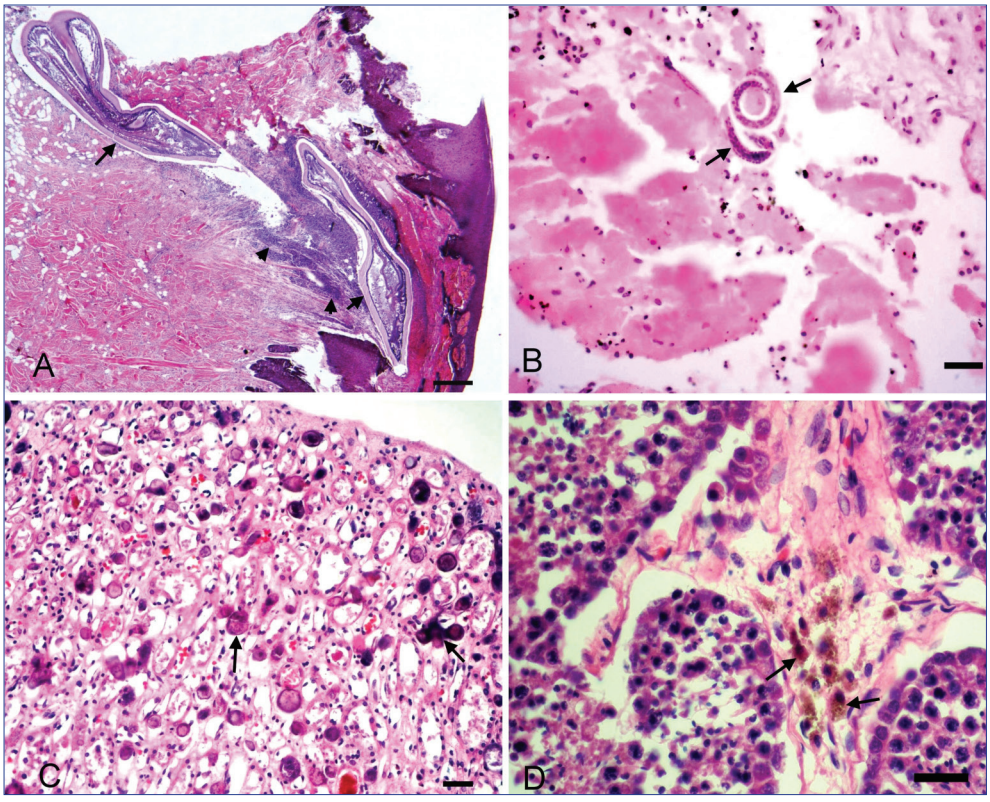

Fig 2. A. Mononuclear cell infiltrations *(arrowheads)* around the parasites *(arrows)* in the skin. H.E. Bar: 150 µm. B. *S. minor larvae* in the lung tissue *(arrows)*. H.E. Bar: 30 µm. C. Calculi formation varying in sizes in the lumen of the renal tubules *(arrows)*. H.E. Bar: 50 µm. D. Pigment granules in the Leydig cells in testis *(arrows)*. H.E. Bar: 30 µm

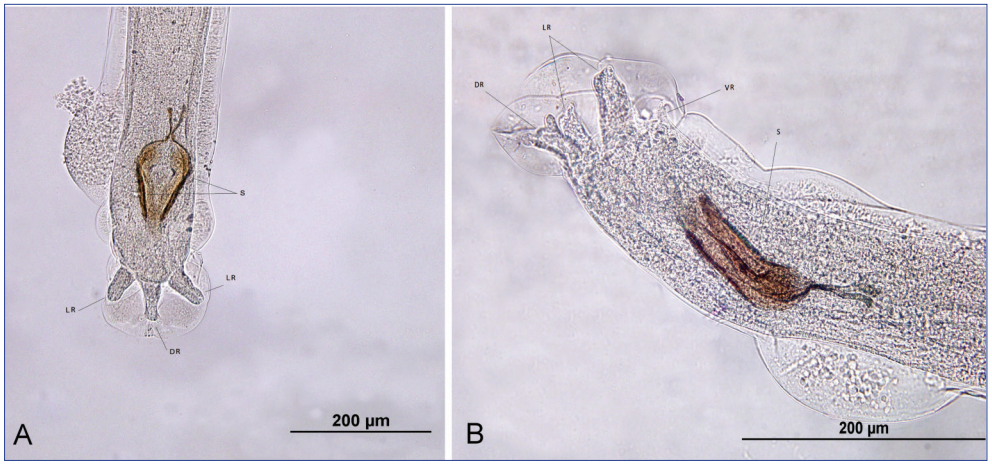

Fig 3. A. *S. minor.* Caudal end of male, ventral view; S: Spicule, LR: Lateral ray, DR: Dorsal ray. B. *S. minor.* Caudal end of male, lateral view; S: Spicule, LR: Lateral ray, DR: Dorsal ray, VR: Ventral ray

hemorrhages in the intestinal serosa. In the lungs, edema, macrophages, and few parasites larvae were seen in the alveolar lumens *(Fig. 2B)*. Diffusely, severe pneumoconiosis (anthracosis) was seen throughout bronchi, bronchioles and alveolar walls. In kidneys, interstitial and glomerular fibrosis were observed. In the medulla, calculi formation *(Fig. 2C)* in the lumen of the renal tubules were detected. There were diffuse dark brown pigment granules within Leydig cells in testes *(Fig. 2D),* and these granules were positively stained with melanin removal method II for melanin, but gave a negative reaction for PAS.

The tissues that underwent pepsin-aided digestion were obtained without damaging the outer layer and parasites. However, since the tissues were not extracted from deeper layers (localized just above the head and anterior thorax of the parasite), the parasite could be characterized only at the family level and morphological characteristics and histological appearance of the parasite show similarities with those of Pennellidae family [6,7]. The parasitological examinations of the lungs showed the presence of one male nematode, and the morphological characteristics were found compatible with those of *S. minor* in the light of previous studies [2,8] *(Fig. 3A, B)*.

DISCUSSION

In the previous studies, five species belonging to the Pennellidae family were reported in the seas of Turkey [3,9-12]. *Lernaeolophus sultanus* (in the mouth of Black Sea bream, *Diplodus vulgaris*) [9], *Pennella instructa* (in the anal/pectoral fins and abdominal muscle of swordfish) [10], *P. balaenopterae* (in the skin of fin whale, *Balaenoptera physalus)* [11], *Peniculus fistula* (in the ventral fin of a dolphin species, which is called lambuka, *Coryphaena hippurus)* [12] and *P. filose* (in the fins, body surface and gills of the fishes known as yellowtail, *Seriola dumerili)* [3] were found in the Turkey. *P. balaenopterae* was also found in a harbor dolphin *(Phocoena phocoena)* on the southern Aegean Sea coast of Turkey [6]. Although the parasites in this case could not be characterized at the species level, morphological characteristics and histological appearance of the parasite show similarities with those of Pennellidae family [6,7]. Besides detecting lesions and parasites, it is very important to highlight that the presence of this parasite has never been reported earlier to this case, and this is the first report of the presence of ectoparasites that belonging to the family Pennellidae from Bottlenose Dolphin *(T. truncatus)* in Turkey.

Intestinal volvulus is one of the most common changes in the intestines of marine mammals. It was reported that abnormal peristaltic movements, inflammation, parasitism, neoplasia, foreign bodies, vigorous exercise or violent rolling are the major risk factors for intestinal volvulus [13]. In the present case, no underlying etiology of the volvulus was determined on gross and histopathologic examination; however, it is suggested that verminous pneumonia and severe skin parasites may be predisposing factors that triggered intestinal volvulus [13,14].

Lungworms (Metastrongyloidea: Pseudaliidae) have been implicated as the main factor of natural mortality of marine mammals [2]. In one study, the prevalence of lungworm infections was determined to be 77% in the stranded bottlenose dolphins [15]. To date, *Stenurus ovatus, Halocercus lagenorhynchi, Pharurus alatus,* and *Skrjabinalius cryptocephalus* related verminous pneumonia [1,15,16] was reported in the bottlenose dolphins; but there was no case related to *S. minor.* It is reported that the prevalence of *S. minor* infection of harbor dolphins on the coast of Norway, Iceland, Greenland and South America was 66-100% [17,18]. In Turkish Seas, *S. minor* was detected in the lungs of the harbor dolphins on the coast of Black Sea [19], and in the stomach content of the striped dolphins in Eastern Mediterranean coast [20]. Cranial air sinuses, the inner ear, and supracranial airways are the most common locations of *S. minor*, and the parasite has been also found in the lungs, stomach and middle ear [17,21]. The pathological effects of *S. minor* in tissues have not been explained clearly yet. However, it has been reported that parasite-dependent severe inner ear infections may cause dysregulation in hearing and navigation abilities [21]. Although some parasitic larvae were also seen in alveoli in the case

of *S. minor*, not detecting any chronic lesion or adult parasite sections was interpreted as a sign of early infection in this case [14-16,19].

In conclusion, the presence of rasped and extracted teeth together with pneumoconiosis was suggested that this dolphin was likely involved in human interaction activities for a while and then left to the open sea.

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Case Report

First Report of *Trichodectes melis* (Phthiraptera, Trichodectidae) from the Eurasian Badger *Meles canescens* (Carnivora, Mustelidae) in Turkey [1]

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[1] This study was presented in 4th Vet İstanbul Group Congress Almaty - Kazakhstan, 11th-13th May 2017 and published in abstract book (titled as " First Report of Trichodectes melis (Phthiraptera, Trichodectidae) from the European Badger Meles meles (Carnivora, Mustelidae) in Turkey")

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Abstract

The study was performed to first report *Trichodectes melis* in Turkey. Research material was obtained from one female Eurasian badger brought to the Kafkas University, Wildlife Conservation, Recovery, Rehabilitation Research and Application Center. The badger was examined for the presence of ectoparasites. The lice specimens were preserved in a vial containing 70% ethanol, cleared in lactophenol for a few days, mounted on slides in the Canada balsam medium and examined under a microscope. All of specimens diagnosed as *T. melis* is the first record for ectoparasite fauna of Turkey.

Keywords: *Trichodectes melis, Ischnocera, Meles canescens, Turkey*

Türkiye'de Asya Porsuğu *Meles canescens* (Carnivora, Mustelidae)'te *Trichodectes melis*'in İlk Tespiti

Özet

Bu makale, *Trichodectes melis*'in Türkiye'den ilk bildirim olması nedeniyle hazırlanmıştır. Çalışma materyalini Kafkas Üniversitesi Yaban Hayatı Koruma, Kurtarma, Rehabilitasyon, Araştırma ve Uygulama Merkezi'ne getirilen dişi bir Asya porsuğu oluşturmuştur. Porsuk ektoparazit varlığı yönünden incelenmiştir. Bit örnekleri birkaç gün %70 etanol içinde bekletilmiş, laktofenol içinde şeffaflandırılmış, Kanada balsamı ile lama sabitlenmiş ve mikroskop altında incelenmiştir. *Trichodectes melis* olarak kaydedilen tüm bit örnekleri Türkiye'de bit faunası için ilk kayıttır.

Anahtar sözcükler: *Trichodectes melis, Ischnocera, Meles canescens, Türkiye*

INTRODUCTION

The Eurasian badgers *(Meles* spp.), Carnivora-Mustelidae, have a geographical range from the British Islands in the west to the Japanese Islands in the east, including the Scandinavia, Southwest Asia, southern China and Turkey [1,2]. The badgers have black, white, brown or grey fur, small head, stocky body, black eyes and short tail. Its weight varies between 6.6-14 kg in females and between 9.1-16.7 kg in males. Head-body length ranges between 56-90 cm and tail length ranges from 11.5 cm to 20.2 cm. Badgers *(Meles meles)* are nocturnally active wild animals, so they sleep during all day [3-6].

The badgers are commonly infested with ectoparasites such as lice *(Trichodectes melis)* [7-11].

A small number of studies have been undertaken about the infestation of badgers with ectoparasites especially

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with lice in Turkey and World [6-13]. This is the first report of *Trichodectes melis* from Eurasian badger *(Meles canescens)* in Turkey.

CASE HISTORY

Research material was obtained from one female Eurasian badger brought to the Kafkas University, Wildlife Conservation, Recovery, Rehabilitation Research and Application Center on 29 March 2016. The badger was examined for the presence of ectoparasites. The lice specimens collected on the fur were preserved in a vial containing 70% ethanol, cleared in lactophenol for a few days, mounted on slides in the Canada balsam medium and examined under a stereo-microscope. The parasites were measured and recorded.

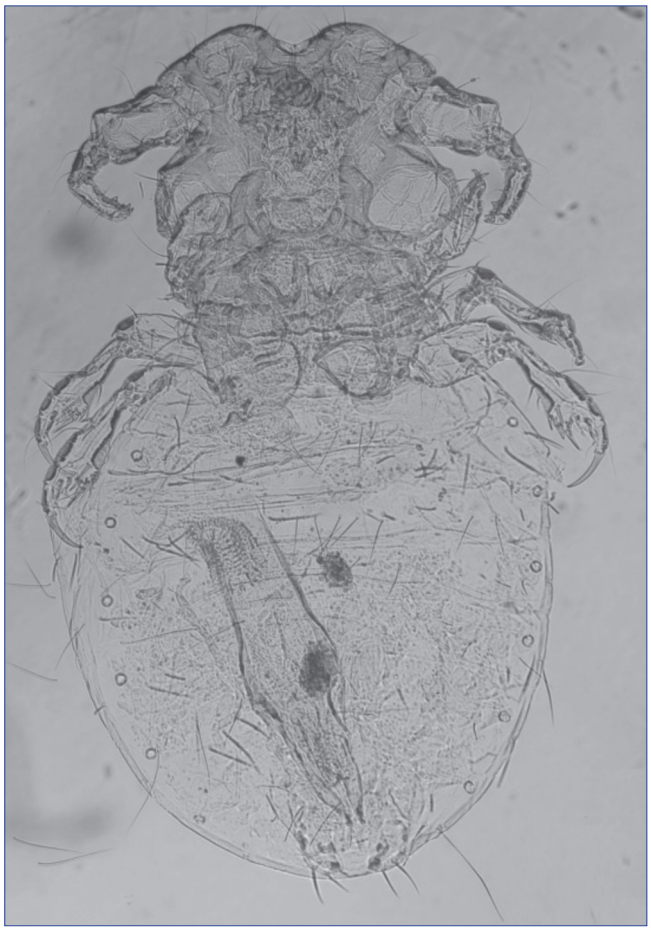

Fig 1. *Trichodectes melis*, male

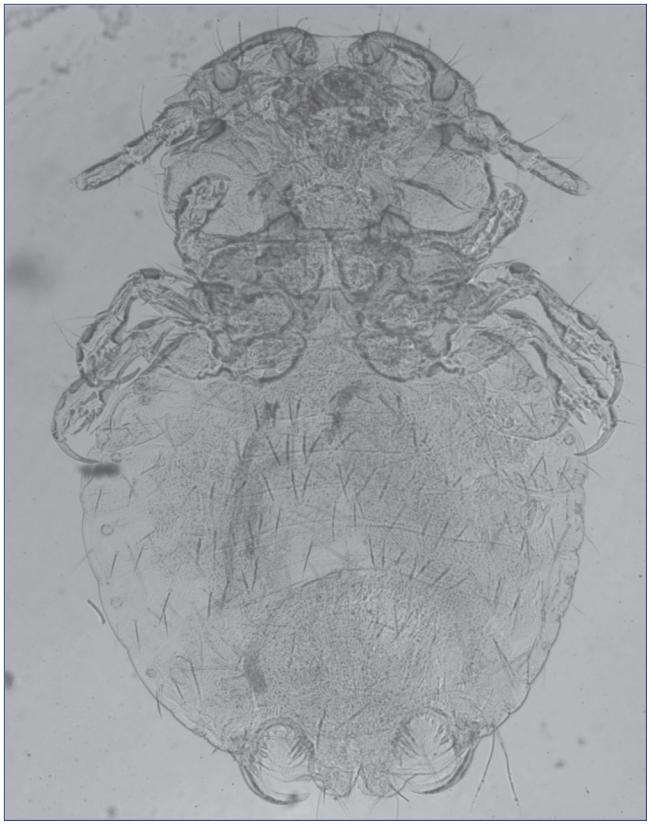

Fig 2. *Trichodectes melis*, female

In this research, 7 specimens of *T. melis* were found on the skin of badger. Three of them were males *(Fig. 1)*, and remaining 4 were females *(Fig. 2)*. The morphometric measurements of *T. melis* specimens were shown in *Table1*.

Table 1. *Mean body size (mm) of T. melis from badgers in our study and the others*

Mean Body Size	Present Study		Perez-Jimenez et al.[14]		Kozina et al.[11]	
	Male (n=3)	Female (n=4)	Male (n=18)	Female (n=48)	Male (n=7)	Female (n=44)
Length of head	0.46-0.49	0.46-0.54	0.50-0.53	0.52-0.58	0.45-0.51	0.48-0.61
Width of head	0.61-0.83	0.67-0.83	0.79-0.84	0.84-0.94	0.57-0.83	0.69-0.80
Length of thorax	0.31-0.33	0.30-0.36	0.30-0.35	0.27-0.35	0.21-0.27	0.18-0.30
Width of thorax	0.47-0.57	0.51-0.64	0.55-0.61	0.62-0.69	0.38-0.50	0.37-0.63
Length of abdomen	0.95-1.09	0.88-1.04	1.01-1.12	1.05-1.26	0.82-0.90	0.86-1.22
Width of abdomen	0.73-0.97	0.92-1.01	0.95-1.06	1.11-1.30	0.82-0.94	0.85-1.21
Total lenght of body	1.77-1.81	1.65-1.88	1.83-1.98	1.88-2.13	1.57-1.67	1.58-2.04

DISCUSSION

It was reported that *T. melis* is a common and important ectoparasite of the badgers in Spain. In a study, two badgers were examined and both of them were found infected with *T. melis* [13]. In another study, 66 adult lice (18 males, 48 females) were collected on two badgers [14]. Dominguez [12] reported that the parasitic intensity of infestation were found with over 300 lice per animal. In another study, 6 of 7 badgers were found to be infected with *Trichodectes melis* [10]. In England, an adult female badger was found as infested with a total of 754 chewing lice in 1979 [7]. There is one parasitological study on badgers that carried out in Turkey but no lice species were determined [6]. Although there are numerous lice species have been reported on wild animals in Turkey [15], no data was able to found on the existence of lice species neither on badger nor on another animal belongs to Mustelidae family.

It is known that many pathogenic agents are carried and infected by domestic and wild animals which are infested by ectoparasites such as *T. melis*. This is the first report of *T. melis* from Eurasian badger *(Meles canescens)* in Turkey. This study emphasizes the importance of detailed examination of these and similar wild animals which are frequently coming to our clinics, and also it is thought that this study will be supposed to support the studies which will be done later about ectoparasites of wild or domestic animals.

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Review

A Historical Overview of Turkey's Animal Welfare Legislation

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Abstract

There were a number of legal codes which aimed to protect animal welfare in Turkish history, but there was no separate animal welfare law until 2004. Although *Animal Protection Law* (5199) had been accepted on July 01, 2004, little progress had been shown until 2010, except for *"Regulation on the Protection of Experimental Animals Used for Experimental and Other Scientific Purposes and the Procedures and Principles of the Establishment, Operation and Inspection of Experimental Animal Production Premises and Laboratories Performing Animal Experiments", "Regulation on the Procedures and Principles of the Work of Zoos"* and *"Implementing Regulation on Animal Protection"*. The framework of the adhesion process to the EU the regulation has been made under Law No. 5996 on June 13, 2010 and it has a separate animal welfare article. After this law went into effect, two new regulations were issued on December 23, 2011: *The Regulation on Welfare of Farm Animals* and, *The Regulation on the Welfare and Protection of Animals during Transport*. But, at the end of the 2014, *The Regulation on welfare of Farm Animals* was issued and former one repealed. All these legislations are quite similar to the EU's regulations. However, there were no subject specific legislations on slaughtering and any species-specific stipulations except for welfare standards for the protection of calves and laying hens.

Keywords: *Animal protection, Animal welfare, Legislation, Turkey*

Türkiye'de Hayvan Refahı Mevzuatına Tarihsel Bir Bakış

Özet

Türk tarihinde hayvanların refahını korumaya yönelik olarak çıkarılmış bazı önemli hukuki düzenlemelere rastlamak mümkünse de, 2004 yılına kadar, bağımsız bir hayvan refahı düzenlemesi yoktur. Hayvan refahı ile ilgili ilk bağımsız yasa olan 5199 sayılı *Hayvanları Koruma Kanunu* 01 Temmuz 2004 tarihinde yürürlüğe girmiştir. Ancak, bu kanunun yürürlük tarihi 2004 olmasına rağmen, bu alanda, 2010 yılına gelinceye kadar, *"Deneysel ve Diğer Bilimsel Amaçlar için Kullanılan Deney Hayvanlarının Korunması, Deney Hayvanlarının Üretim Yerleri ile Deney Yapacak Olan Laboratuvarların Kuruluş, Çalışma, Denetleme, Usul ve Esaslarına Dair Yönetmelik", "Hayvanat Bahçelerinin Kuruluşu ile Çalışma Usul ve Esasları Hakkında Yönetmelik"* ve *"Hayvanların Korunmasına Dair Uygulama Yönetmeliği"* dışında ilerleme kaydedilmemiştir. Avrupa Birliği uyum sürecinde yapılan çalışmalar çerçevesinde çıkarılan ve hayvan refahı ile ilgili bir bölüm içeren diğer yasa 13 Haziran 2010 tarihinde yürürlüğe giren 5996 sayılı 2010 tarihli *Veteriner Hizmetleri, Bitki Sağlığı, Gıda ve Yem Kanunu*'dur. Bu yasanın yürürlüğünden sonra çıkarılan yönetmelikler ise; 23 Aralık 2011 tarihli *Çiftlik Hayvanları Refahına İlişkin Yönetmelik ve Hayvanların Nakilleri Sırasında Refahı ve Korunması Yönetmeliği'dir. Çiftlik Hayvanları Refahına İlişkin Yönetmelik* 2014 yılında yürürlükten kaldırılmış ve yerine yenisi getirilmiştir. Tüm bu düzenlemeler Avrupa Birliği mevzuatıyla oldukça benzerlik taşımaktadır. Ancak, kesim süreçlerinde hayvan refahına yönelik bir düzenleme ve ayrıca buzağıların ve yumurtacı tavukların refah standartlarını belirleyen düzenlemeler dışında tür-spesifik düzenlemeler bulunmamaktadır.

Anahtar sözcükler: *Hayvan koruma, Hayvan refahı/gönenci, Mevzuat, Türkiye*

INTRODUCTION

Turkey is a nation of people who have lived intimately with animals for hundreds of years of their history. Based on this association, they have a deep-rooted, animal-based cultural heritage [1]. It is extremely likely to find many examples of animal love, animal welfare and animal protection subjects in this cultural history.

In the western world, the first legal code aimed at the protection of animals is referred to as the 92nd and 93rd provisions of the Body of Liberties[1], which took effect in 1641 in the Massachusetts Colony [2,3]. However, nearly 150 years earlier this enactment (at the beginning of the 16th Century), the Law of Istanbul Municipality *(Kanunname-i*

1 *Off the Bruite Creature -92. No man shall exercise any Tirranny or Crueltie towards any bruite Creature which are usuallie kept for man's use. -93. If any man shall have occasion to leade or drive Cattel from place to place that is far of, so that they be weary, or hungry, or fall sick, or lambe, It shall be lawful to rest or refresh them, for competant time, in any open place that is not Corne, meadow, or inclosed for some peculiar use*

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Ihtisab-i Istanbul) was enforced during the Ottoman Empire. It read as follows:

"Pack-horses with any hoof problem should not be forced to labour. Care should be taken of the hooves and packsaddles of horses, mules and donkeys. These animals should not be forced to carry excessive load as they are mute creatures. It should be ensured that any lacking need of an animal is fulfilled by its owner. Those who disregard these matters should be punished" [4].

These statements are very significant in that they provide valuable insight into both the approach of the Turkish society for the protection of animals, and the cultural grounds of animal protection in Turkish society [4].

Similarly, the cultural heritage that has survived with regard to birds, animals can be considered important to give an idea about the love of animal of the Turkish people. Birdhouses (or bird shelters), built as part of Turkish architecture, began in the Seljuks era, along with pigeon and stork hospitals built for the treatment of sick birds. Charitable foundations were established to take care of hungry animals during the winter season. Similarly, The Migratory Birds Foundation was founded to treat animals taken sick on their route of migration. All these organizations are examples of the daily efforts of the Turkish people to protect animals [5-9]. Pre-nineteenth century Turkish history is full of such striking and impressive representations of Turks' caring nature for animal life [5,6,8,10].

Unfortunately, developments following the 18th century are not as exemplary. Despite the presence of the Society for the Protection of Animals, which was founded in 1912, and some other examples that might be referred to as regards animal protection, the adverse circumstances that overwhelmed the Ottoman territory throughout the 19th Century and during the first half of the 20th Century, also affected animals unfavourably. It was not until the start of the 21st Century that this unfortunate trajectory ended for animals [6,8,10,11]. Many studies have been conducted on animal rights, animal protection and animal welfare in Turkey. In these articles, concept of animal welfare has been evaluated in terms of historical [7,11,12], philosophical [13-17], sociological [18,19], educational [20,21], theoretical [22], practical [23-33] context. Also, there are one book [34], one *European Union expertise thesis* [35] *and* two PhD thesis [36,37] relating animal welfare in Turkish. In addition to these, animal welfare legislation has been evaluated by a number of authors [4,34,38-43]. But, some regulations on farm animal welfare were enacted after 2010; therefore this topic should re-evaluate in the light of new regulations and new developments. The aim of this review is to re-evaluate the existing developments and sources of animal welfare legislation in Turkey in recent history.

ANIMAL WELFARE LAWS

In Turkish history, the first attempt to enact a separate law for the protection of animals was made in 1932. However, the draft law titled *"How Can We Protect Animals?"* could not get through the Parliament [8]. The second attempt was made in mid 1980s. This draft bill, which titled *"Animal Protection Draft Bill"*, waited to be discussed, sitting at the bottom of Parliament's draft bill list more than 10 years. Despite Mrs. Imren Aykut's supports and efforts, who was the Environment Minister of Turkey, she had not been able to succeed in drawing the attention of the deputies to this draft bill. Consequently, the second draft bill could not get through the parliament, either [6].

Until the second millennium, the legal regulations enacted were primarily related to the increase of animal production, the control of contagious animal diseases, and veterinary services. There is scarcely any reference to animal welfare in these regulations. Legal provisions, related to the mistreatment of animals and damage caused by animals have been included under the penal code. Articles 521 and 577 of the former Turkish Penal Code[2] are such legal provisions. On the other hand, Articles 151 and 181 of the new Turkish Penal Code (Law 5237) address the direct human persecution and harm of animals [3].

The year 1999 was a turning point for animal welfare developments in Turkey. In 1999, Turkey was officially recognized by the EU as a candidate country [44]. Legislative developments that occurred in the field of animal welfare following the recognition of the EU candidate country status of Turkey outnumber those in prior periods [34,42].

Today, in Turkey, three laws contain provisions directly related to the protection and welfare of animals. Of these, the first, "*Law 4934 on the Approval of the Ratification of the European Convention for the Protection of Pet Animals*", was enacted in 2003[4]. Shortly after the enactment of this law, the first *"Animal Protection Law"* of Turkish history took effect in 2004[5]. This law contains the articles of the

2 *Article 520. "Whoever as causing unjustified as belonging to someone else, an animal killed or if he puts it not going to work up on the complaint to four months with prison and, up to a hundred Liras heavier fines would be doomed ...". Article –577. "A person who acts unfairly towards animals or unduly beat or sores or if the obvious by force to the extent that tired inordinately up to ten Liras lighter fines would be doomed ..."* Türk Ceza Kanunu (Abolished). http://www.ceza-bb.adalet.gov.tr/mevzuat/765.htm; *Accessed:* October 27, 2016.

3 *Article 151-* (2) *"Any person who kills or harms an animal with an owner, without a justified reason, in such a way not to be used any more or to lower its value, is punished with imprisonment from four months to three years, or imposed punitive fine upon complaint of the aggrieved party".* Article 181 (5): *"In case of commission of offenses defined in first and second subsections by processing of refuses or wastes in such a way to result with incurable disease both in human and animals, or deterioration of fertility and change natural characteristics of animals and plants; the offenders are punished with imprisonment not less than five years and also imposed punitive fine up to thousand days".* Türk Ceza Kanunu, *Wipo.* http://www.wipo.int/ edocs/lexdocs/laws/en/tr/tr171en.pdf; *Accessed:* October 17, 2016.

4 4934 sayılı Ev Hayvanlarının Korunmasına Dair Avrupa Sözleşmesinin Onaylanmasının Uygun Bulunduğu Hakkında Kanun. *Resmi Gazete*. http://www.resmigazete.gov.tr/eskiler/2003/07/20030722.htm#7; *Accessed:* October 28, 2016.

5 5199 sayılı Hayvanları Koruma Kanunu. *Resmi Gazete*. https://www.

"European Convention for the Protection of Pet Animals" under separate chapters. The purview of these two laws is to *"ensure the comfort of animals, provide for their fair and proper treatment, and secure their protection against the infliction of pain, suffering and stress."* While the *Animal Protection Law* mostly focuses on the protection of pet animals and stray animals, it also includes provisions related to farm animals and wild animals. Finally, the third law, the *"Law on Veterinary Services, Plant Health, Food and Feed"*, was enacted in 2010 and includes a chapter specific to animal welfare[6]. Article 9 of the *Law on Veterinary Services, Plant Health, Food and Feed* is directly related to animal welfare:

"Article 9 - (1) To ensure animal welfare, the owners or keepers of animals are responsible for satisfying the sheltering, care, feeding, health and other needs of animals, and taking necessary measures against possible adverse effects the animals under their responsibility may cause on human, animal and environmental health.

2) The slaughter and culling of animals for disease control purposes shall be performed without causing unnecessary fear, pain and distress and by using appropriate tools.

3) Animals shall not be euthanized. However:

a) in cases where animals have a painful and distressing or incurable disease,

b) For the purpose of the prevention or eradication of an acute contagious animal disease or in cases of threat to human health,

c) In cases where their behaviour poses threat to the lives and health of humans and animals and where their negative behaviour cannot be controlled a veterinarian may decide to perform euthanasia. Euthanasia shall be performed by a veterinarian or under the supervision of a veterinarian.

(4) The rules for animal welfare during their sheltering, transport, pre-slaughter and slaughter are determined by the Ministry. Animals shall be slaughtered in slaughterhouses approved by the Ministry.

(5) Principles and procedures related to the implementation of this article shall be laid down in an implementing regulation to be promulgated by the Ministry"[7]

In addition to these, Terrestrial Hunting Law (Law 4915)[8], Fishery Law (Main Law 1380; Amending Law 3288)[9], and The National Parks Law (Law 2873)[10] has some articles which are related protecting the welfare of wild animals. Thus, these laws should be accepted in the animal welfare legislation. Also, Turkey has been a party of Cites[11], Bern[12], Ramsar[13] and Convention on Biological Diversity[14]. These conventions might be accepted as a supplementary part of national legislation [33].

(IMPLEMENTING) REGULATIONS ON ANIMAL WELFARE

The first regulation, which included provisions on the protection of animals, was enforced one month before the enactment of the *Animal Protection Law*, on the basis of the Animal Health and Surveillance[15] (Law 3285), Decree *Law no. 441* on Establishment and Duties of *Ministry of Agriculture* and Rural Affairs,[16] and the Pursuit of Veterinary Medicine Profession, Establishment and Duties of Turkish Veterinary Medical Association and Veterinary Medicine Chambers[17] (Law 6343), was the *"Regulation on the Protection*

tbmm. gov.tr/kanunlar/k5199.html; *Accessed:* October 17, 2016.

6 5996 sayılı Veteriner Hizmetleri, Bitki Sağlığı, Gıda ve Yem Kanunu. *Resmi Gazete.* http://www.resmigazete.gov.tr/eskiler/2010/06/20100613-12.htm; *Accessed:* October 28, 2016.

7 5996 Law on Veterinary Services, Plant Health, Food and Feed. http://www.lawsturkey.com/law/5996-law-on-veterinary-services-plant-health-food-and-feed; *Accessed:* October 28, 2016.

8 4915 sayılı Kara Avcılığı Kanunu. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2003/07/20030711.htm&main=http://www.resmigazete.gov.tr/eskiler/2003/07/20030711.htm; *Accessed:* May 24, 2017.

9 1380 Sayılı Su Ürünleri Kanunu. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/arsiv/13799.pdf&main=http://www.resmigazete.gov.tr/arsiv/13799.pdf. *Accessed:* May 24, 2017; 3288 Sayılı "1380 Sayılı Su Ürünleri Kanununda Değişiklik Yapılmasına Dair Kanun". *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/arsiv/19120.pdf&main=http://www.resmigazete.gov.tr/arsiv/19120.pdf; *Accessed:* May 24, 2017.

10 2873 Nolu Milli Parklar Kanunu. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/arsiv/18132.pdf&main=http://www.resmigazete.gov.tr/arsiv/18132.pdf; *Accessed:* May 24, 2017.

11 CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) is an international agreement between governments. Its' aim is to ensure that international trade in specimens of wild animals and plants does not threaten their survival. Cites (The Convention on International Trade in Endangered Species of Wild Fauna and Flora) webpage. https://www.cites.org/eng/disc/parties/index.php; *Accessed:* June 2, 2017.

12 Bern Convention: The Bern Convention is a binding international legal instrument in the field of nature conservation, covering most of the natural heritage of the European continent and extending to some States of Africa. http://www.coe.int/en/web/bern-convention; *Accessed:* June 2, 2017.

13 Ramsar Convention: The Convention on wetlands, called the Ramsar Convention, is an intergovenrmental treaty that provides the framework for national action and international cooperation for the conservation and wise use of wetlands and their resources. http://www.ramsar.org/; *Accessed:* June 2, 2017.

14 Convention on Biological Diversity: Its' aim is to (1) the conservation of biological diversity; (2) the sustainable use of the components of biological diversity; (3) the fair and equitable sharing of the benefits arising out of the utilization of genetic resources. https://www.cbd.int/intro/defaults.html; *Accessed:* June 02, 2017.

15 3285 sayılı Hayvan Sağlığı ve Zabıtası Kanunu. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/arsiv/19109.pdf&main=http://www.resmigazete.gov.tr/arsiv/19109.pdf; *Accessed:* June 29, 2017.

16 441 Sayılı Tarım ve Köyişleri Bakanlığının Kuruluş ve Görevleri Hakkında Kanun Hükmünde Kararname. Resmi Gazete. http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/arsiv/20955_1.pdf&main=http://www.resmigazete.gov.tr/arsiv/20955_1.pdf; *Accessed:* June 29, 2017.

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Table 1. *Regulations regarding animal welfare and legal basis*		
Regulation	**Date**	**Legal Basis**
The Regulation on the Execution of Services for the Feast of Sacrifice by the Presidency of Religious Affairs (and amending regulation)	2002 (2005)	Law on The *Act* on Organization and Missions of the Directorate of *Religious Affairs (Law 633) and* Animal Protection Law (Law 5199)
Regulation on the Protection of Experimental Animals Used for Experimental and Other Scientific Purposes and the Procedures and Principles of the Establishment, Operation and Inspection of Experimental Animal Production Premises and Laboratories Performing Animal Experiments (Abolished)	2004	Animal Health and Surveillance (Law 3285), Decree *Law no. 441* on Establishment and Duties of *Ministry of Agriculture* and Rural Affairs and The Pursuit of Veterinary Medicine Profession, Establishment and Duties of Turkish Veterinary Medical Association and Veterinary Medicine Chambers (Law 6343)
Implementing Regulation on Animal Protection	2006	Animal Protection Law (Law 5199)
Regulation on the Procedures and Principles of the Work of Ethics Boards for Animal Experiments (Abolished)	2006	Animal Protection Law (Law 5199)
Regulation on the Establishment, and Procedures and Working Principles of the Zoos	2007	Animal Protection Law (Law 5199)
Regulation on the Places of Breeding, Sales, Housing and Training of Pet and Ornamental Animals	2011	The Pursuit of Veterinary Medicine Profession, Establishment and Duties of Turkish Veterinary Medical Association and Veterinary Medicine Chambers (Law 6343) and Law on Veterinary Services, Plant Health, Food and Feed (Law 5996)
Regulation on the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes	2011	Law on Veterinary Services, Plant Health, Food and Feed (Law 5996); Biosecurity Law (Law 5977)
Regulation on the Welfare of Farm Animals (Abolished)	2011	Law on Veterinary Services, Plant Health, Food and Feed (Law 5996)
Regulation on the Welfare and Protection of Animals during Their Transport	2011	Law on Veterinary Services, Plant Health, Food and Feed (Law 5996)
The Regulation on Procedure and Principles of Authorization and Inspection of Livestock Markets	2011	Law on Veterinary Services, Plant Health, Food and Feed (Law 5996)
The Regulation the Official Controls of Food and Feed	2011	Law on Veterinary Services, Plant Health, Food and Feed (Law 5996)
The Regulation on Food Hygiene	2011	Law on Veterinary Services, Plant Health, Food and Feed (Law 5996)
The Regulation Laying Down Specific Rules for the Official Controls of Food of Animal Origin	2011	Law on Veterinary Services, Plant Health, Food and Feed (Law 5996)
The Regulation on Specific Hygiene Rules for Food of Animal Origin	2011	Law on Veterinary Services, Plant Health, Food and Feed (Law 5996)
The Regulation on General Provisions Related to the Welfare of Farm Animals	2014	Law on Veterinary Services, Plant Health, Food and Feed (Law 5996)
The Regulation on Minimum Standards for the Protection of Calves	2014	Law on Veterinary Services, Plant Health, Food and Feed (Law 5996)
The Regulation on Minimum Standards for the Protection of Laying Hens	2014	Law on Veterinary Services, Plant Health, Food and Feed (Law 5996)
Regulation on the Procedures and Principles of the Work of Ethics Boards for Animal Experiments	2014	Animal Protection Law (Law 5199)

of Experimental Animals Used for Experimental and Other Scientific Purposes and the Procedures and Principles of the Establishment, Operation and Inspection of Experimental Animal Production Premises and Laboratories Performing Animal Experiments" (published in the Official Gazette dated the 16th of May 2004 and numbered 25464)[18]. This regulation, repealed by the *"Regulation on the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes"*, was enforced on the 13th of December in 2011[19] *(Table 1)*.

Another regulation on animal welfare is the *"Regulation on the Procedures and Principles of the Work of Ethics Boards for Animal Experiments"*(published in the Official Gazette dated the 6th of July 2006 and numbered 26220)[20], and was enforced on the basis of the *Animal Protection Law*. This regulation was prepared in compliance with *Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes*, and was repealed by the regulation with the same title, enforced on the 15th of February in 2014[21] *(Table 1)*. These two regulations laid down the

Birliği île Odalarının Teşekkül Tarzına ve Göreceği Işlere Dair Kanun. *Resmi Gazete*. http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/arsiv/8661.pdf&main=http://www.resmigazete.gov.tr/arsiv/8661.pdf; *Accessed:* June 29, 2017.

18 Deneysel ve Diğer Bilimsel Amaçlar için Kullanılan Deney Hayvanlarının Korunması, Deney Hayvanlarının Üretim Yerleri ile Deney Yapacak Olan Laboratuvarların Kuruluş, Çalışma, Denetleme, Usul ve Esaslarına Dair Yönetmelik. *Resmi Gazete*. http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2004/05/20040516.htm&main=http://www.resmigazete.gov.tr/eskiler/2004/05/20040516.htm; *Accessed:* October 28, 2016.

19 Deneysel ve Diğer Bilimsel Amaçlar İçin Kullanılan Hayvanların Refah ve Korunmasına Dair Yönetmelik. *Resmi Gazete*. http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2011/12/20111213.htm&main=http://www.resmigazete.gov.tr/eskiler/2011/12/20111213.htm; Accessed: October 17, 2016.

20 Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esaslarina Dair Yönetmelik (Abolished). *Resmi Gazete*. http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2006/07/20060706.htm&main=http://www.resmigazete.gov.tr/eskiler/2006/07/20060706.htm; *Accessed:* October 17, 2016.

21 Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esaslarına Dair Yönetmelik. *Resmi Gazete*. http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2014/02/20140215.htm&main=http://www.resmigazete.gov.tr/eskiler/2014/02/20140215.htm; *Accessed:* October 28, 2016.

implementing principles for the fulfilment of animal welfare standards during the use of animals for research and educational purposes in Turkey.

Although the *Animal Protection Law* stipulated that the implementing regulation should be enforced within a one-year period, the *"Implementing Regulation on Animal Protection"*[22] (published in the Official Gazette dated the 12th of May 2006 and numbered 26166) was enforced two years after the enactment of the law and is of particular importance with respect to the implementation of the law *(Table 1)*. In this regulation:

a) Conditions set for keeping and owning pet and ornamental animals, and animals under control, which are defined in the regulation as identified and registered pet and ornamental animals that are owned by real and legal persons or institutions and organisations, and are vaccinated and periodically checked for their health

b) Details related to the rehabilitation of stray animals

c) Procedures and principles to be followed for the use of animals in all kinds of commercial shows, advertisements, films and photo shootings

d) Procedures and principles to be followed in the killing of animals

e) Procedures and principles related to the work of Provincial Animal Protection Boards

f) Inspections to be carried out on the basis of Law 5199 and the qualifications to be sought for inspectors are laid down in detail.

Regulation on the Establishment, and Procedures and Working Principles of the Zoos (published in the Official Gazette dated the 11th of August 2007 and numbered 26610) was enforced on the basis of the 22nd article of *Animal Protection Law (Table 1)*. The aim of this regulation is to promote welfare standards of animals in zoos.[23]

Another regulation aimed at improving the welfare of pet and ornamental animals is the *"Regulation on the Places of Breeding, Sales, Housing and Training of Pet and Ornamental Animals"* (published in the Official Gazette dated the 8th of October 2011 and numbered 28078) [24] *(Table 1)*. This regulation regulates the technical and sanitary conditions set for places where pet and ornamental animals are bred, bought and sold, housed and trained.

Moreover, as secondary legislation related to the welfare of farm animals was enforced only after the enactment of Law 5996 in 2010, the publication of implementing regulation mainly concentrates on the period after 2010.

The provisional clauses of Law 5996 required that: *"The regulations foreseen in this Law and the procedures and principles related to implementation are enforced within 18 months following the enactment of the Law, at the latest".*

In fact, nearly 18 months after the enactment of Law 5996, two regulation were published. The first was related to the welfare of farm animals and the second was related to the welfare of animals during transport.

At the first glance, the *"Regulation on the Welfare of Farm Animals"*[25], which was published in the Official Gazette dated the 23rd of December 2011, was not a species-specific regulation *(Table 1)*. However, apart from the implementing principles applicable to all farm animals, this regulation also laid down minimum welfare standards for the protection of laying hens and calves. This regulation was repealed by three new regulations, which include:

"The Regulation on General Provisions Related to the Welfare of Farm Animals" (published in the Official Gazette dated the 22nd of November 2014 and numbered 29183),[26]

"The Regulation on Minimum Standards for the Protection of Calves" (published in the Official Gazette dated the 22nd of November 2014 and numbered 29183),[27]

"The Regulation on Minimum Standards for the Protection of Laying Hens" (published in the Official Gazette dated the 22nd of November 2014 and numbered 29183) [28] *(Table 1)*.

The content of these three regulations is almost the same as those of the former regulation. The only difference is in the date set for the ban of the use of conventional cage systems for laying hens. The former regulation included a provision stipulating the ban of the use of conventional cage systems from the 1st of January 2015. As a result of the pressure of the egg producers on the Ministry of Food, Agriculture and Livestock, this date has been postponed to 2023 in the new regulation [9]. This postponement must have been the main motive underlying the amendment to the regulation.

22 Hayvanların Korunmasına Dair Uygulama Yönetmeliği. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2006/05/20060512.htm&main=http://www.resmigazete.gov.tr/eskiler/2006/05/20060512.htm; *Accessed:* October 17, 2016.

23 Hayvanat Bahçelerinin Kuruluşu ile Çalışma Usul ve Esasları Hakkında Yönetmelik. *http://www.resmigazete.gov.tr/eskiler/2007/08/20070811-7.htm; Accessed:* June 5, 2017.

24 Ev ve Süs Hayvanlarının Üretim, Satış, Barınma ve Eğitim Yerleri Hakkında Yönetmelik. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2011/10/20111008.htm&main=http://www.resmigazete.gov.tr/eskiler/2011/10/20111008.htm; *Accessed:* October 28, 2016.

25 Çiftlik Hayvanlarının Refahına İlişkin Yönetmelik. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2011/12/20111223.htm&main=http://www.resmigazete.gov.tr/eskiler/2011/12/20111223.htm; *Accessed:* October 31, 2016.

26 Çiftlik Hayvanlarının Refahına İlişkin Genel Hükümler Hakkında Yönetmelik. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2014/11/20141122.htm&main=http://www.resmigazete.gov.tr/eskiler/2014/11/20141122.htm; *Accessed:* October 31, 2016.

27 Ibidem.

28 Ibidem.

Another regulation enforced on the basis of Law 5996 is the *"Regulation on the Welfare and Protection of Animals during Their Transport"* (published in the Official Gazette dated the 24th of December 2011 and numbered 28152)[29] *(Table 1)*. This regulation lays down the procedures and principles to be followed in order to ensure the protection and welfare of all live vertebrate animals during their transport within the territory of Turkey.

From one year ago, preparation of this article, Food, Agriculture and Livestock Ministry --in an interview with the author who had been informed that two further preparations for the enforcement of regulations-- was underway. In fact, of these two prospective regulations, the one related to the protection of broiler chickens was expected to be enforced by the end of 2015. The second regulation, which has been drafted and submitted to the Presidency of Religious Affairs for consultation, is related to the welfare of animals before and at the time of slaughter. In fact, it has been mentioned that these two regulations have to be enforced since 2005 [26,39]. But, as yet, these regulations haven't been enforced.

In addition to all of these, there are a number of regulations that, currently in force in Turkey, include provisions either directly or indirectly relate to the welfare of animals before and at the time of slaughter. However, these provisions are not compiled under a separate legislative regulation. The regulations, which include provisions related to the welfare of animals before and at the time of slaughter, are:

"The Regulation on Procedure and Principles of Authorization and Inspection of Livestock Markets" (published in the Official Gazette dated the 24th of December 2011 and numbered 28152)[30], *"The Regulation the Official Controls of Food and Feed"* (published in the Official Gazette dated the 17th of December 2011 and numbered 28145)[31], *"The Regulation on Food Hygiene"* (published in the Official Gazette dated the 17th of December 2011 and numbered 28145)[32], *"The Regulation Laying Down Specific Rules for the Official Controls of Food of Animal Origin"* (published in the Official Gazette dated the 17th of December 2011 and numbered 28145)[33] and *"The Regulation on Specific Hygiene Rules for Food of Animal Origin"* (published in the Official Gazette dated the 27th of December 2011 and numbered 28155)[34] *(Table 1)*. In fact, *"The Regulation on the Execution of Services for the Feast of Sacrifice by the Presidency of Religious Affairs"* (published in the Official Gazette dated the 18th of August 2002 and numbered 24850)[35] *(Table 1)* can also be included in this group of legislation.

CONCLUSION

The aim of this article is to re-evaluate recent developments and sources of animal welfare legislation in Turkey. The result today, as happens in many countries in animal welfare as in Turkey, is a situation that is largely guaranteed by laws. The retrospective evaluation of animal welfare legislation in Turkey shows that most of these developments have occurred in the past 15 years. These developments have coincided with the efforts of Turkey to align with the EU *acquis* is not by chance. Furthermore, on the basis of the animal welfare regulations, currently in force in Turkey being in line with EU legislation, it is suggested that legislative work in the field of animal welfare has resulted from the obligations of Turkey as a negotiating EU candidate country, rather than from sociological evolution. It can be deduced that recent developments seem to be supporting this claim. For example, the regulation dictating the traditional cage system to be banned as of 1 January 2015 has been postponed to 2023 due to the resistance of the sectoral representatives [45]. The farmers are not complying with the regulations that have been put in place so far due to the increase in expenses. In addition, the authorized offices are not inspecting their practices as they should [46]. Also, this claim has been supported by European Commission Turkey Progress Reports 2006-2016 [42,47-57]. Although a number of laws and some implementing regulations were enacted until 2010, according to 2006-2010 European Commission Reports [47-51]; *"Turkey has made no progress on zootechnical issues or on animal welfare, the latter being a key element for the accession negotiations..."*. After 2010, some progress has been made on alignment with the animal welfare *acquis*, relating to welfare of animals during transport and on farm. But, these progress were not found enough by European Commission Reports [47-52]: *"...full implementation of the acquis in this area will require*

29 Hayvanların Nakilleri Sırasında Refahı ve Korunması Yönetmeliği. *Resmi Gazete*. http://www.resmigazete. gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2011/12/20111224. htm&main= http://www.resmigazete.gov.tr/eskiler/2011/12/20111224.htm; *Accessed:* October 31, 2016.

30 Hayvan Satış Yerlerinin Ruhsatlandırılma ve Denetleme Usul ve Esasları Hakkında Yönetmelik. *Resmi Gazete.* http://www.resmigazete.gov.tr/eskiler/2011/12/20111224-1.htm; *Accessed:* March 16, 2017.

31 Gıda ve Yemin Resmi Kontrollerine Dair Yönetmelik. *Resmi Gazete*, http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2011/12/20111217.htm&main=http://www.resmigazete.gov.tr/eskiler/2011/12/20111217.htm; *Accessed:* October 18, 2016.

32 Gıda Hijyeni Yönetmeliği. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2011/12/20111217.htm&main=http://www.resmigazete.gov.tr/eskiler/2011/ 12/20111217.htm; *Accessed:* October 18, 2016.

33 Hayvansal Gıdaların Resmi Kontrollerine İlişkin Özel Kuralları Belirleyen Yönetmelik. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2011/12/20111217.htm&main=http://www.resmigazete.gov.tr/eskiler/2011/12/20111217.htm; *Accessed:* October 18, 2016.

34 Hayvansal Gıdalar İçin Özel Hijyen Kuralları Yönetmeliği. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2011/12/20111227.htm&main=http://www.resmigazete.gov.tr/eskiler/2011/12/20111227.htm; *Accessed:* October 18, 2016.

35 Kurban Hizmetlerinin Diyanet İşleri Başkanlığınca Yürütülmesine Dair Yönetmelik. *Resmi Gazete.* http://www.resmigazete.gov.tr/main.aspx?home=http://www.resmigazete.gov.tr/eskiler/2002/08/20020818.htm&main=http://www.resmigazete.gov.tr/eskiler/2002/08/20020818.htm; *Accessed:* October 18, 2016.

significant further efforts... Turkey should in particular: take further steps to adapt and enforce rules on animal welfare and animal by-products. Further structural and administrative efforts are required to fully implement the acquis on animal welfare. In addition to all these, it can be said that this speculation is supported by the results of some studies [36,58] which were conducted in Turkey. One of these is Izmirli's PhD thesis [36]. According to Izmirli, even if participants show positive attitudes about animal welfare in generel, he claims that the participants have a lack of information about animal welfare and its true meaning [36]. Similarly, in the study conducted by Şeker et al.[58], the participants who say that they relate animal welfare and its implications on society really don't understand it thoroughly enough. So, it is difficult to say that there is a strong social accaptence about the necesity of animal welfare among Turkish people. In the same vein, Bozkurt et al.[59] underscore the realization that social acceptance of the minimum standards regarding animal welfare is more important than the legal implementation and enforcement of the relevant legislation. Yet, it needs to be recognized that this is something new and needs to be appreciated by Turkish society as a whole.

When compared to EU legislation, animal welfare standards in slaughtering process, animal welfare standards for pigs, broiler chicken, turkey, sheep, goat, dairy cows or beef cattle-- primarily main areas of animal breeding-keeping-- there is a significant lack of species-specific animal welfare regulations absent. This perspective reveals that Turkey's record on animal welfare needs to be rewritten: there is room for substantial improvement. Nevertheless, even if not sufficient, these regulations being in force is important and satisfying.

As a result, it may be stated that the efforts making legislation regarding animal welfare and the inspections related to the current regulation have not reached a proper level of satisfaction.

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