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Research Article

Classification of Biochemical and Biomechanical Data of Diabetic Rats Treated with Magnetic Field By PCA-Supported J48 Algorithm

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Abstract

The aim of this study was to investigate the J48 mediated decision tree algorithm from the principal component analysis - PCA, which is more complex, one of the statistical algorithms of diabetic metabolic disorders of Wistar albino rats' biochemical values and magnetic field application. Wistar Albino rats were examined under 4 different groups including the control group. When the results were examined, it was observed that PCA increased the success rate of classification from 96.25% to 97.50% when used with J48 decision tree algorithm. Thus, the PCA-supported J48 algorithm demonstrated that Wistar albino rats could be successfully used on the data obtained from more complex diabetic metabolic values.

Keywords: *Diabetes mellitus, Magnetic field, J48, PCA*

PCA Destekli J48 Algoritması İle Manyetik Alanla Tedavi Edilen Diyabetik Sıçanların Biyokimyasal ve Biyomekanik Verilerinin Sınıflandırılması

Öz

Bu çalışmanın amacı, Wistar albino türü sıçanların diyabetik biyokimyasal değerleri ve Manyetik Alan Uygulamasıyla kasılma değerleriyle daha karmaşık hale getirilen veri setinin, istatistiksel algoritmalarından biri olan temel bileşen analiz -PCA ile etkili karar ağacı makinesi öğrenme algoritması-J48 aracılığı ortaya konulmasıdır. Wistar Albino türü sıçanlarkontrol grubu da dahil olmak üzere 4 farklı grup altında incelenmiştir. Sonuçlar incelendiğinde, PCA'nın karar ağacı makinesi öğrenme algoritması J48 ile birlikte kullanıldığında sınıflandırmadaki başarı oranı %96.25'den %97.50'e arttırdığı gözlenmiştir. Böylece, PCA ile desteklenen J48 algoritmasının, Wistar albino türü sıçanlarının daha karmaşık hale getirilmiş diyabetik metobolik değerlerinden elde edilen veriler üzerinde başarılı bir şekilde kullanılabileceğini ortaya koymuştur.

Anahtar sözcükler: *Diyabetes mellitus, Manyetik alan, J48, PCA*

INTRODUCTION

Diabetes mellitus (DM) is a chronic autoimmune disease where either the pancreas does not produce enough insulin or the body cannot effectively use the insulin produced. Insulin regulates the blood sugar level by enabling entry of glucose into cells. Hyperglycemia or increased blood sugar is a common effect of DM leading to long-term vascular complications such as retinopathy, neuropathy and nephropathy [1]. There are essentially two types of DM. Type 1 DM usually results in the autoimmune-mediated destruction of pancreatic beta cells and absolute insulin deficiency. Type 2 DM is characterized by insulin resistance or relatively insufficient insulin release [2]. The number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014 [3]. The global prevalence of diabetes among adults over 18 years of age rose from 4.7% in 1980 to 8.5 in 2014 [4]. In 2016, an estimated, 1.6 million deaths were directly caused by diabetes. Another 2.2 million deaths were attributable to high blood glucose in 2012 [5]. Almost half of all deaths attributable to high blood glucose occur before the age of 70 years. WHO estimates that diabetes was the seventh leading cause of death in 2016 [6].

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The muscles are the basis of the movement system. The fact that muscle diseases affect the quality of life in patients with and without diabetes is a condition that we all observe. Diabetes is a metabolic disease affecting various organ systems, including skeletal muscles. These changes are thought to involve both structural and metabolic defects. Experimentally induced diabetes is associated with changes in the contractile and electrical properties of skeletal muscles. The soleus and extensor digitorumlongus (EDL) are important skeletal muscles that play important roles in standing to the gravity force and exercises [7]. At the same time, atrophy may develop (muscle loss) in skeletal muscles and results in decreased muscle strength. The parameters are dependent on the distribution of muscle fiber types since the electrical and contractile functions of the skeletal muscles change. Therefore, the effect of diabetes mellitus on different skeletal muscles varies due to the distribution of muscle fibers [8].

There are many findings that electromagnetic waves have effects on organ systems, cell membranes and even the biological structure of enzymes [9]. Basset Previous studies have reported that magnetic field applications, which are used as a non-invasive method in the treatment of many diseases, may produce positive effects on different tissues [10-13]. It has been reported that low-frequency magnetic field applications help peripheral blood mononuclear cells to warn of diseases such as angiogenesis and diabetic foot ulcer [14]. Pulsed Magnetic Field (PMF) applications are considered to be a very important alternative treatment method for inflammatory pain resulting from pharmacological agents with toxicity and side effects [15-17]. It has been observed in research that PMF has an effect on healing in neuropathic pain induced by diabetes PMF application increased regeneration by approximately 22% after sciatic nerve crush injury [18,19]. We can easily say there is a relationship between diabetes and muscle strength and between muscle strength and the magnetic field [18].

In order to research diabetes and its complications of DM, we need to have advanced information technology. Therefore, data mining technology is an appropriate study field for us. Data mining, also known as Knowledge Discovery in Database, is defined common study as the computational process of discovering patterns, large dataset involving methods of artificial intelligence, machine learning, statistics, and database system [20]. Recently, Zou and co-workers reported that Support Vector Machine (SVM), Decision Trees (DT) are the most common classification tools for predicting diabetes. The j48 algorithm is one of the most successful algorithms used in DT classification [21]. Models are also based on the baseline estimates of medical decision-making systems in diabetes diagnosis. They are based on the underlying statistical analysis of the results of studies and are thus more meaningful in relation to the outcome. For high accuracy of forecasting success, it is necessary to create the best model using headed algorithms.

In this study, Wistar type albino rats were used for experiments. Biomechanical, biochemical values, body weights, and other parameters were measured in the all group end of the experiments. Based on these parameters data mining was used in this research. Data mining, decision tree and j48 algorithms with the help of machine learning through the data provided by diabetes classification were made. J48 algorithm was utilized from decision tree algorithms including 7 attributes. Then, attribute selection (PCA) was chosen for just three attributes, and the results were compared with these three attribute values. Using the data obtained from the experimental rats, machine learning and estimation were applied to the computer with the help of DT and J48 Algorithms, then, it was aimed to compare the data obtained from the experiment with the data obtained as a result of machine learning.

MATERIAL and METHODS

The ethics committee approval was provided from Animal Experiments Domestic Ethic Committee of Çukurova University (No. B.30.2.ÇKO.0.5L.00.001). The model organisms studied were Wistar albino rats. Wistar Albino rats were examined under 4 different groups including the control group: Group I (n=20, control), Group II (n=20, control with magnetic field), Group III (n=20, streptozotocin-induced diabetes), and Group IV (n=20, streptozotocin-induced with magnetic field), where *n* denotes the number of rats in each group. We have recorded the animals' weight (*g*), biomechanical and biochemical parameters. Biomechanical parameters included twitch and tetanic parameters. Twitch (mN/mm^2) is a mechanical reaction to a solitary incitement of the muscle. Tetanic contraction force (mN/mm^2) is created at a high rate by a solitary muscle unit. Biochemical parameters included blood glucose level (in mg/dL; the ordinary estimate for rodents is under 300 mg/dL, and greater than 300 mg/dL is called diabetic), high density lipoprotein (HDL; in mg/dL), low density lipoprotein (LDL; in mg/dL), and triglyceride (in mg/dL). Blood glucose level is the measure of glucose exhibited in the blood of people and different creatures. HDL is known as the "good" cholesterol since it conveys cholesterol from different parts of the body back to the liver. The liver at that point expels the cholesterol from the body. LDL is known as the "bad" cholesterol on the grounds that a high LDL level prompts development of cholesterol in the supply routes. Triglycerides are the most widely recognized form of fat in the body; they store overabundant calories from the dietary regimen. A high triglyceride level conjoined with low HDL cholesterol or high LDL cholesterol is connected with fat accumulation in blood vessels. This increases the risk of heart attack and stroke.

Magnetic Field Application

The MF was created by a couple of 60 cm-wide Helmholtz

curls that were 30 cm apart. The loops were placed in a 90-90-50 cm-sized Faraday confine to forestall natural electromagnetic collaboration. Helmholtz curls were associated with a power supply and a chip-controlled recurrence generator created by the Department of Biophysics, Çukurova University, Adana, Turkey. For estimating the force of the MF, a pivotal test of a Tesla Meter (PHYWE System GmbH, Gottingen, Germany) was placed inside the MF confine. MF power was 1.5 mT, and there were no temperature changes caused by the MF. The rats were placed into a 30-30-25 cm plastic cage between the two curls where they could roam freely. The introduction was connected for a month for Groups II and IV. Five experimental animals were placed within MF exposure inside this cage, exposure was invariably applied to separate regions, excluding groups I and III. Therefore, the animals were not exposed to electrical transitions once the sphere was turned on and off.

Biomechanical Recordings

After 30 min of thermoregulation and equilibration, the muscle length was resolved (the length provides the most extreme muscle strain). Amid the entire preliminary timeframe, the muscles were specifically fortified for 20 min supramaximally by applying square frequencies of 0.05 Hz (15-20 V) of 0.5-ms intervals. To fortify the muscle and to record the reaction, a force-displacement transducer (FDT 10-A 500 g, Commat, Ankara, Turkey), a stimulator (STPT02-A, Commat), an exploratory tissue organ bath and circulator (WBC 3044, Commat), and the Biopac Systems (Goleta, CA) Student Lab System (MP30) were utilized. The muscle strain (Ps; in mN/mm^2), contraction times (CT) and half-relaxation times (HRT, in ms), and the contraction and relaxation rates (±dP/dt; mN/mm^2. ms) were recorded. After utilization of the beat trains of 10, 20, 50, and 100 Hz frequencies for times of 200-400 ms, the greatest muscle strain was recorded. These parameters were repeated for every one of the groups (I-IV). The muscle cross-sectional zone was evaluated utilizing muscle weight and length. The length was estimated before the distal ligaments were cut.

Principal Component Analysis (PCA)

One of the data pre-processing methods before the use of machine learning is the PCA [22]. The aim is to discover variables that best represent the data; with fewer variables, PCA is a useful statistical technique for understanding the relationship of multiple dimensions during data analysis and, thus reduces the size of the data set (dimension reduction). At the same time, intensive clustering in data mining can also result in more rapid processing of some operations (e.g., training of the classification algorithm) by converting a dataset with n features into a *k* (k<n) dimensional dataset.

Clearly, some of the features of the data will be lost during these operations since the PCA can also clarify the data sets and the relationship of the data to each other, so the data can also be used to calculate the weights of the effects. The major aim is to be able to work with a minimum loss by maintaining high variance.

Decision Tree -J48

The J48 decision tree, also known as C4.5, is an alternative Dichotomizer-3 (ID3) -based machine learning model which is based on the Quilan side; the model identifies and predicts a target value (dependent variable) of a new instance based on the various property values of the existing data J48 [23]. It is quite a popular algorithm that is ranked #1 in "top 10 algorithms in data mining" [24].The decision tree classifier follows a simple divide-and-conquer algorithm. To classify a new substance, it is first necessary to form a decision tree based on the property values of the existing training data. For this reason, when compared to the number of items (training set), the nature that distinguishes the various examples is clearly determined. Different attributes of a decision tree define the final value (classification) of a dependent variable in nodes, while branches between nodes report the possible values that these attributes may adopt in the observed instances. The property to be estimated is known as the dependent variable because the value depends on the values of all other properties or is related to the values of all other properties. Other features that assist in predicting the value of the dependent variable are known as arguments in the data set.

Model Evaluation

Root mean square error (RMSE), mean absolute error (MAE), RRSE are regularly employed in model evaluation studies. Experimental results are evaluated for both precision and accuracy, in medical data, it is common to express accuracy as a percentage. While magnetic field application made the data set more complex, PCA was applied to reduce the data.

Accuracy = (correctly predicted class /n) × 100% (Equation 1)

The Kappa test is presently a standard statistical method that measures the reliability of fit between two or more classes. The Kappa value ranges between (-) 1 and (+) 1, the positive value is shown as better interpreted, and kappa values between 0.81-1.00 indicate a very good level of integration.

When the results are examined, it is seen that the PCA increases the success rate in the tree learning algorithms from the machine learning models in this study. It has been observed that the J48 algorithm improves accuracy when used together with PCA. In the Kappa (K) values, the Mean Absolute Error (MAE), Root Mean Squared Error (RMSE), Relative Absolute Error (RAE) and Root Relative Squared Error (RRSE) values were also found to improve.

The formula of K is given Equation 2.

$$K = \frac{\Pr(a) - \Pr(e)}{1 - \Pr(e)} \quad \text{(Equation 2)}$$

The MAE shows thatthe average of all absolute errors and is a linear scoring that means that all individual differences are equally weighted in the mean. The formula is given in Equation 3.

$$MAE = \frac{1}{n}\sum_{i=1}^{n}|actual - predicted| \quad \text{(Equation 3)}$$

Where n = the number of errors, |actual – predicted| = the absolute errors.

The RMSE should be scored on the second level. which this metric measures the difference between the actual value and the predicted value. The formula is given in Equation 4.

$$RMSE = \sqrt{\sum_{i=1}^{n}\frac{(actual-predicted)^2}{n}} \quad \text{(Equation 4)}$$

The formula of RAE shows the absolute error of the size of the thing being measured. The formula is given in Equation 5.

$$RAE = \frac{\sum_{i=1}^{n}|actual-predicted|}{\sum_{i=1}^{n}|Ave-predcitedæ} \quad \text{(Equation 5)}$$

TheRRSE is relative to what it would have been if a simple predictor had been used. More specifically. this simple predictor is just the average of the actual values. The formula is given in Equation 6.

$$RRSE = \sqrt{\frac{\sum_{i=1}^{n}(Actual-predicted)^2}{\sum_{i=1}^{n}(Average-predicted)^2}} \quad \text{(Equation 6)}$$

RESULTS

J48 classification involves a classifier based on 7 attributes and 4 groups, using J48 as the algorithm, and classifies the muscle contraction parameter by considering the magnetic field specifier in the J48 algorithm. Ten-fold cross-validation mode was used for classification. In terms of data obtained from the diabetic group, it was determined that measured glucose levels were high. The reason for increased glucose levels in diabetic animals was that, in this study, rats were injected with a single high dose of 45 mg/kg/mL STZ (i.v.), which might disrupt the function of the insulin-releasing mechanism according to the widely accepted view of scientists [25-27]. No enhancing treatment was applied to animals for 30 days. All data sets were given in *Table 1*.

Furthermore, the blood glucose levels for diagnosing diabetes in humans during fasting and 2 h postprandial are 7.0 mmol/L (126 mg/dL) or greater and 11.1 mmol/L (200 mg/dL) or greater [28], respectively, and the situation for diabetic rats is that they can be considered as diabetic with blood glucose levels of 300 mg/dL (16.7 mmol/L)[7]. The J48 algorithm used the Twitch (mN/mm^2) feature to classify the data in the set. The values of isometric twitch were <= 45.47 (mN/mm^2) according to the values of Group III and Group IV, and isometric twitch values > 45.47 (mN/mm^2) were classified as Group I and Group II *(Fig. 1* and *Table 2)*. Classification of the J48 algorithm according to the contraction parameters which are magnetic field indicator was defined by these methods. Using PCA, a wider range of variance was generated using the entire data set. The resulting three new features using the J48 algorithm in the new dataset are provided in *Fig. 2* and *Table 3*.

DISCUSSION

Diabetes mellitus is a disease with a wide range of social effectsincludingmuscularandneuromusculardiseasessuch as muscular atrophy and partial paralysis; therefore, it is a

Table 1. *Basic descriptive statistics organized by groups*

Groups (n=20)	Parameter	Weight (g)	Glucose (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	TG (mg/dL)	Twitch (mN/mm^2)	Tetanic (mN/mm^2)
Group I	Std. Dev. Min. Max. Arith. Mean. Std. Error	11.76 322.72 354.94 338.36 2.70	2.36 160.08 167.06 163.652 0.52	1.25 35.57 39.43 37.697 0.28	0.58 15.22 17.08 16.12 0.13	1.40 70.97 75.05 73.06 0.31	0.51 45.62 47.26 46.35 0.11	2.92 199.38 208.18 203.26 0.65
Group II	Std. Dev. Min. Max. Arith. Mean Std. Error	14.63 276.41 324.68 293.48 3.27	4.11 148.65 161.08 155.41 0.92	1.03 36.08 39.37 37.78 0.23	0.51 13.77 15.23 14.61 0.11	2.45 47.89 54.41 51.41 0.55	0.48 51.33 52.89 52.13 0.10	0.72 218.22 220.83 219.93 0.16
Group III	Std. Dev. Min. Max. Arith. MeanStd.Error	4.71 293.69 308.19 300.40 1.05	7.66 584.32 610.64 597.28 1.71	1.55 42.63 47.27 44.40 0.34	2.49 65.15 72.77 68.36 0.55	8.79 245.56 271.18 259.78 1.96	0.23 41.02 41.76 41.40 0.05	0.47 93.65 65.32 94.608 0.10
Group IV	Std. Dev. Min. Max. Arith. Mean. Std. Error	1.65 301.41 306.55 304.11 0.37	10.30 550.17 579.24 567.71 2.30	1.23 41.22 45.03 42.99 0.27	1.07 53.13 56.65 55.05 0.24	7.53 162.68 184.66 173.56 1.66	0.38 44.31 45.47 44.98 0.08	1.19 147.7 151.91 150.30 0.26

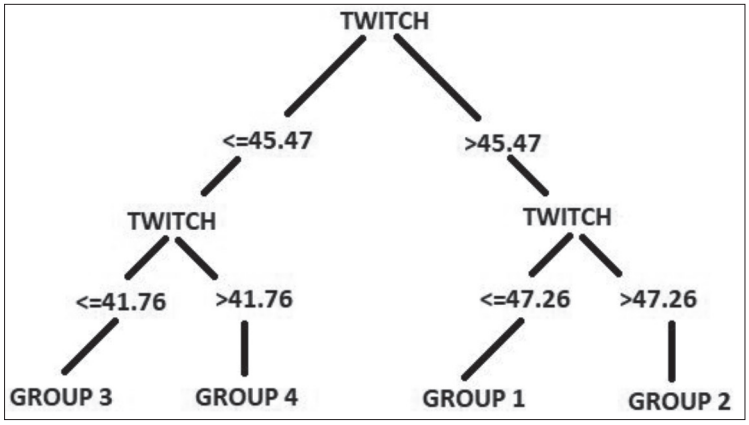

Fig 1. Decision Tree Classification, J48 algorithm in the four groups; It has been classified as to the twitch attributes. (WEKA Version 3.9.2)

Table 2. *Biomechanical parameters for all groups with Decision Tree Classification*

Muscle Contraction	<= 45.47	Twitch > 45.47
Twitch (mN/mm²)	<= 41.76; Group III (20.0)	<= 47.26; Group I (20.0)
Twitch (mN/mm²)	> 41.76; Group IV(20.0)	> 47.26; Group II (20.0)

Fig 2. By decreasing the attributes to PCA with 3-attributes

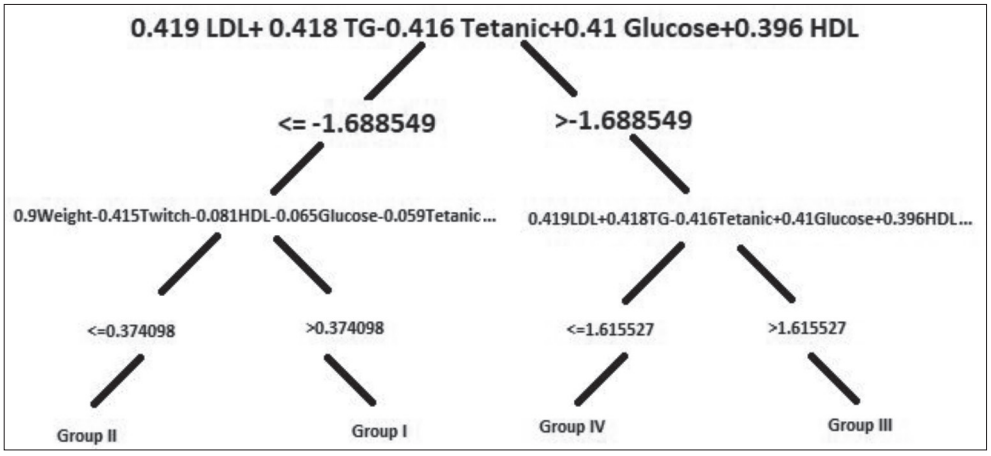

Table 3. *The performance results of J48 Algorithm applied with PCA and without PCA*

Total Number of Ins. =80	Correctly Classified Ins.	Accuracy (%)	Kappa Statistic	Mean Absolute Error (MAE)	Root Mean Squared Error (RMSE)	Relative Absolute Error (%) (RAE)	Root Relative Squared Error (%) (RRSE)
Without PCA	77	96.25	0.95	0.0187	0.1369	5	31.62
With PCA	78	97.50	0.96	0.0125	0.1118	3	25.82

priority in many studies including machine learning and data mining studies. We have applied a controlled learning algorithm in our categorization model because the value of the dependent variable or output variable was estimated through a set of inputs or independent variables. Support Vector Machine (SVM), Artificial Neural Network (ANN) and DT are the most commonly used classification algorithms used in estimation methods, as mentioned in the literature. Generally, controlled learning algorithms and the rules of the relationship between biomarkers are discussed in approximately 85% and 15%, respectively, of DM estimation studies [29]. Numerical results were obtained in parallel with the same classification methods used in DM studies.

Biochemical and biomechanical measurements developed with diabetes model were generated by the hypothesis that variables are related to the PCA of each other, a hypothesis that could be used in data preparation techniques for analyses. These measurements were preferred for the destruction of the dependency structure or for size reduction [30,31]. PCA and Machine learning techniques are known in previous studies for their use in a large number of complex data. In this study, we found that PCA and J48 algorithm can be possible with a limited number of subjects in large and complex data. We believe that this algorithm will increase the accuracy percentage in a high number of subjects. In our study, although the

number of rat subjects was limited due to ethical reasons, biochemical parameters and magnetic field factor were 7 attributes in 4 different groups forming the data set. In fact, Wu et al.[32], using the Weka algorithm in the predictive model of diabetes, Artificial neural networks with J48 and ID3 compared with 89.3%-81.9% showed that they work with higher accuracy. They found that the use of different pre-processing techniques in DM prediction modeling increased accuracy in Bayes and DT classification.

We could compare our results with Quan Zou et al.[21]'s results that they applied many machine learning algorithms on public diabetics datasets. They showed that J48 was 74.75% accuracy on Pima Indian's dataset. But, J48 gives better accuracy results on our dataset.

Diabetes mellitus is a major public health problem allover world. In this study, we applied PCA and J48 two different algorithms in models for predicting diabetes mellitus using 7 important attributes biomechanic and biochemical of the four groups classified by twitch attributes. We may conclude that *Table 3* demonstrates that applying PCA with J48 has increased the predictive success of the diabetic studies that the incremented of accuracy is 0.75% when we compared them with or without PCA. However, this is a significant increment on biological datasets. Therefore, it is shown that J48 could be applied to this kind of biological datasets with PCA.

Although there are constraints (number of subjects, diabetes) in animal experiments, the results are confirmatory to our hypothesis. If the same data set is obtained on people, it is thought that a higher performance rate will be achieved.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

Effects of High Rice Diet on Growth Performance, Nutrients Apparent Digestibility, Nitrogen Metabolism, Blood Parameters and Rumen Fermentation in Growing Goats

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Abstract

This study was conducted to evaluate the effects of high rice grain diet on apparent nutrient digestibility, growth performance, nitrogen digestion, blood parameters and rumen fermentation in goats. Sixteen growing goats were divided into 2 groups and fed a normal-concentrate diet (NC, 55% concentrate of dry matter; n=8) or a high concentrate diet (HC, 90% concentrate of dry matter; n=8) for 5 wk. Growth performance, nutrients digestibility, nitrogen digestion, blood parameters and ruminal fermentation were measured. Total weight gain and average daily gain increased in the HC group (P<0.01). Digestibility of Neutral Detergent Fibre (NDF) and Acid Detergent Fibre (ADF), nitrogen intake and digested nitrogen also increased (P<0.05) by HC feeding. Triglycerides, cholesterol, high density lipoprotein and low-density lipoprotein concentrations in the blood decreased (P<0.05). HC diet feeding decreased (P<0.01) the pH value, acetate level and ratio of acetate to propionate, but increased (P<0.05) the concentrations of propionate, valerate and total volatile fatty acids in the rumen. These findings revealed that the HC diet could promote the growth of growing goats, change the ruminal fermentation pattern and lipid metabolism in the blood, but cause subacute ruminal acidosis, which might increase the risk of body health.

Keywords: *High concentrate diet, Goats, Apparent digestibility, Metabolism, Fermentation*

Büyüme Dönemindeki Keçilerde Pirinç Ağırlıklı Diyetin Büyüme Performansı, Görünür Sindirilebilirlik, Nitrojen Metabolizması, Kan Parametreleri ve Rumen Fermentasyonu Üzerine Etkileri

Öz

Bu çalışma, keçilerde pirinç ağırlıklı diyetin, görünür sindirilebilirlik, büyüme performansı, azot sindirimi, kan parametreleri ve rumen fermentasyonu üzerindeki etkilerini değerlendirmek amacıyla yapıldı. Bu amaçla büyüme dönemindeki on altı keçi, 2 gruba ayrıldı ve 5 hafta boyunca normal konsantrasyon (NC, %55 kuru madde konsantrasyonu; n=8) veya yüksek konsantrasyondaki diyet (HC, %90 kuru madde konsantrasyonu; n=8) verildi. Büyüme performansı, besin sindirilebilirliği, azot sindirimi, kan parametreleri ve ruminal fermantasyon ölçüldü. Sonuçta, HC grubunun toplam kilo artışı ve ortalama günlük kilo artışı yüksek bulundu (P<0.01). Nötral Deterjan Lif (NDF) ve Asit Deterjan Lif (ADF)'in sindirilebilirliği, azot alımı ve sindirilmiş azot oranının da, HC beslemesi ile arttığı belirlendi (P<0.05). Kandaki trigliseritler, kolesterol, yüksek yoğunluklu lipoprotein ve düşük yoğunluklu lipoprotein konsantrasyonları azaldı (P<0.05). HC diyet beslemesi pH değerini, asetat seviyesini ve asetatın propiyonata oranını azaltırken (P<0.01), rumendeki propiyonat, valerat ve toplam uçucu yağ asitlerinin konsantrasyonlarını arttırdı (P<0.05). Bu bulgular, HC diyetinin büyüme dönemindeki keçilerin gelişimini destekleyebileceğini, kandaki ruminal fermentasyon düzenini ve lipid metabolizmasını değiştirebildiğini, ancak subakut ruminal asidoza neden olarak risk oluşturduğunu ortaya koydu.

Anahtar sözcükler: *Yüksek konsantrasyon diyet, Keçiler, Görünür sindirilebilirlik, Metabolizma, Fermantasyon*

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INTRODUCTION

Relative high fiber feeding and stable microbial flora are the necessary conditions for the health of ruminants [1]. However, at present, feeding high concentrate diet is widely adopted to provide adequate protein and energy supply for meeting higher performance needs at finishing stage of ruminants [2]. Non-fibrous carbohydrates in the concentrate of diets can provide ruminants with energy and small intestinal absorbable glucose to meet high production and energy needs. Appropriate increase of dietary concentrate level can improve the production performance of ruminant animals [3]. It is well known for feeding excessive amounts of non-structural carbohydrates and highly fermentable roughages easily result in a series of metabolic diseases in high-yield ruminants [4,5]. For instance, previous studies have shown that feeding high concentrate diets can induce subacute ruminal acidosis (SARA) in ruminants [6]. The presence of SARA is an important concern in terms of both productivity and animal welfare. The high rumen digestibility of most grains in the concentrate improved the ruminal VFA production, and the accumulation of volatile fatty acids (VFA) causes a decrease in ruminal pH [7]. Typically, when rumen pH is lower than normal level, then gram-negative bacteria in the rumen and intestinal tracts releases a lot of lipopolysaccharide (LPS) [3], also known as endotoxin, is a part of the outer membrane of the gram-negative bacteria [8], seriously disturbing the normal physiological function of gastrointestinal tracts and body health. Additionally, ruminal fermentation and digestion processes mediated by symbiotic microorganisms can convert dietary carbohydrates into available nutrients such as VFA and sugars [9].

Black goat is common in the south of China, by a wide range of breeds, mainly located in Hunan, Jiangxi, Guizhou and surrounding provinces and cities. Liuyang black goat is a rare pure black goat breed in China. Liuyang black goat has tender meat, delicious taste, leaner meat, less fat, high nutritional value and strong disease resistance. Therefore, Liuyang black goat has good research value. Previous evidence has suggested that rapid growth can be achieved by feeding the diets exceeding 50~65% proportions of concentrate (mainly containing ground corn grain) to ruminants [10]. In fact, because corn grain is commonly scarce in the traditional rice cropping region of southern Asia, rice grain is thereby used as an alternative feed applied in the goat diets. Howbeit few studies were conducted to explore the effects of dietary high rice grain proportion on the gastrointestinal nutrient digestion and metabolism. The aim of this research is to conduct a feeding trail to investigate whether a diet with high unhusked rice grain proportion would affect the growth performance, nutrients apparent digestibility, nitrogen metabolism, blood metabolites and ruminal fermentation, and to seek for the rational and practical approaches of rice grain in a ruminant production system.

MATERIAL and METHODS

The study was approved by the Animal Care Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China and the Animal Care and Use Committee of Hunan Agricultural University (HAU201408).

Animals, Diets and Management

Sixteen Liuyang Black goats (6 months old, a local breed in the south of China), with an average BW of 15.33±1.67 kg were randomly divided into two groups and fed normal -concentrate diet (NC, the ratio of concentrate to forage was 55:45) and high-concentrate diet (HC, the ratio of concentrate to forage was 90:10), respectively. The ingredients and nutrient levels of the diets are given in *Table 1*. Unhusked rice was in the form of dried powder and provided by the Hunan LiFeng Bio-Technology Company Ltd. (Changsha, China). Before formulating the diets, the rice straw was chopped to approximately 2 cm in length.

The experiment duration consisted of 35 days, with 7 days for diet adaptation and 28 days for sampling. Diets were equally offered at approximately 08:00 and 18:00 h,

Table 1. *Ingredients and nutrient levels of the experimental diets (air-dried basis)*

Item	NC[1]	HC[2]
Ingredients composition (%)		
Forage		
Rice straw	45.0	10.0
Concentrate		
Rice with shell	33.2	54.3
Soybean meal	9.60	15.7
Wheat bran	6.00	9.80
Fat powder	3.20	5.20
Calcium carbonate	0.50	0.80
Calcium bicarbonate	1.10	1.80
Sodium chloride	0.60	1.00
Premix[3]	1.00	1.40
Nutrient levels[4], % of DM		
Crude protein	13.5	17.6
Crude ash	9.34	9.12
Crude fat	4.18	6.01
Neutral detergent fiber	49.8	38.4
Acid detergent fiber	36.5	9.51
NFC[5]	14.83	24.35

[1] NC: normal-concentrate diet; [2] HC: High-concentrate diet; [3] Premix composition per kg diet: 68 mg $FeSO_4 \cdot H_2O$, 44 mg $CuSO_4 \cdot 5H_2O$, 411 μg $CoCl_2 \cdot 6H_2O$, 1.70 mg KIO_3, 211 mg $MnSO_4 \cdot H_2O$, 126 mg $ZnSO_4 \cdot H_2O$, 56 μg Na_2SeO_3, 462 mg $MgSO_4 \cdot 7H_2O$, 737 IU Vit. A, 8.29 mg Vit. E, 4.0 g $NaHCO_3$, 5.1 g carrier zeolite powder; [4] Nutrient levels were measured values; [5] NFC: Non-fibrous carbohydrate. NFC was calculated in accordance with *NFC = DM - (CP + EE + Ash + NDF)*

respectively. All goats were fed in separate cages. Goats had free access to water and feed intake of each goat was recorded daily.

Sampling and Collection

The goats were kept in metabolic cages which enabled the separation of urine from feces. Feces and urine were collected from goats twice daily before feeding (08:00 h and 18:00 h) and lasted for 7 days. For feces samples, further subsamples (2% of total weight) were acidified with 10% H_2SO_4 and stored at -20ºC for the determination of dry matter (DM), organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and nitrogen (N). The fecal subsamples were dried at 65ºC for 48 h and stored in plastic bags until laboratory analysis. For each goat, the total volume of urine was recorded daily. A 10% urine subsample was taken, in which 10% H_2SO_4 was added to keep pH less than 3.0 for the determination of N. Samples of the diets were collected after feeding in the morning each week, and urine was stored at -20ºC until analysis.

Blood samples were collected aseptically in tubes with heparin sodium from the jugular vein of goats before feeding in two consecutive days (d34 and d35) of the sampling period, respectively. Plasma samples were separated by centrifugation by 1000 g for 15 min at 4ºC, and stored at -20ºC for further analysis. Rumen fluid samples were taken at 0h (before feeding in the morning), 3 h (3 h after feeding in the morning) and 6 h (6 h after feeding in the morning) on day 35 through the oral cavity. Meanwhile, pH values of rumen fluid were immediately determined after sampling using a pH meter (Model PHS-3C, Shanghai Precision Science Instrument Co., Ltd., China). Further subsamples were centrifuged at 15.000 g for 10 min and the supernatant fluids were acidified with 25% (w/v) metaphosphoric acid in a ratio of 10:1, vortexed and stored at -20ºC for VFA determination.

Chemical Analysis

The samples of diets and feces were dried at 105ºC overnight and ignited at 550ºC for 6 h for measuring DM, OM and Ash (method 942.05; AOAC, 1995), respectively [11]. Crude protein content was calculated as 6.25×N which was determined using the Kjeldahl method [12]. The NDF and ADF were measured by using the procedures of Van Soest et al.[13]. The plasma biochemical components including lactate dehydrogenase (LDH), lactate (LACT), glucose (Glu), triglycerides (TG), cholesterol (CHOL), high density lipoprotein (HDL), low density lipoprotein (LDL), total protein (TP), and albumin (ALB)were determined using an Automatic Biochemistry analyzer (Cobas c 311, Roche). Insulin-like growth factor 1 (IGF-1) in the plasma was measured by ELISA kits (Cusabio, Wuhan, China) according to the instructions of the manufacturer. LPS and growth hormone (GH) in the plasma were also detected using corresponding ELISA kits (Jian Cheng Bioengineering Institute, Nanjing, China).

The ingested N (IN, g/d), fecal N (FN, g/d) and urinary N (UN, g/d) were used to calculate N balance as following:

Digested N (DN) [g/d] = IN–FN,

Retained N (RN) [g/d] = IN–FN–UN,

Availability of RN [%] = (IN–FN–UN)/IN × 100%,

Availability of DN [%] = (IN–FN–UN)/(IN–FN) × 100%,

Apparent N digestibility [%] = (IN–FN)/IN × 100%.

The rumen fluid samples were thawed and centrifuged at 10.000 g and 4ºC for 15 min. The supernatant solution was used for VFA determination by gas chromatography (HP5890, Agilent Technologies Co. Ltd., USA).

Statistical Analyses

Statistical analyses of data were evaluated through independent sample T-test, and animal were used as experimental unit. Values are expressed as the mean ± standard error of the mean (SEM). Statistical significance was set at $P<0.05$ and tendencies at $0.05 \leq P \leq 0.10$. All statistical analyses were conducted with SPSS 19.0 (SPSS Inc., Chicago, IL, USA, 2009).

RESULTS

As shown in *Table 2,* the total weight gainand average daily gain wereincreased by HC ($P<0.01$). But HC diet significantly decreased the FCR (feed conversion ratio). Mean while, no significant differences in final weight and DMI were noted between groups ($P>0.10$). As shown in *Table 3,* the intake, fecal output and apparent digestibility of OM were not affected ($P>0.05$) by HC. HC decreased the intake of NDF ($P<0.01$), but increased the fecal output ($P<0.01$), and finally reduced the digestibility of NDF ($P<0.01$). ADF intake was not influenced by HC ($P>0.05$), but the fecal output was greater ($P<0.01$) and the digestibility was reduced ($P<0.01$) in the HC group.

Nitrogen intake, fecal nitrogen, urinary nitrogen excretion and DN were increased ($P<0.05$) by the HC diet *(Table 4)*. However, there were no significant differences in the RN, DN and their availability, and N digestibility ($P>0.05$).

Table 2. *Effects of high-concentrate diet on growth performance in goats*

Items	NC[1]	HC[2]	SEM	P-value
Initial weight/kg	15.5	15.2	0.51	0.78
Final weight/kg	17.4	18.7	0.64	0.34
Total weight gain/kg	1.88	3.50	0.33	<0.01
Average daily gain (g/d)	67.1	125	12	<0.01
DMI (g/d)	572	602	13	>0.10
FCR[3]	11.46	7.27	1.09	0.032

[1] NC: normal-concentrate diet, [2] HC: High-concentrate diet, [3] FCR: feed conversion ratio

Table 3. *Effects of high-concentrate diet on nutrients apparent digestibility in goats*

Items	NC[1]	HC[2]	SEM	P-value
OM[3]				
Intake, g/d	523	563	12	0.12
Fecal output, g/d	131	168	11	0.078
Apparent digestibility, %	74.9	70.0	1.7	0.15
NDF[4]				
Intake, g/d	317	265	10	<0.01
Fecal output, g/d	72.2	131	12	<0.01
Apparent digestibility, %	77.2	50.6	5.0	<0.01
ADF[5]				
Intake, g/d	173	166	3.4	0.36
Fecal output, g/d	58.2	88.6	5.3	<0.01
Apparent digestibility, %	66.3	46.6	3.3	<0.01

[1] NC: normal-concentrate diet; [2] HC: High-concentrate diet, [3] OM: organic material; [4] NDF: neutral detergent fiber, [5] ADF: acid detergent fiber

Table 4. *Effects of high-concentrate diet on nitrogen digestion in goats*

Items	NC[1]	HC[2]	SEM	P-value
Nitrogen intake, g/d	12.1	17.4	0.93	<0.01
Fecal nitrogen, g/d	2.66	4.88	0.51	0.018
Urinary nitrogen, g/d	1.95	4.77	0.63	0.014
Digested nitrogen, g/d	9.47	12.5	0.67	0.014
Retained nitrogen, g/d	7.53	7.70	0.72	0.91
Availability,% of RN[3]	62.0	44.0	4.8	0.055
Availability,% of DN[4]	79.4	60.2	5.3	0.067
Digestibility,%	78.1	71.7	2.3	0.18

[1] NC: normal-concentrate diet, [2] HC: High-concentrate diet, [3] RN: retained nitrogen, [4] DN: digested nitrogen

HC increased the concentration of LACT (P=0.040), while LACT was not detected in the NC group *(Table 5)*. Meanwhile, no significant differences were observed (P>0.05) between the dietary treatments in contents of LPS, LDH, Glu, TP, ALB and GH. Concentrations of TG (P<0.01), CHOL (P=0.029), HDL (P=0.035) and LDL (P=0.034) in blood were decreased in HC goats.

Variation of pH values in the rumen fluid was shown in *Fig. 1*. It is obvious that goats fed the NC diet had greater ruminal pH than that of the HC group (P<0.01). Ruminal pH in the HC group was affected by sampling hour (P<0.01). The pH values in rumen samples of two groups were the same before feeding in the morning (0 h), but it was declined in the HC group at 3 h or 6 h after feeding (P<0.01). During the time period from 3 h after the feeding until the sampling at 6 h, the average ruminal pH was below 5.7 in the HC group and remained significantly lower than that of the NC goats.

Data of metabolites in the ruminal fluid are displayed in

Table 5. *Effect of high-concentrate diet on blood parameters in goats*

Items	NC[1]	HC[2]	SEM	P-value
LPS[3] (EU/mL)	1.84	1.77	0.049	0.52
LDH[4] (U/L)	289	305	33	0.83
LACT[5] (mmol/L)	0	1.02	0.25	0.040
Glu[6] (mmol/L)	4.09	4.05	0.087	0.85
TG[7] (mmol/L)	0.683	0.378	0.061	<0.01
CHOL[8] (mmol/L)	2.64	1.72	0.23	0.029
HDL[9] (mmol/L)	1.76	1.19	0.14	0.035
LDL[10] (mmol/L)	1.02	0.480	0.13	0.034
TP[11] (g/L)	73.0	69.6	2.4	0.51
ALB[12] (g/L)	30.3	34.6	2.3	0.39
IGF-1[13] (ng/mL)	29.8	64.4	8.5	0.040
GH[14] (ng/mL)	1.57	1.41	0.13	0.56

[1] NC: normal-concentrate diet, [2] HC: High-concentrate diet, [3] LPS: lipopolysaccharide, [4] LDH: lactate dehydrogenase, [5] LACT: lactate, [6] Glu: glucose, [7] TG: triglycerides, [8] CHOL: cholesterol, [9] HDL: high density lipoprotein, [10] LDL: low density lipoprotein, [11] TP: total protein, [12] ALB: albumin, [13] IGF-1: insulin-like growth factor 1, [14] GH: growth hormone

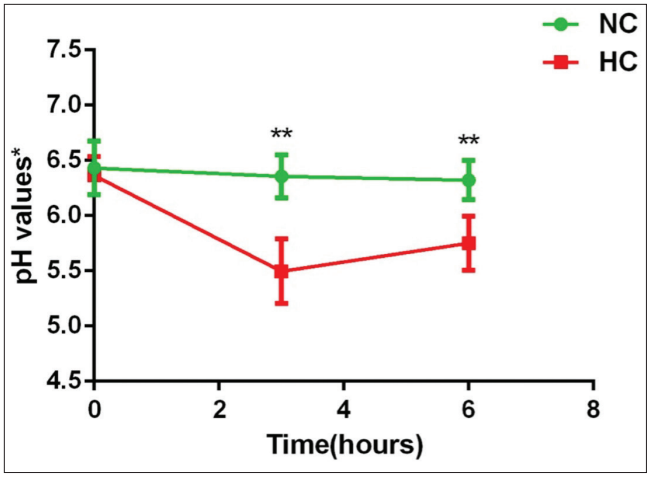

Fig 1. Effects of high-concentrate diet on pH values in ruminal fluid of goats

* The pH value was measured on day 35, ** indicates a very significant difference in two groups at the same time

Table 6. Ther e was no difference in the LACT concentration of ruminal fluid in two groups (P=0.77). The acetate level was lower in the HC group (P<0.01), but the propionate (P<0.01), valerate (P=0.023), and total VFA (P<0.01) concentrations were higher in the HC group. There were no differences in isobutyrate, butyrate, and isovalerate between the two groups (P>0.05). Additionally, the ratio of acetate and propionate in the ruminal fluid in the HC group was lower than that of the NC group (P<0.01).

DISCUSSION

In the present study, the HC diet increased the total weight gainand average daily gain of goats, indicating that HC

Table 6. *Effects of high-concentrate diet on the ruminal lactate, molar proportions of VFAs in goats*

Items	NC[1]	HC[2]	SEM	P-value
LACT[3] (mmol/L)	0.125	0.144	0.030	0.77
Total VFA[4] (mmol/L)	97.4	164	11	<0.01
Acetate: Propionate	4.24	2.20	0.34	<0.01
Individual VFA (mol/100 mol total VFA)				
Acetate	73.3	61.9	1.7	<0.01
Propionate	18.0	28.6	1.7	<0.01
Isobutyrate	0.642	0.692	0.039	0.55
Butyrate	6.92	7.27	0.61	0.79
Isovalerate	0.636	0.849	0.074	0.16
Valerate	0.530	0.732	0.046	0.023

[1] NC: normal-concentrate diet, [2] HC: High-concentrate diet, [3] LACT (lactate), [4] VFA (volatile fatty acids)

diet can promote the growth performance of goats. In our study, the total live weight gain of HC diet group was increased by a large amount of concentrate containing rich carbohydrates, and more carbohydrates produced more protein and energy for the goats, which led to a higher weight gain in the HC diet group. Higher total weight gain also leads to higher average daily gain. Meanwhile, the result is consistent with that of Mahgoub that study proved that the final weight and dry matter intake are not affected by the ratio of dietary concentrates to forge [14]. Because the NC group fed a large amount of forge to the goat in the study and a little concentrate, contrary to HC group fed a large amount of concentrate, finally, the FCR of the HC group was lower than NC group. We speculate that an increase of VFA in the rumen might partly be contributed to the overall weight and average daily gain of goats. The rumen absorbs energy material (i.e., SCFA), which accounts for about 70% of the energy required for ruminant growth, maintenance and production [15]. Additionally, we found that DMI in the HC group was not varied, which is agreed with previous research which has performed in Omani goats [16].

Dietary nutrients, such as fiber, is also essential for digestion mediated by chewing nutrients, microbial fermentation in the rumen and the rate of passage in the gastrointestinal tract [17]. In this study, the decreases in NDF intake and apparent digestibility and increase in fecal output of NDF were observed in the HC group. ADF fecal output was greater and the digestibility was reduced in the HC group. These results suggest that growing goats are less able to digest and degrade NDF and ADF of diet in the HC group. Our data agree with that Walsh et al.[18] which observed that the digestibility of fiber decreased with the increase of concentrate. The explanation of the decrease in NDF and ADF digestibility can be ascribed to two points. Firstly, changes in the rumen pH does not help to fiber digestion [19], since we also observed the less pH value in the rumen of HC goats. Low pH values in rumen leads to changes in rumen microbial population and reduces fiber digestion [20]. Secondly, the high levels of fat in the diet decrease the digestibility of the total digestive tract of NDF, ADF and cellulose in lambs [21] and dairy cows.

We find significant changes in fecal and urinary N among the two groups. Losses of N in feces and urine may be the result of low efficiency of protein utilization in the rumen. Meanwhile, according to Nocek and Russell [22], when protein degradation rate exceeds carbohydrate fermentation rate, a large number of N can be used as ammonia loss, and excess ammonia in the rumen may have been excreted in the urine. Additionally, the HC diet tends to reduce the availability of RN. The fecal N, urinary N, digested N and retained N were directly related to dietary N levels. According to Lallo [23], N retention showed a curvilinear response to energy intake but showed a linear response to increasing N levels. This means that N retention is more closely related to N intake rather than energy intake. Besides, urea can also recirculate through saliva and reenter the rumen [24]. Usually ruminants synthesize urea Nbeyond the N apparently digested [25], and it suggested that the N balance in ruminants is positive. Nitrogen recirculated to the gastrointestinal tract can be used to synthesize the microbial proteins in the rumen and provide amino acids to the host [26].

In this study, HC did not influence plasma LPS concentration. Gozho et al.[27] also did not observe increased LPS in the peripheral blood plasma of lactating dairy cows with SARA. This is partly attributable to the fact that lipoprotein can neutralize LPS in the body. There was evidence that lipoproteins can neutralize LPS *in vivo* [28]. Several experiments have also demonstrated that plasma lipoprotein, especially HDL, can be combined with LPS and preferentially shunt liver cells away from liver macrophages, thereby increasing LPS excretion through the bile and preventing immune responses [29]. HC diet reduced TG, CHOL, HDL and LDL, indicating that the metabolism of lipid in blood was changed. These data are very important in preventing hyperlipidemia and the heart and liver diseases [30].

Ruminal propionate produced during ruminal fermentation is used as a substrate in the gluconeogenesis pathway to produce glucose [31]. In the current study, although propionate in the rumen feeding HC was higher than NC, there were no significant differences on the concentration of glucose between NC and HC suggesting that ALB is the main protein produced in the liver and usually accounts for more than 50% of the total blood protein content [32]. Both TP and ALB were not influenced by HC in our study. TP is more sensitive to the effects of nutrition, but the changes are often subtle and difficult to detect and explain. The level of serum protein depends on a number of factors, including the presence of inflammatory or metabolic liver processes and other organ diseases [33].

Hormones can regulate various physiological and behavioral activities. The synthesis, storage and secretion of GH by the somatotropin cells, stimulate the growth of animals and the production of IGF-1 [34]. There is a positive correlation between the concentration of IGF-1 and the growth rate of animals [35]. Though the HC diet did not rise the plasma GH level, there is a significant increase in IGF-1, suggesting that the HC diet promotes growth development in goats, which can explain the increases in the total weight gainand average daily gain. The enzyme LDH is widely found in body tissues. As a marker of common injury and disease, it is released during tissue damage. In particular, feedback inhibition by LDH can reduce the rate of conversion of pyruvate to lactic acid at high lactate concentration [36]. Our data display the significant rise in plasma LACT content in response to the HC diet, but there was no significant influence in LDH between treatments. Further exploration is needed to explain this phenomenon.

The pH value is an extremely important indicator of the epithelial barrier and the ruminal metabolic state [10]. In this study, the rumen pH of NC group ranged from 6.07 to 6.73, and it was ideal for optimum rumen metabolism [37]. The rumen pH thresholds of 5.8 or lower were usually used to define a clinical diagnosis of SARA [38]. In this study, the data in *Fig. 1* indicated that NC-fed goats have a higher rumen pH. We observed that the duration of rumen pH below 5.8 in the SARA group was approximately 4 h after the first feeding, which meant that SARA occurred in goats by feeding the HC diet. The sampling time also affected the rumen pH. Briefly, the data indicated that HC-raised induced SARA in growing goats.

LACT is one of the main products in the rapid fermentation of rumen, and a large number of LACT can cause SARA has been widely recognized [39]. As we know, LACT can cross the rumen wall and dissolve in the blood to cause an increase in plasma. Although the concentration of LACT was found to be negligible in the rumen, we found a significant difference in the blood. This may mean that LACT was produced and immediately converted to VFA as suggested by some authors, thus preventing the accumulation of LACT in the rumen [40]. Previous studies have shown that long-term HC feeding increased the production of VFA in the rumen. Accumulation of these acids reduces the pH of the rumen and may cause SARA [9]. For many years, researchers have been looking for markers of SARA caused by HC feeding. Feeding goats with the HC diet can cause abnormal fermentation of the rumen. Data of the ratio A:P showed a decrease in HC fed goats compared to the NC group. The result was in agreement with Zervas [21] that the higher fat content in the diet reduces the rate of A:P, indicating that the rumen fermentation was developing towards propionate production. It was reported that the lower proportion of A:P was caused by the lower fermentation of cellulose in the rumen [41]. HC diet not only increased the concentration of propionate and valerate, but also increased the concentration of total VFA. The concentration of VFA in this study is similar to that reported previously, which indicated that the concentration of total VFA increases with the HC diet [42]. These data showed that the HC diet increased the production of VFA in the rumen. Accumulation of VFA decreased the ruminal pH and led to SARA. Therefore, an HC diet destroyed the balance of the rumen fermentation.

This study is the first in using unhusked rice as HC diet to survey the growth performance, nutrients apparent digestibility, nitrogen digestion, blood parameters and rumen fermentation of growing goats. In summary, these findings revealed that the HC diet could promote the growth of growing goats, changed the ruminal fermentation pattern and lipid metabolism in the blood, but cause subacute ruminal acidosis, which increases the risk of body health. The exact mechanism of this relationship between the blood parameters and the rumen microbial metabolites is needed to explore.

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Conflict of interest

There is no conflict of interest.

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Research Article

Study of Non-Specific Reactive Hepatitis in Stray Dogs

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Abstract

The purpose of this paper was to study the possible causes of Non-Specific Reactive Hepatitis (NSRH) in tissue samples of housed dogs that were collected from different cities of Andalucia (Spain). Histologically, this disease was characterized by the presence of lymphocytes and plasma cells spread throughout the liver parenchyma and in the portal stroma, with no evidence of hepatocyte necrosis. These animals showed non-specific chronic reactive hepatitis that varied from moderate to severe. In order of prevalence, the more common pathologies associated with NSRH were gastrointestinal and renal diseases, as well as pneumonia mainly of parasitic or infectious origin.

Keywords: *Liver, Hepatitis, Stray dog, Retrospective case series*

Sokak Köpeklerinde Spesifik Olmayan Reaktif Hepatit Çalışması

Öz

Bu çalışmanın amacı, Endülüs'ün (İspanya) farklı şehirlerinden toplanmış olan barınak köpeklerine ait doku örneklerinde Spesifik Olmayan Reaktif Hepatitin (NSRH) olası nedenlerini araştırmaktı. Hastalık, histolojik olarak, hepatosit nekrozu bulgusu olmadan, karaciğer parankimi boyunca ve portal stroma içinde yayılan lenfositlerin ve plazma hücrelerinin varlığı ile karakterize edildi. İncelenen hayvanlarda, derecesi orta ila şiddetli arasında değişen, spesifik olmayan kronik reaktif hepatit belirlendi. Görülme oranı yönünden değerlendirildiğinde, NSRH ile ilişkili olarak en sık görülen patolojiler gastrointestinal problemler ve böbrek hastalıklarının yanı sıra parazitik veya bulaşıcı kökenli pnömonilerdi.

Anahtar sözcükler: *Karaciğer, Hepatit, Sokak köpeği, Retrospektif olgular*

INTRODUCTION

The liver is a vital organ to investigate in any individual animal, but also to determine the health status of a population of domestic or stray dogs, which may be reservoirs of diseases for humans, domestic and other free-living animals. Liver has important roles in homeostasis, metabolism, catabolism and immune function [1]. Diseases within the hepatobiliary system for any reason could lead to decreased metabolism of toxins and therefore an increased risk of immune suppression or reproductive failure in the animal [2,3].

Inflammation in the liver parenchyma is termed hepatitis and it can be classified as acute or chronic hepatitis [3]. Acute hepatitis is characterized by random distribution of necrosis and apoptosis, with neutrophils accumulate and minimal infiltrations of lymphocytes. This type of hepatitis is usually related to infectious organisms such as bacteria, like *Escherichia coli* that in neonates usually seed the liver via the umbilical veins or less often the portal venous or hepatic arterial systems [3,4]. Chronic hepatitis is the most common form of hepatitis and is characterized by fibrosis, accumulation of mononuclear inflammatory cells, including lymphocytes, macrophages and plasma cells [2-4]. In veterinary medicine, chronic liver disease may develop following chronic bile duct obstruction, infection with hepatotropic infectious agents, familial or hereditary metabolic diseases, or may be toxic, drug-induced, or possibly autoimmune in origin [1-4]. Nonetheless, little information has been reported about this type of pathologies in domestic or stray dogs, among

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which parasitic infestations [5], bacterial infections [6], and viruses, such as those that affect the digestive system have been frequently described [7].

Non-Specific Reactive Hepatitis (NSRH) is another form of hepatitis that has been well described in humans [8], but little is known in domestic or free-living wild mammals [2,9]. NSRH is distributed throughout the liver parenchyma in response to some systemic illness, most often in the gastrointestinal tract or as the residuum of prior liver inflammation. Typically, there is a mild inflammatory infiltrate in the portal tract and without evidence of necrosis [8,9]. NSRH can be further classified into two different types according to the nature of inflammatory infiltrate; when there is an infiltrate of neutrophils within the connective tissue of the portal tracts it is called non-specific reactive active hepatitis; whereas when mononuclear cells, primarily lymphocytes and plasma cells are present it is termed as non-specific chronic reactive hepatitis [4,8].

There are only few studies done on this new entity in veterinary medicine [2,9-11]. The aim of this study was to analyze the histopathological changes and nature of the inflammatory infiltrates in dogs with NSRH and to correlate it with different extrahepatic diseases.

MATERIAL and METHODS

A total of 104 stray dogs that were housed in rescue shelters of Andalucia (Spain) were necropsied in the Faculty of Veterinary Medicine of Cordoba University between 2011 and 2016. They followed standard protocol that included macroscopic and microscopic evaluation of a range of organs, including the liver. The study was conducted with authorization of the Spanish Ministry of Interior (Protocol 2012) and the control of the Ethical Commission of Veterinary Medicine of the University of Las Palmas de Gran Canaria (agreement MV-2017/05).

Of these 104 carcasses, 58 cases were selected due to the evidence of macroscopic or microscopic liver changes noted in the reports. Cases with moderate to severe histologic distortion due to autolysis were eliminated. After review, only 23 cases of different breed, age and sex that fit the selection criteria were included in the study *(Table 1)*. These criteria were used in other studies performed in marine and terrestrial mammals [10-12], and included mild liver enlargement and presence of inflammatory infiltrates in the liver parenchyma. The tissue samples were fixed in 10% neutral-buffered formalin, dehydrated through graded alcohols, and embedded in paraffin wax. Sections (4 µm thick) were cut and stained with haematoxylin and eosin for histopathological examination. The number of infiltrating neutrophils, lymphocytes and plasma cells observed in the liver parenchyma were counted in randomly selected fields at 400x, which also included hepatic sinusoids and portal areas. NSRH was classified as mild (cell counts ranging from 20 to 50 cells per field), moderate (50 to 75 cells per field) or severe (more than 75 cells per field), following the WSAVA classification for liver inflammation [12]. In addition, diagnoses of extrahepatic causes in animals with different NSRH severities were evaluated to see the possible relation with the degree of NSRH diagnosed.

Table 1. *Age, sex and species of dogs included in the study*

Case	Age	Sex	Breed
1	Adult	M	Mixed-breed
2	Young	M	Mixed-breed
3	Young	M	French Bulldog
4	Adult	M	Golden Retriever
5	Young	F	Mixed-breed
6	Adult	F	Yorkshire Terrier
7	Adult	M	Yorkshire Terrier
8	Young	F	Spanish Water Dog
9	Young	F	Mixed-breed
10	Adult	M	Mixed-breed
11	Adult	F	Mixed-breed
12	Adult	F	Boxer
13	Young	M	Canary Mastiff
14	Young	M	Neapolitan Mastiff
15	Adult	M	Mixed-breed
16	Young	M	Mixed-breed
17	Young	M	English Bulldog
18	Young	F	Mixed-breed
19	Adult	F	Mixed-breed
20	Young	F	Mixed-breed
21	Adult	M	Labrador Retriever
22	Adult	M	Mixed-breed
23	Adult	F	Mixed-breed

Statistical Analysis

Variables were summarized as frequencies and percentages in each extrahepatic disease. Chi-squared test ($\chi 2$) with Yates correction was used to compare prevalence between age (adult/young), sex, as well as the degrees (mild, moderate, severe) of the groups in the set of studied diseases. Differences were considered significant at $P<0.05$.

RESULTS

The histopathological findings of the lesions presented in liver were in accordant with the diagnosis of this disease. All animals studied showed non-specific chronic reactive hepatitis. No other hepatic conditions such as hepatic lipidosis or pigment accumulation were seen. Two out of 23 were diagnosed as severe, five out of 23 as moderate and the rest of sixteen animals as mild NSRH. The histologic exam revealed an inflammatory infiltrate

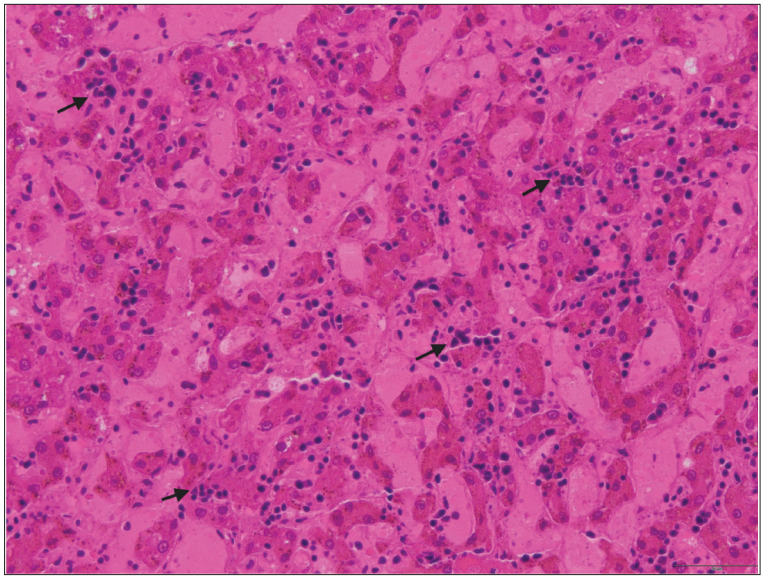
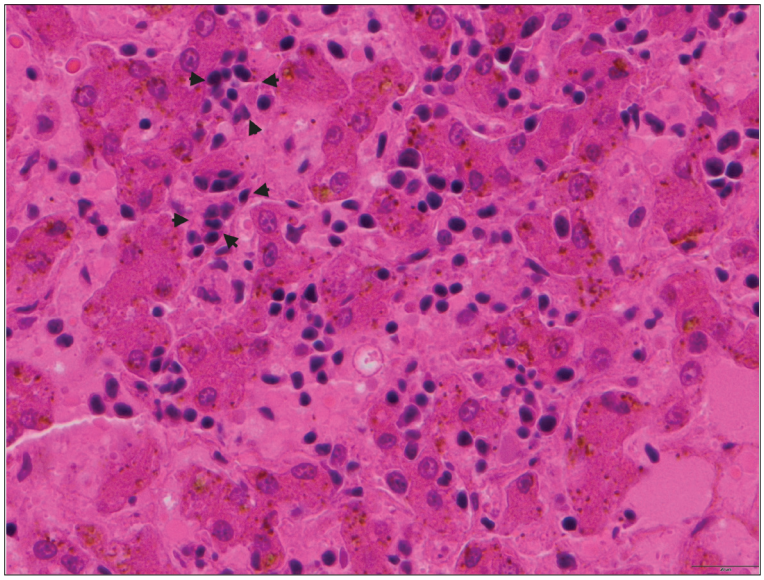


Fig 1. Moderate NSRH showing inflammatory infiltrate in portal spaces and hepatic sinusoids composed by lymphocytes and plasma cells *(arrows),* HE x20

Fig 2. Severe NSRH with inflammatory infiltrate in the hepatic sinusoids, and clusters of lymphocytes and plasma cells *(arrows head),* HE x40



composed of plasma cells and lymphocytes in the portal areas, around the hepatic veins and within the sinusoids, without evidence of hepatocellular necrosis or fibrosis *(Fig. 1)*. Those dogs with severe NSRH also showed marked dilatation of the hepatic sinusoids as a result of increased number of erythrocytes, lymphocytes and plasma cells *(Fig. 2)*.

These stray dogs showed some pre-existing extrahepatic disorders that might have accounted for NSRH *(Table 2)*. Therefore, gastrointestinal diseases caused by parasites and viruses were the most common pathologies observed (10/23, 43.48%). Parasites involved in these processes were *Ascaris* spp. and *Ancylostoma* spp. (3/23, 13.04%), whereas Parvovirus (2/23, 8.7%) was the only virus related to gastrointestinal processes. The remaining five cases of gastrointestinal diseases were inflammatory diseases such as inflammatory bowel disease. Renal disease was the second extrahepatic pathology more commonly found in this study (5/23, 21.74%), and consisted mainly of glomerulonephritis with thickening of the glomerular basement membrane and increased cellularity by proliferation of mesangial cells, and tubulonephritis with predominantly lymphocytic infiltrate associated with tubular degeneration and necrosis. Fibrinosupurative pneumonia produced by *Pasteurella* spp. was also observed in 2/23 dogs. In addition, widespread granulomas due to fungal infection *(Cryptococcus neoformans)* were found in 2/23 animals. Other viruses identified were Adenovirus I (2/23, 8.7%) and *Morbillivirus* (2/23, 8.7%). Pathologies such as meningitis, demodicosis, *Ehrlichia canis* infection, melanoma and cirrhosis were also diagnosed in the dogs of the present study *(Table 2)*.

The statistical analysis revealed no differences between age, sex, and degree of NSRH considering P values significant at $P<0.05$. Gastrointestinal disease was the most prevalent process, with p value of 0.55 when trying to find differences

Table 2. *Samples studied and overall prevalence of extrahepatic diseases in dogs according to age, sex and degree of NSRH*

Extrahepatic Diseases	Age		Sex		Degree			
	Adult (n=12)	Young (n=11)	Male (n=13)	Female (n=10)	Mild (n=16)	Moderate (n=5)	Severe (n=2)	Moderate - Severe (n=7)
Fungal granulomas	8.3% (n=1)		7.7% (n=1)		6.3% (n=1)			
Adenovirus	8.3% (n=1)	9.1% (n=1)	7.7% (n=1)	10% (n=1)		40% (n=2)		28.57% (n=2)
Virus infection		9.1% (n=1)	7.7% (n=1)				50% (n=1)	14.3% (n=1)
Inmunodeficiency		9.1% (n=1)		10% (n=1)	6.3% (n=1)			
Gastrointestinal disease	33.3% (n=4)	54.6% (n=6)	38.5% (n=6)	40% (n=4)	43.7% (n=7)	40% (n=2)	50% (n=1)	42.9% (n=3)
Nematodes	8.3% (n=1)	18.2% (n=2)	15.4% (n=2)	10% (n=1)	12.5% (n=2)	20% (n=1)		14.3% (n=1)
Parvovirus		18.2% (n=2)	7.7% (n=1)	10% (n=1)	12.5% (n=2)			
Others	25% (n=3)	18.2% (n=2)	23.1% (n=3)	20% (n=2)	18.75% (n=3)	20% (n=1)	50% (n=1)	28.57% (n=2)
Renal disease	33.3% (n=4)	9.1% (n=1)	15.4% (n=2)	30% (n=3)	25% (n=4)	20% (n=1)		14.3% (n=1)
Heart failure		9.1% (n=1)	7.7% (n=1)		6.3% (n=1)			
Meningitis		9.1% (n=1)	7.7% (n=1)		6.3% (n=1)			
Foreign body obstruction	8.3% (n=1)		7.7% (n=1)		6.3% (n=1)			
Demodicosis		9.1% (n=1)	7.7% (n=1)		6.3% (n=1)			
Morbillivirus		9.1% (n=1)		10% (n=1)		20% (n=1)		14.3% (n=1)
Pneumonia	8.3% (n=1)	9.1% (n=1)	7.7% (n=1)	10% (n=1)	6.3% (n=1)	20% (n=1)		14.3% (n=1)
Peritonitis		9.1% (n=1)		10% (n=1)		20% (n=1)		14.3% (n=1)
Mycotic tracheobronchitis		9.1% (n=1)		10% (n=1)	6.3% (n=1)			
Ehrlichia canis infection	8.3% (n=1)		7.7% (n=1)				50% (n=1)	14.3% (n=1)
Cirrhosis	8.3% (n=1)		7.7% (n=1)		6.3% (n=1)			
Melanoma	8.3% (n=1)			10% (n=1)	6.3% (n=1)			

according to age, and 0.9 and 0.67 in the case of gender and degree of NSRH, respectively.

DISCUSSION

Histologic features observed in the 23 stray dogs were similar to those described in cetaceans [10,11] and dogs with reactive hepatitis [9]. Thus, NSRH was the only hepatic change observed in the present study. Identical results were described in other studies of the canine liver [9] or in numerous studies of humans with the hepatitis C virus [13]. Although this form of hepatitis is one of the most common forms of hepatopathy, only a few detailed descriptions have been reported [9-11]. Interestingly, this process is often not taken in consideration and misdiagnosed as chronic hepatitis. All these authors describe NSRH as an unspecific reaction of the liver that may result from a variety of causes, such as bacterial endotoxins resulting from sepsis, or a bystander reaction of the liver to any systemic reaction, as well as febrile illnesses and inflammation in the splanchnic bed [2,8,11].

Non-specific chronic reactive hepatitis was the unique form of NSRH detected in these dogs. They were seen in the different age, sex and breed groups of the study population, and often associated with chronic inflammatory lesions

in other organ systems, most commonly gastrointestinal disease (10 cases), renal disease (5 cases) or pneumonia (2 cases). Similar results were seen in other studies performed in dogs [2,9]. Nevertheless, the other form of NSRH termed as non-specific reactive active hepatitis was only observed in cetaceans [11], but not in any of the animals of our study.

The degree of inflammation observed in the dogs of the present study was mild to moderate and the distribution of the inflammatory infiltrates in this organ was diffuse or periportal. Therefore, most of the hepatitis cases had mild portal inflammatory infiltrates (16/23, 69.57%). This pattern was also a common finding in a case series of hepatic lesions in cetaceans stranded on the Canary Islands [10,11], as well as in other studies performed in dogs [9], where they described slight to moderate infiltration of neutrophils in acute cases and plasma cells and lymphocytes in chronic cases [9-11]. In contrast, 2 out of 23 animals of our study showed severe inflammatory infiltrate in the hepatic sinusoids forming clusters. It has been suggested that it could be associated with local or systemic antigen stimulation of the immune system caused by infection or infestation [11]. In other reports done in large number of animals, similar hepatitis was found in combination with other hepatic findings such as lipidosis or peracute hepatitis [1]. Nonetheless, this type of hepatic findings was not found in any animal of our study. Only one animal of 23 showed hepatic cirrhosis that was related to exposure to toxic substances. Moderate (5/23, 21.74%) and severe (2/23, 8.7%) NSRH were often associated with inflammatory and non-inflammatory diseases in other organ systems. Nevertheless, cases with mild inflammatory infiltrates were associated with gastrointestinal and renal disease, pneumonia, and other pathologies like melanoma, dermatitis, *Ehrlichia canis* infection or meningitis. However, other reports associated reactive hepatitis with a primary problem in the heart such as cardiac insufficiency, diseases of the reproductive system as endometritis and endocrine disorders, mainly diabetes mellitus [9].

Studies done in large number of stray dogs have also showed that high number of animals were parasitized with these helminths [5], or similar viruses [7]. Despite this fact, it is interesting to highlight that no information has been described about NSRH in stray dogs, among which parasites [5], bacteria [6], and viruses, such as those found in the digestive tract have been frequently reported [7]. Moreover, recent studies emphasized the relevance to evaluate stray dogs since can have an epidemiological importance in relation to different zoonotic diseases [14] due to they share the same environment with humans and thus can be host or reservoir of a wide variety of zoonosis [15].

The statistical analysis showed no significant differences between age or gender and degree of NSRH in each extrahepatic disease.

In conclusion, the results of the present study pointed that disorders of a wide variety of organs inside and outside the splanchnic bed could be associated with NSRH. Hence, within this collection of 23 cases of liver disease, there was evidence NSRH of variable severity that could be related to different stages of extrahepatic disorders such as parasitic or infectious diseases. The grading system used in this study provided more detailed information than was typically described in the case record and has the added value of uniform designations of lesion classification and severity. Larger studies must be done in order to obtain a better estimation of the relation between NSRH and extrahepatic diseases, as well as to learn about the specific mechanism underlying these phenomena.

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Research Article

Yitirilen Bir Servet: Tiftik Keçisi [1] [2]

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Öz

Anadolu'daki geçmişi çok eskiye dayanan tiftik keçisi, başka ülkelerde de yetiştirilene kadar yüzyıllarca sadece Orta Anadolu'da, özellikle de Ankara ve çevresindeki illerde yetiştirilmiştir. Tiftik keçisinden elde edilen tiftik üretimi de yalnızca bu bölgede yapılmıştır. Ankara Sancağında 1905 yılı sayımında, yaklaşık 1 milyon beş yüz bin baş tiftik keçisi bulunduğu bildirilmiştir. Bu keçilerden elde edilen tiftik "Ankara sofu, çorap, boyun atkısı ve başörtüsü" gibi ürünlerin işlenmesinde kullanılmış ve tiftik sanayii, 16-18. yüzyılları kapsayan dönemde Ankara halkına geniş iş olanakları ve gelir sağlamıştır. Bu araştırma, 1800-1918 yılları arasında tiftik keçisi ihracatı ve tiftik ticaretine ilişkin arşiv kayıtlarının saptanması ve sonuçlarının değerlendirilmesi amacıyla gerçekleştirilmiştir. Konuya ilişkin belgeler Ankara, İstanbul, Konya ve Manisa'da bulunan arşivlerde yapılan araştırmalar sonucu elde edilmiştir. Tiftik keçisinin, 19. yüzyılın ortalarından itibaren çeşitli yollarla ülke dışına çıkarılan damızlıklarla, diğer ülkelerde de yetiştirilmeye başlanması, tiftik ticaretindeki ekonomik kaybın gerek halk ve gerekse Osmanlı yönetimi tarafından yoğun biçimde hissedilmesine neden olmuştur. Tiftiğin okkası 19. yüzyılın sonlarına doğru 50 kuruştan 10 kuruşa kadar gerilemiştir. Bu durum, tiftik keçisi dış satımının çeşitli dönemlerde yasaklanmasına, yıllar süren yazışmalar ve çabaların ardından 1918 yılında "Damızlık Tiftik Keçilerinin Harice Men'i İhraç ve Teksiri Adadı Hakkında Kanunu"nun kabul edilmesine yol açmıştır. Sonuç olarak, tiftik keçisi yetiştiriciliğinin canlandırılması için geç de olsa alınan önlemlere rağmen, beş yüz yıl boyunca Anadolu topraklarında yetiştirilen önemli bir ticaret tekeli yitirilmiştir.

Anahtar sözcükler: *Tiftik keçisi, Tiftik ticareti, Tiftik sanayii, Ankara*

A Lost Wealth: Angora Goat

Abstract

The Angora goat, which has a history deeply rooted in Anatolia, has been raised for centuries exclusively in the middle regions of Anatolia, especially in Ankara and the surrounding cities. The mohair that is produced from the Angora goat, too, has only been produced in this region. It has reported that there were approximately 1.500.000 Angora goats in the Ankara district in 1905 census. The mohair obtained from these goats was used to process Ankara cloth, socks, neck scarves and head scarves, and in the period between the 16th and 18th centuries, the mohair industry provided a wide range of jobs and income to the people of Ankara. This research was carried out in order to determine the records of Angora goats and mohair trade and to evaluate the results between 1800 and 1918. Related documents were obtained from the archives in Ankara, İstanbul, Konya and Manisa. With the expulsion of angora goat breeding to other countries starting from the middle of the 19th century in different ways, the economic loss in mohair trade has been felt intensely by both the people and the Ottoman administration. By the end of the 19th century, the okka of mohair fell from 50 kuruş to 10 kuruş. In this case, the prohibition of the export of Angora goat in various periods; and after many years of correspondence and efforts, in 1918, the Law on the "Prevention of the sale of breeding Angora goats abroad and its improvement" was adopted. As a result, despite the late preventions to revitalize the cultivation of Angora goats, an important source of trade for five hundred years in Anatolia has been lost.

Keywords: *Angora goat, Mohair trade, Mohair industry, Ankara*

GİRİŞ

Tiftik keçilerinin Anadolu'da yetiştirilmesine ait bilinen en eski kayıtlar Hitit dönemine götürülebilmektedir. Bazı Hitit eserleri üzerinde tiftik keçisi figürü bulunması ve tiftiğin dokumacılıkta kullanılmış olması bu durumu ortaya koymaktadır [1]. Batu [2], tiftik keçilerinin 13. yüzyılda Türk göçerler tarafından Ankara civarına getirildiklerini ve iklim

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uygun olduğu için burada geliştiklerini öne sürmektedir. Anadolu'yu 15-17. yüzyıllar arasında dolaşan gezginler, tiftik keçilerinin Kızılırmak ve Sakarya nehirleri arasındaki bölgede yetiştirildiğini bildirmişlerdir. Bu keçilerin tüylerinin ipek gibi ince ve parlak, yapağılarından üretilen kumaşın[1] ise çok zarif olduğunu vurgulamış ve bu sof kumaşın dokunuşu, işlenişi ve ticareti hakkında bilgi vermişlerdir [2-5]. Evliya Çelebi, 1648 yılında gittiği Ankara Vilayetinin sof yeri olduğunu ve bu sofun başka yerde bulunamayacağını belirtmektedir [6]. İngiliz gezgin William James Hamilton 1820'li yıllarda Ankara'da tiftik keçisi ticaretinin çoğunlukla Ermeniler tarafından yürütüldüğü belirtmiş; Beypazarı yöresinin 1845 yılı resmi kayıtlarında, halkın önemli bir kısmının tiftik keçisi yetiştirdiği ve İspanyol merinosuyla melezlenmesi sonucu çok daha fazla verim alındığı vurgulanmıştır [7]. Fransız hükümeti tarafından Anadolu'nun tarihi coğrafyasını incelemek üzere 1833 yılında gönderilen mimar-arkeolog Texier'in, Beypazarı'nda karşılaştığı ticari hareketliliği Avrupa kentlerine benzetmesinin arka planında sof ihracatının bulunduğu ifade edilmektedir. Ayrıca, Batılı şirketlerin bu tarihlerde Ankara'daki irtibat büroları vasıtasıyla tiftik alımı yaptığı bilinmektedir [3,8].

Bahse konu dönemde, tiftik keçisinin uzun, hafif yağlı olan yünlerinden eğrilerek yapılan iplerle dokunan sof kumaştan yapılan kıyafetlerin, gösterişli ve dökümlü olması nedeniyle tercih edildikleri görülmektedir. Tiftik tek başına ya da yünlü ve ipekli kumaşlara katılarak, düğme, ilik, sırma şerit, giysi, aksesuar, peruk, peruka ve postiş imalatı ile iç dekorasyon ve askeri amaçla kullanılmıştır [8,9].

Osmanlı İmparatorluğu, tiftik üretiminde 19. yüzyılın ilk yarısına kadar rakipsiz kalmıştır. Ankara bölgesindeki 1.355 tiftik tezgâhında yapılan üretim ile, her yıl 20 bin ton kumaşın Avrupa'ya ihraç edildiği ifade edilmektedir [10]. Ancak, 1839 yılından sonra İngiltere'de kurulan modern sanayiye dayalı tiftik endüstrisi, tiftiklerin daha iyi değerlendirilmesini sağlamış ve buna paralel olarak Amerika ve Güney Afrika'da modern yöntemlerle yetiştirilen tiftik keçisi sayısında artış görülmüştür. Bu ortamda, çiftçilere tarım yöntemlerinin öğretilmesi ve tiftik keçilerinin ıslahı amacıyla kurulan "Ankara Numune Tarlası" ve "Çoban Mektebi" 1898 yılı sonlarında açılmıştır. Fakat, tiftik piyasasında İngilizler ve Amerikalıların söz sahibi olması; keçi yetiştiriciliğinin kârlı bir iş olmaktan çıkması; halkın ilgisizliği ve kısıtlı kadro ve eğitim kalitesinin düşüklüğü nedeniyle ağıl bölümü 1908'de kapatılarak Ziraat Ameliyat Mektebine dönüştürülmüştür [11]. I. Dünya Savaşı ve dünyadaki ekonomik kriz döneminde tiftik ihracatı tamamen durmuş ve elde edilen tiftiklerin iç piyasada da değerlendirilememesi nedeniyle, tiftik üreticisi büyük bir sıkıntı içine düşmüştür [11,12].

Bu araştırma, beş yüzyıl boyunca Osmanlı İmparatorluğu'nun tekelinde kalan tiftik keçisi yetiştiriciliği ile tiftik sanayindeki öncü konumun kaybedilmesi, bu sektördeki egemenliğin sona ermesi ve Osmanlının bu sektörü yeniden canlandırma çalışmalarını belgelerle incelemek amacıyla gerçekleştirilmiştir.

[1] Ankara sofu, Ankara keçisinden elde edilen tiftikten dokunan bir çeşit kumaştır

MATERYAL ve METOT

Araştırmada, Ankara, İstanbul, Konya ve Manisa'da bulunan T.C. Cumhurbaşkanlığı Devlet Arşivleri Cumhuriyet ve Osmanlı dönemi belgeleri ile Milli kütüphanedeki kaynaklarda, tiftik keçisi ihracatı, tiftik ticareti, Osmanlı'da tiftik keçisi ve tiftiğin durumuna ilişkin belgeler saptanmış ve tasnif edilerek değerlendirilmiştir. Aynı zamanda, kronolojik olarak incelenen arşiv belgelerinin özetlenmesi ile konunun tarihsel panoraması çıkartılmaya çalışılmıştır.

BULGULAR

Araştırmanın kapsadığı yıllara (1800-1918) ilişkin ulaşılabilen ilk belgeler tiftik ipliği vergileri hakkında olup, 1811 yılına tarihlenmektedir. Bu belgelerde[2,3,4] tiftik ipliği ticaretinde "gümrük vergisi" dışında "Resm-i miri" adındaki vergi, 1 okka (1.238 gr) tiftik için İstanbul'da 10, İzmir ve Halep gibi illerde ise dokuz akçe olarak bildirilmiştir. 1865 yılında; İzmirli bir tüccarın "Çengelli" ve "Dehrem" olarak bildirdiği tiftiklerin vergilendirilmesine ilişkin bir belge[5] saptanmıştır. Yine aynı yıla ait bir belgede[6] Bolu, Mudurnu, Gerede, Dörtdivan, Çağa, Mengen ve Viranşehir'deki tiftik keçisi sayısının artması nedeniyle, 69.450 kuruş olan verginin yükseltilmesi gerektiği bildirilmektedir. Ayrıca, tiftik keçisi yetiştirilmesi ve vergilendirme konularında yazışmalar[7] mevcuttur. 1869 yılında; yukarıda adı geçen İzmirli tüccarın "Çengelli" ve "Dehrem" olarak ifade ettiği tiftiklerin, Ankara'nın fenik tiftiğinden daha kalitesiz olmasından dolayı, gümrük vergisi indirimi yapılması talep edilmiştir. 1872 yılında; ticari bir şirketin, tiftik keçilerinin derileri ve değişik kalitede tiftikler için verilen fiyatları içeren yazışmalarında[8], 22 balya tiftik için 4.269 kuruş perakende fiyat önerisi verildiği görülmektedir. 1882 yılında; tiftik ve tiftik keçilerine ilişkin sorunlar ve çözüm önerileri Ziraat Müfettiş-i Evveli Aramyan'ın 8 Nisan 1882 tarihli raporunda[9] detaylı bir şekilde verilmiştir. Söz konusu raporda; "*Osmanlı'da yalnız altı vilayette tiftik keçileri olup bunların en kalitelisi Ankara, Kastamonu ve Bursa vilayetlerinde ve kalanı da Konya, Van ve Sivas vilayetlerinde bulunur. Her birinden birer okka tiftik alınmaktadır. Bu keçiler* ülke dışında *yalnız Ümit Burnunda yetiştirilir, bir milyon olup 1.5 milyon okka tiftik alınmaktadır. Ankara, Kastamonu vilayetleri dahilinde* Çorpa, *Yabanabad, Kıbrısı ve Gerede tiftiği gibi ince ve uzun ve parlak ve beyaz nevi tiftik elde edilememektedir.*" denilerek tiftik

[2] OA., CML.00430.17410.001
[3] BOA., CML.00430.17410.002
[4] BOA., CML00677.27765.001
[5] BOA.A.MKT.DV.00069.00015.002
[6] BOA., A. MKT. MHM.00335.00098.002
[7] BOA., A.MKT.MHM.00335.00098.003
[8] BOA., HR.TO.00558.00126.001
BOA., HR.TO.00558.00126.001
BOA., HR.TO.00558.00125.001
[9] BOA., A.MKT.MHM727/6, S. 2-1, 27 Mart 1298 (8 Nisan 1882)

üretimine ilişkin genel coğrafi bilgiler verilmiştir. Raporun devamında, Avrupalılar tarafından, otuz sene (1850'li yıllar) tiftik tekesi satın alınarak Amerika, Afrika ve Avusturalya'ya ithal edildiği; ancak yalnız Afrika'nın Ümit Burnunda pek çok adi keçi bulunduğu için tiftik tekeleri ile melezleme çalışmalarının kısa sürede başarılı sonuç verdiği vurgulanmıştır. Ümit Burnu'nda Osmanlı'dan alınan 2.800 tekenin bir milyon baş keçiye ulaştığı, tiftik veriminin hayvan başına bir okkadan bir buçuk okkaya çıktığı belirtilmiştir. Osmanlı'dan teke ihracatı yasaklansa bile, Ümit Burnu'nda ulaşılan sayısal büyümenin engellenemeyeceği, aksine ihracata izin verilirse güzel tekeler aranılacağından doğal olarak hayvan sahiplerinin bunları yetiştirip satacağı ve böylece ülkeye para geleceği hususu üzerinde durulmuştur. Ümit Burnu'nda iklim ve arazi koşullarının uygunluğu ve hayvanların iyi beslenme ve bakım şartlarından dolayı hem üretimin arttığı hem de hastalıklardan ölümlerin engellendiği belirtilmiştir. Ayrıca, tiftiğin İngiltere'ye deniz yolu ile gönderilmesinin masrafları azalttığı, bununla birlikte vergi alınmadığı gibi devlet tarafından belirlenen koşullara uygun hayvanlara yirmiden elli liraya kadar teşvik verildiği vurgulanmıştır. Ankara'da tiftik yetiştiriciliğinin daha zor koşullarda daha az kârla yapılabilmesinden dolayı ve özellikle Nisan ayında ödenen vergiler nedeniyle, üreticinin borcunu ödeyebilmek için tiftiğini bazen de hayvanını ucuza elden çıkartmak zorunda kaldığı belirtilmektedir. Söz konusu dönemde tiftik fiyatının düşmesinin nedeninin Ümit Burnu'ndaki hasılatın artmasından kaynaklanmadığı sayısal verilerle *(Tablo 1)* desteklenmektedir.

Raporda, halkın eğitilerek hayvan başına alınan tiftik miktarının yükseltilmesi ile Ümit Burnu'nda üretilen tiftik ile rekabet edilebileceği önerilmektedir. Ayrıca, tiftik keçilerinin bilimsel yöntemlerle bakım beslemelerinin yapılabilmesi ve ıslah edilmesi için Ankara'da 4.000-5.000 dönümlük bir çiftlik içinde çoban okulu kurulması istenmektedir. 1886 yılı; İngiltere sefaretinden gelen yazıda[10], tiftik keçisinin dört asırdır ihraç edildiği ve 1877 yıllında çıkartılan nizamname ile getirilen yasağın yeniden değerlendirilmesi istenmektedir. Buna gerekçe olarak, soğuk geçen kış mevsiminde yalnızca dişi ve kuzularda yüksek oranda kayıp olduğu ve kalan erkeklerin ihraç edilmesinin ya da kesilmesinin gerekliliği bildirilmiştir. Afrika, Amerika ve Asya'da zaten tiftik keçisi yetiştirildiği ve Osmanlı ihraca izin versin ya da vermesin bunların zaten çoğaltılacağı vurgulanmaktadır. Ayrıca, resmi ihracat olmadığında kaçak yollardan bunun yapılacağını ve bir keçinin değeri 2-3 lira iken keçi başına beşten 10 liraya kadar kazanç sağlanacağını belirterek, yasağın kaldırılmasını önermektedir. 1893 yılı; Ticaret ve Nafia Nezareti ile Orman, Maadin ve Ziraat Nezaretinin yazışmalarında[11] "numune tarlasının" 10 bin kuruşa alındığı (fakat sıkıntılar olduğu ve sorumluların bulunamadığı), Ankara'da alınan bu tarlanın satışı ile kurulacak olan "numune ağılının" 220 bin kuruşa mal olacağı ve bir an evvel kurulması için Ziraat Bankası'na başvurulmasının gerektiği belirtilmektedir. 1894 yılı; Çoban mektebi, "numune tarlası" ve "ağılı" için uygun yer seçimi, alımı, komisyon kurulması ve 1882 yılında Komisyon Başkanı Aramyan Efendinin verdiği rapora atıfta bulunulmuş, Ankara'ya bir çeyrek mesafede Çubukabad çayının çevresindeki 500 dönümlük yerin alınması ile daha önce alınan 350 bin dönümlük arazinin de işe yarayacağı bildirilmiştir[12]. Öte yandan aynı yılın sonlarına doğru, 24 Kasım 1894 tarihinde Dâhiliye Nezareti'nden gönderilen yazıda[13] Ankara Vilayeti tiftik ticaretinin daha önce yıllık 600 bin lira iken, Ümit Burnu'nda tiftik keçilerinin yetiştirilmesinden dolayı 15-20 bin lira azaldığı vurgulanmıştır. Ümit Burnu'ndaki hayvanların damızlıklarının Ankara'dan gittiği, beş yıl sonra yeni damızlıklara ihtiyaç duyulduğu için 15-20 yıl ihracatın yasaklanması ve yalnız Ankara'da değil sahil kasabalarında da dikkatli olunmasını isteyen "Şurayı Devlet" kararı bildirilmiştir. 1895 yılı; İngiltere Sefaretinden gönderilen yazıda[14], Ümit Burnu'nda tiftik keçisi yetiştirilmesi sonucunda doğan rekabetten dolayı okkası yarım altın olan tiftiğin 10-12 kuruşa düşmüş olmasına karşın, yeniden damızlık ihtiyacı doğduğu için ihracata izin verilmesi, aksi takdirde bir miktar damızlığın hediye edilmesi istenmektedir. Aynı konu, Ankara Vilayet Gazetesinde[15] yayımlanan köşe yazısında ele alınmıştır. 1896 yılı; Mart ayına ait bir belgede,[16] dış

[10] BOA.HR.TO.00260.00013.001

[11] BOA., BEO.000361.027034.001; BOA., BEO.000361.027034.002
[12] BOA., ŞD.00518.00002.001-002
[13] BOA., DH.MKT.02069.00007.001
[14] BOA., A.MKT. MHM. Nr 727\6,10
[15] Milli Kütüphane 1961 SC 34-36 Sayı 0206
[16] BOA., BEO.000766.057381.002

Tablo 1. *Yıllara göre tiftik üretimi ve fiyatı**

Yıl	Osmanlı'da Üretilen Tiftik (Okka)	Ümit Burnunda Üretilen Tiftik (Okka)	Toplam	Birim Fiyat (Kuruş)
1872	950.000	250.000	1.200.000	16
1873				30**
1874				42
1878	950.000	776.000	1.700.000	38-46
1878				30
1879	784.000	1.270.000	1.900.000	33
1882	-	-	2.150.000	17-20

* BOA., A.MKT.MHM727/6, S. 2-1, 27 Mart 1298 (8 Nisan 1882); ** Tiftiğin kullanımına ilişkin değişiklikler

rekabet yüzünden fiyatların düştüğü, aynı zamanda vergilerden dolayı çiftçinin zor durumda olduğu, hatta tefecilerden para aldıkları, bu nedenle vergilerin Mart ve Nisan ayları yerine Temmuz ayında ve üç taksitte ödenmesinin kabul edilmesi istenmektedir. 1 Eylül 1896 tarihli bir belgede[17] de, Ankara'da numune tarlası kurulması için arazinin hazır olduğu, ancak gerekli binaların yapımı için ödemelerde aksaklıklar yaşandığından bahsedilmektedir. 1899 yılı; 23 Mart 1899 tarihinde Amerikalıların keçi alma talebi ve bununla ilgili izin konusunu içeren bir belge[18] vardır. Bu istek üzerinden çok vakit geçmeden 15 Nisan 1899[19]'de Kıbrıs'ta bulunan tiftik keçilerinin ıslahı için Kıbrıs'a 100 baş keçinin gönderilmesi istenmiş ve keçilerin nakline 1 Mayıs 1899[20] tarihinde müsaade edilmiştir. 1900 yılı; Anadolu'dan Avrupa'ya gönderilen tiftiklerin ıslatılması vb. hileler yapıldığı için Ümit Burnu tiftiklerinin tercih edildiği vurgulanmış, bu konuda önlem alınması[21] istenmiştir. 1901yılı; Konya'daki keçilerin ıslahı için Ankara'dan satın alınacak keçileri konu alan bir belge[22] saptanmıştır. 1904 yılı; 8 Ağustos 1904 yılında Dâhiliye Nezareti, tiftik keçisi sevkinin yasaklanmasına rağmen halen İstanbul'a sürüler halinde sevkin devam ettiği, bununla ilgili önlem alınması gerekliliğine dair bir tezkere[23] göndermiş; bu tezkereye 11 Ağustos'ta[24] sağlıklı, genç ve döl verimine elverişli keçilerin kesilmediği ve sürüde kullanıldığı, hasta olanların da kasaplık olarak kullanıldığı ve özel izin olmadan yurtdışına kesinlikle çıkartılmadığı şeklinde yanıt verilmiştir. 1905 yılına ait sekiz adet belge mevcuttur. Dâhiliye Nezareti'nden, 29 Mart 1905 tarihinde Kastamonu, Bursa, Ankara ve Konya vilayetlerine hitaben yazılan bir yazıda[25], tiftik keçilerinin ihracı yasak olduğu bildirilerek İstanbul'a sevk edilen keçilerin neden ve ne kadar sevk edildiği sorulmuştur. 5 Nisan 1905 tarihli Ankara Vilayeti'nin Dahiliye Nezareti'ne yazdığı cevabi yazıda[26] özetle; "sevk edilen hayvanların damızlık olmadığı ve kışa dayanamayacak yaşlı hayvanların kesim için başka illere gönderildiği, 1904 yılında gelen emre uygun olarak damızlık keçilerin sevkine kesinlikle izin verilmediği ve Ankara Vilayeti ve kazalarında yasağa uyulduğu ve keçilerin 7 yaşından sonra tüyleri bozulduğu için kesildikleri 1.5 milyon Tiftik Keçisi olduğu" bildirilmiştir. Kastamonu Vilayeti'nden ise; "ihracat yasağına" uyulduğu ve keçilerin 7 yaşından sonra tüyleri bozulduğu için kesildikleri bildirilmiştir[27]. Aynı yıldaki diğer belgeler[28,29,30,31] ise yakalanan sürülere ilişkin yazışmalardır. Bir başka belgede[32] ise Ankara Vilayeti ve kazalarında tiftik keçisi sayıları tek tek verilmiş ve vilayette toplamda 1.488.507 adet tiftik keçisi bulunduğu bildirilmiştir. 1906 yılında; elde edilen belgelerde tiftik keçisi ihraç yasağının uygulanmasında zorluk çekildiği ve sorumluların kontrolleri zamanında yapması[33], yapmayanlara ceza verilmesi istenmiştir[34,35]. Aynı yıl kasaplık keçi miktarı 1905 Ağustos-Aralık aylarında Konya, Ankara, Eskişehir, Bolu, Gerede ve Çerkeş'te toplam 20.166[36] olarak bildirilmiştir. Sahrayı Cedid tahaffuzhanesinin yazısında kasaplık keçi sayısı, Ağustos ayına kadar 2.585 olarak bildirilmiştir. 1 Ocak 1906 tarihinde Ziraat Nezaretinin Dâhiliye Nezaretine yazdığı yazıda[35] yasağa rağmen İstanbul'a sürüler halinde sevkin yapıldığı bunların geri gönderilmesinin mümkün olmadığı için yakalanan hayvanların alıkonulması gibi bir ceza uygulanması önerilmiştir. Ayrıca yine aynı yazıda kısırlaştırılmamış erkek keçilerin 4, dişilerin ise 5-6 yaşına kadar tüylerinin nefasetini koruduklarından, bu nedenle kısırlaş-tırılmamış erkek keçilerin ve 5-6 yaşındaki dişi keçilerin İstanbul'a ve diğer vilayetlere gönderilmesinin engellenmesi istenmiştir. 1908 yılı; Avusturya Konsolosluğunun 50 baş tiftik keçisi almak için Hariciye Nezareti ile Ticaret ve Ziraat Nezareti'ne yazdığı yazılar[37,38] görülmektedir. 1909 yılı; Ticaret ve Ziraat Nezareti'nden, damızlık tiftik keçilerinin ihracatının yasaklanmasına ilişkin bir kanun[39] hazırlanması istenmiştir. İzleyen dönemde Avusturya'ya 50, Ümit Burnu'na da 500 baş[43] tiftik keçisinin ihracatına izin verildiğinin duyulması üzerine Ankara'dan[40,41], Bilecik-Eskişehir'den[42], Beypazarı'ndan[43] ve Bursa'dan[44] gelen tepki telgrafları Dâhiliye Nezareti'ne iletilmiştir. Bu yazılarda *"... umum vilayetimiz ahalisinin servet-i umumisini sekteye uğratacağından, değil elli baş, bir baş tekenin ihracına katiyen müsaade edil-memesini umum ahalimiz namına istirham ederiz[42]"* talebi ile *"Tiftik keçisi ihracatı memleket servetinin can damarını kanatacağı[45]"* vurgusu dikkat çekmektedir. Bu telgraflara "*Avusturya Sefareti müracaatı üzerine istisnai surette olmak üzere yavrularıyla beraber yalnız 50 keçinin ihracına izin verilerek irade-i seniyyece müsaade olunmuş, şimdi geri alınması muğayir-i nezaket bir hareket olacağı ve bu miktar keçinin çıkarılmasında mühim bir zarar vermeyeceği"* yanıtı verilmiş ve keçilerin *"bu defalık"* ihracı 24 Ağustos 1909 tarihinde Sadrazam tarafından onaylanmıştır. Bu durum 7 Aralık 1909 tarihinde *"Mecli-si Mebusan"* da yoğun bir şekilde tartışılmıştır[45]. 1910 ve 1911 yılları: 1909 yılında, Avusturya'ya ihraç edilmesine izin verilen keçiler hakkında Avusturya Sefareti, Hariciye ve Dâhiliye Nezaretleri ile ilgili iller arasında yoğun bir yazışma trafiği tespit edilmiştir.

[17] BOA., ŞD.00522.00052.001
[18] BOA., HR.İD.02120.00046.001
[19] BOA., YA.RES.00100.00004.004
[20] BOA., YA.RES.00100.00004.001
[21] BOA., DH.MKT.02351.00038.001
[22] BOA., BEO.001682.126110.001
[23] BOA., DH.MKT.00882.00042.002
[24] BOA., DH.MKT.00882.00042.003
[25] BOA., DH.MKT.00882.00042.007
[26] BOA., DH.MKT.00882.00042.009
[27] BOA., DH.MKT.00882.0042.004
[28] BOA., DH.MKT.00882.00042.010
[29] BOA., DH.MKT.00882.00042.012
[30] BOA., DH.MKT.00882.00042.011
[31] BOA., DH.MKT.00882.0042.013
[32] BOA., DH.MKT.00882.00042.008
[33] BOA., DH.MKT.00882.00042.018
[34] BOA., DH.MKT.00882.00042.023
[35] BOA., DH.MKT.00882.00042.020
[36] BOA., DH.MKT.00882.00042.015
[37] BOA., BEO.003293.246951.001
[38] BOA., H BOA., R.İD.02120.00110.001
[39] BOA., BEO.003689.276610.001
[40] BOA., DH.İD.00104.1.00001.003
[41] BOA., DH.İD.00104.1.00001.002
[42] BOA., DH.İD.00104.1.0001.005
[43] BOA., DH.İD.00104.1.00001.003
[44] BOA., DH. MUİ.00022.2.00029.002
[45] BOA., DH.İD.00104.1.00001.017

1913 yılı; Ekim ayında yapılan yazışmalarda Amerika Birleşik Devletleri Washington sefaretinin[46] Amerika'ya gümrüksüz tiftik keçisi ihraç talebi ve bunun için tiftik keçisi ihracatı yasağının kaldırılması isteği Osmanlı hükûmetince ret edilmiştir[47]. 1914 yılı; Bakanlar Kurulunda Tiftik ihracına izin verilmesi konusu görüşülmüştür[48]. 1918 yılı; 11 Nisan1918 tarihinde Osmanlı Meclis-i Mebûsan'ı tarafından kabul edilen bir yasayla, "*Damızlık Tiftik Keçilerinin Harice Men'i İhraç ve Teksiri Adadı Hakkında Kanun*" kabul edilmiştir[49].

TARTIŞMA ve SONUÇ

Çalışmamızda elde edilen belgeler incelendiğinde, 1882 yılındaki rapora[9] kadar olan kayıtların[4-10] tiftik ve tiftik keçisinden alınacak vergi miktarları ile satış fiyatı hakkında olduğu görülmektedir.

Bu tarihten sonraki belgelerde tiftik keçisi ihracatından doğan sıkıntılar ve ihracatın engellenmesi konusunun öne çıktığı saptanmıştır. Ümit Burnu'nda Tiftik Keçisi yetiştirilmesinin olumsuz ticari sonuçlarının 1800'lü yılların sonundan itibaren hem Anadolu halkı hem de Osmanlı idaresi tarafından şiddetle hissedildiği ve Devletin konuya dair ciddi önlemler almaya çalıştığı anlaşılmaktadır[43-48]. Buna karşın, tiftik keçisi ihracatının serbest bırakılması yönünde yoğun bir siyasi baskı olduğu görülmektedir[13,40,41,49].

Aramyan'ın 8 Nisan 1882 tarihli raporunda[9], Ümit Burnu ile Osmanlı'da tiftik keçisi yetiştiriciliğinin karşılaştırıldığı görülmektedir. Tiftiğin Ümit Burnu'ndan İngiltere'ye deniz yolu ile gönderilmesinin masrafları azalttığı, bununla birlikte vergi alınmadığı gibi devlet tarafından belirlenen koşullara uygun hayvanlara yirmiden elli liraya kadar teşvik verildiği vurgulanmıştır. Buna karşın, Osmanlı'da tiftik yetiştiriciliğinin daha zor koşullarda daha az kârla yapıldığı, aynı zamanda erken alınan vergiler yüzünden[17,51] üreticinin zor durumda kaldığı belirtilmektedir. Raporda üreticinin eğitilebilmesi için *"Çoban Mektebi"* açılması ve bir "*Numune Tarlası"* yapılması önerisinde bulunulmuştur. İzleyen dönemde 1893[12], 1894[13] ve 1894[18] yıllarına ait belgelerde çoban mektebi ve numune ağılı kurulması çalışmalarına dair yazışmaların olması da Aramyan'ın önerilerinin yetkili makamlarca dikkate alındığını göstermektedir. Ancak, çalışmamızda mektebin açılışına ve kapanmasına dair belgelere ulaşılamamıştır. Araştırma bulgularımıza paralel olarak Keskin [11] "*Numune Tarlası"* ve *"Çoban Mektebi"* kuruluş çalışmaları hakkında bilgi vermiş, ancak Aramyan'ın raporundan söz etmemiştir. Tiftik keçisi yetiştiriciliğini geliştirmek amacıyla yapılan bu çalışmaların hedeflenen sonucu sağlamadığı düşünülebilir.

Tiftik keçisinin yurt dışına çıkışına 1860 yılından sonra bazı engellemeler getirilmeye başlandığı [13], İngiliz konsolosluğundan[13] gelen bir yazıda yasaklama tarihi 1877 olarak geçerken, bazı kaynaklarda ise [2,5,9] tiftik keçisinin dış satımının 1881 yılında Padişah fermanıyla yasaklandığı bildirilmektedir. Ancak, arşiv çalışmalarımızda bahsedilen her iki yasaklama belgesine de ulaşılamamıştır.

1886 yılında İngiliz Sefaretinden gelen bir yazıda[11], tiftik keçisi ihracatının açılma talebinin gerekçelerinden biri soğuk geçen kış mevsiminde dişi ve kuzularda görülen ölümler olarak belirtilmiştir. İzleyen dönemlerde kuraklık ve kıtlık nedeniyle 1870 ve 1874-1875 senelerinde tiftik keçisinin soyunun tükenme tehlikesi ile karşı karşıya kalındığı rapor edilmiştir [13]. Bu dönemde yaşanan Ankara keçisi ölümlerinin, uluslararası pazarlarda İngilizlerin öncülüğünde ortaya çıkan yeni rakiplerle yarışta, Osmanlı devletinin elini zayıflattığı ve coğrafi öncülüğe sahip olmanın bu ürün üzerinde rekabet avantajı getirmediği ileri sürülebilir.

Tiftik keçisi ihracatına yasak getiren kanun teklifi[4,12] için 1909 yılında başlayan çalışmalar, 1918 yılında, Osmanlı Meclis-i Mebûsan'ında "*Damızlık Tiftik Keçilerinin Harice Men'i İhraç ve Teksiri Adadı Hakkında Kanun*"nun[52] kabul edilmesiyle sonuçlanmıştır. Ancak, bu süreçte Osmanlı Devleti'ne gerek Avrupa ülkelerinden gerekse Amerika Birleşik Devletleri'nden çok yoğun siyasal baskıların yapıldığı belirlenmiştir. 1909 yılında Tiftik keçisi ihracatındaki maddi kaybın etkisinin yetiştirici tarafından hissedilmeye başlanılmasıyla beraber, Devletin halk tarafından *"protesto"* edildiği ve Meclis-i Mebûsan'da[48] tartışmalar yapıldığı görülmüştür. Özellikle, ihraç edilecek keçilerin sevki konusunda yoğun yazışmaların olduğu belirlenmiştir. Bu durum ihraç yasağının siyasi baskılara karşı gelemeyen Devlet tarafından bizzat ihlal edildiğini göstermektedir.

Müftüoğlu ve Öznacar [12], dünyanın çeşitli bölgelerinde titizlikle yapılan yetiştiriciliğe rağmen, buralarda elde edilen tiftiklerin yumuşaklık ve incelik gibi önemli özellikleri bakımından, Anadolu'da üretilen tiftiklerin seviyesine ulaşamadığını bildirmişlerdir. Aramyan'ın raporunda da[9] bu durum açıkça belirtilmiştir. Utkanlar [14], Prof.Dr. Samuel Aysoy'un bu özelliği Anadolu'daki havanın iyonizasyonuna bağladığını ifade etmektedir. Okaner [15]**:** *"Kap tiftiğinin dayanıklığı ve esnekliği Türk tiftiğine göre azdır. Kap tiftiklerinin rengi sarımtıraktır oysaki Türk tiftiği bembeyazdır. Amerika tiftiğinde ise tiftikte istenmeyen kemp* kılı denilen küçük kıllar Türk tiftiğinden yüksektir. Aynı zamanda 1/3 oranında daha kalındır" demektedir. Bu verilerden hareketle, yetiştirme şartları, basit dokuma tezgâhları gibi tüm olumsuzluklara rağmen tiftik kalitesini etkileyen coğrafi bir üstünlüğün bulunduğu iddia edilebilir.

Osmanlı Devleti'nin, Tiftik keçisinin başka coğrafyalarda yetiştirilmesine kadar, tiftik keçisi ve bundan elde edilen moher (yün) iplik ve onun işlenmesi ile elde edilen sof kumaşı üretimi konusunda bölgesel pazarlarda rakipsiz gelir elde etme avantajını yakaladığı söylenebilir.

[46]BOA., BEO .004224.316734.002
[47] BOA., HR.HMŞ.İŞO.00059.0025.002
[48] BOA., DH.İ.UM.00077.2.00001.05.004
[49] BOA., İ.DUİT.00090/8

Sonuç olarak, tiftik keçisinden elde edilen tiftik, Osmanlıda çok önemli bir ekonomik değer oluşturmuş ve dünyaya Anadolu'dan yayılmıştır. Tiftik keçisi bazen padişahların hediye vermesi, bazen kaçak yollardan ve bazen de siyasal baskılar ile Anadolu topraklarından çıkarılmıştır ve götürüldüğü her yerde ana vatanından çok daha iyi bakım ve beslenme yöntemleri uygulanmasına rağmen, istenilen ürün kalitesi elde edilememiştir. Tiftik keçisinin zirai, ticari, coğrafi ve tarihî yönlerinin yansıra önemli bir kültür mirası olarak da değerlendirilmesi gerektiği ileri sürülebilir.

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Research Article

Investigation of Genetic Structures of Coloured Horses by mtDNA D-loop Sequence Analysis in Turkey [1][2]

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Abstract

The aim of this study was to determine the genetic structure of Coloured horses in Turkey by analysis of the D-loop sequence of mitochondrial DNA (mtDNA). A total of 28 Coloured horses were examined. DNA was extracted from blood samples using DNA isolation kit; 519 bp long mtDNA D-loop region was amplified by PCR and sequenced by capillary electrophoresis system. Population parameters and phylogenetic trees were drawn by using MEGA4 software package. It was also compared with the DNA sequences of horse populations from different countries. In this study, 42 different polymorphism regions and 10 different haplogroups were detected. Additionaly, Tajima D value was found to be -0.31 and population expansion was determined. It was determined that the base differences among the horses ranged between 0.000 and 0.032. It was detected that the horses formed different clusters from each other and they were intertwined with the populations of different countries. Moreover, it was also observed that some horses formed their own populations at different points from the other countries' horses. As a result, it was observed that the genetic structures of horses which used in population were different from each other and they originated from different mothers. According to the results obtained, it is considered that Coloured horses can be a native horse breed of Turkey.

Keywords: *Coloured horse, D-loop region, Haplogroup, Mitochondrial DNA*

Türkiye'deki Alaca Atların mtDNA D-Loop Dizi Analizi İle Genetik Yapılarının Araştırılması

Öz

Bu çalışma, Türkiye'deki Alaca atların mtDNA D-Loop dizi analizi ile genetik yapılarının belirlenmesi amacıyla yapılmıştır. Araştırmada, toplam 28 Alaca at incelenmiştir. Alınan kan örneklerinden DNA izolasyon kiti kullanılarak DNA elde edilmiş ve 519 bp uzunluğundaki mtDNA D-loop bölgesi PZR ile çoğaltılmış ve kapiller elektroforez sisteminde dizileme işlemi yapılmıştır. MEGA4 paket programı kullanılarak populasyon parametreleri ve filogenetik ağaçları çizilmiştir. Ayrıca farklı ülke at populasyonlarına ait DNA dizileri ile de karşılaştırılması yapılmıştır. Araştırmada, 42 farklı polimorfizm bölgesi ve 10 farklı haplogrup elde edilmiştir. Ayrıca Tajima D değeri -0.31 elde edilmiş ve populasyon genişlemesi olduğu belirlenmiştir. Örnekler arasındaki baz faklılıklarının 0.000 ile 0.032 arasında değiştiği belirlenmiştir. Çizilen filogenetik ağaçlar sonucunda örneklerin birbirlerinden farklı kümeler oluşturduğu ve farklı ülke populasyonları ile de iç içe girdiği belirlenmiştir. Ayrıca, bazı örneklerin farklı ülke atlarından tamamen ayrı noktalarda kendi populasyonunu oluşturduğu da gözlenmiştir. Sonuç olarak çalışılan populasyondaki örneklerin genetik yapılarının birbirinden farklı olduğu ve farklı analardan köken aldıkları görülmüştür. Elde edilen sonuçlara göre; Alaca atların Türkiye'ye ait yerli bir ırk olabileceği düşünülmektedir.

Anahtar sözcükler: *Alaca at, D-loop bölgesi, Haplogrup, Mitokondrial DNA*

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INTRODUCTION

In the archaeological and genetic studies, the origins of many species were clearly stated [1]. Although there is no clear information about the time and place where the horses were domesticated, it is reported that they were domesticated in different places at different times [2]. It was stated that Eurasian steppes are belived to be an important domestication center [3]. Different horse populations were also compared in other studies, and it was found that horses were domesticated in different regions since the Iron Age [4].

As a result of the phylogenetic analysis, no relationship could be found between domestication of horses and geographical places where they were bred [5]. It was reported that at least 17 haplotypes of the oldest ancient horse breeds have become extinct in the last 5500 years [4]. In a study carried out in indigenous stallions, which were found in Europe for many years, it was reported that genetic diversity has decreased [6].

Native horse breeds of Turkey are decreasing in terms of number and genetic resources and pure breeding is interrupted as a result of the cross-mating of breeds. For this reason, it is important to take measures for preserving these breeds and to conduct molecular genetic analyses to guide these preservation programs. Due to genetic bottleneck, the population faces the danger of extinction. It is also very important to be able to maintain purebred breeding and to develop economically important traits [7].

It was reported that populations created by using haplotypes obtained from horse studies may be used to classify ancient remains, to assess haplogroup variation in modern breeds, and to evaluate possible roles of horses in race performance [8].

Recently, Y chromosome (for paternal history) and the mitochondrial DNA (mtDNA) (for maternal history) were used in the evolutionary, phylogeographic and genetic diversity studies [9-13]. In a study conducted in the United States, mtDNA sequence analyses were found to be useful in eliminating doubts about the origin of horses, other than biological characteristics, and they may also be used in solving problems based on traditional assumptions about Arabian horses with a close common ancestry [14].

In a study conducted for genetic characterization of different horse breeds in Turkey using D-loop region of mtDNA and establishing a preservation program, it was determined that haplotype diversity was high; however, there was low nuclotide diversity. In addition, no valid genetic separation was detected among the breeds. Moreover, a phylogenetic tree was created using 22 horses representing seven haplogroups previously published and indigenous horses. mtDNA analysis of Turkish horses confirmed that many ancestral mare breeds were involved in the domestication of the horses [15].

In a study conducted using the mitochondrial control region to determine the genetic variation in Ayvacık Pony, Malakan, Hınıs and Canik horses; the haplogroups showed high diversity [16]. As a result of mtDNA sequence analysis of 5 horse breeds in Turkey, 68 polymorphic regions and 151 haplotypes (Haplotype diversity, Hd: 0.9866±0.0017, nucleotide diversity, Pi: 0.021±0.00036, and average nucleotide diversity, k: 8,006) were found [17].

Coloured horses are bred in a limited region especially in Ardahan, Kars and Iğdır provinces and have a small number of population in Turkey. In the literature review, there is no study found to define genetic identification of Coloured horses in Turkey by using the mtDNA D-loop sequence analysis. The aim of this study was to reveal the genetic structure of Coloured horses by analysis of the mtDNA D-loop sequence.

MATERIAL and METHODS

Preliminary Study and Determination of Specimens

In the present study, a preliminary field works were conducted to determine the number and characteristics of the animals as research materials to be investigated. Below are some photographs of Coloured horses taken during preliminary work in the field *(Fig. 1, 2, 3, 4)*.

In the field studies conducted, it was found that the total number of Coloured horses in Turkey is 250-300, that horse owners usually have one or two horses, and that horses frequently change hands among the breeders in the region. In the field works, it was determined that some of the Coloured horses sire or dam were not Coloured, and there could be some challenges in collecting samples and data due to the difficulties in reaching some plateaus where horses are found. In samples collected for the study, attention was paid to the fact that horses were not close relatives.

Fig 1. Coloured horses in Ardahan-Turkey

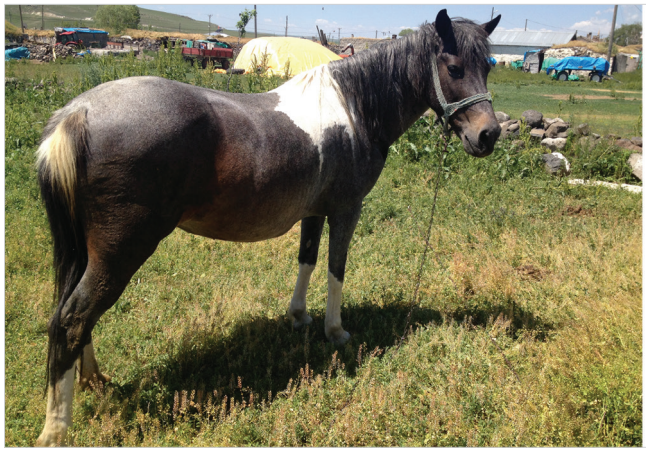

Fig 2. Coloured horse in Kars-Turkey

Fig 3. Coloured horses in Malatya-Turkey

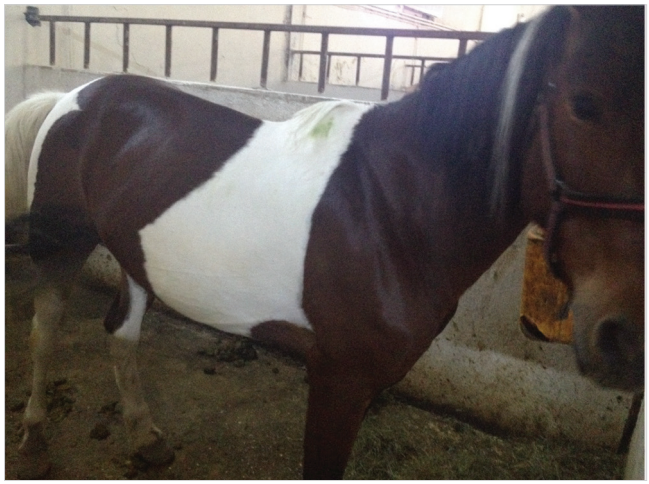

Fig 4. Coloured horse in Erzurum-Turkey

Collection of Blood Samples and Isolation of DNA

Blood was drawn from the *V. jugularis* of 28 Coloured horses (bred in Ardahan, Kars, Iğdır, Erzurum and Malatya provinces) and added into the anticoagulant (K_3EDTA) tubes. Blood samples were kept in the cold chain and delivered to the laboratory. In samples collected for the study, we paid attention to the fact that horses were not close relatives. DNA isolation was performed by using an automated Qiagen Biorobot M48 DNA isolation system and MagAttract DNA Mini M48 kit (Catalog No. 953336).

Amplification and Genotyping of Control Region (D-loop) in Mitochondrial DNA

The control region (D-loop) in mitochondrial DNA (mtDNA) was amplified using the forward primer F7 (5'-CCA TCA ACA CCC AAA GCT GAA-3') and the reverse primer R525 (5'-GTG AGC ATG GGC TGA TTA GTC-3'). Primers were designed using reference sequence (GenBank accession number AF064628.1) with FastPCR Professional 6.2.1 software [18]. A 25 µL PCR mixture consisted of 40 ng DNA, 6 µM of each primer, 2.4 mM $MgCl_2$, 1×PCR buffer, 0.2 mM dNTP mix and 1 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, U.S.A.). Pre-denaturation phase of PCR was programmed to be at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min by 35 cycles and final extension at 72°C for 10 min.

For DNA sequencing analysis, a total of 20 µL reaction mixture was prepared containing 2 µL of Big Dye 3.1, 11 µL of 1X Sequencing Buffer, 5 µL of forward or reverse primer (1 pmol) and 2 µL of PCR product. The PCR was programmed for 30 sec at 96°C for pre-denaturation, 10 sec at 96°C for separation, 15 sec at 50°C for annealing and 4 min at 60°C for extention by 30 cycles. PCR products were cleaned with Bigdye XTherminator and sequencing was performed by Genetic Analyzer (ABI 3500).

Statistical Analysis

As a result of DNA sequencing analysis, D-loop region of mtDNA sequences were edited and assembled using Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). Subsequently, all of sequences were aligned in BioEdit 7.0.9 Sequence Alignment software [19].

The evolutionary relationship between the horses studied was carried out using the UPGMA method [20] with the 1000-iterations Bootstrap test [21]. In addition, D-loop region of mtDNA reference sequences belonging to these populations were obtained from the National Center for Biotechnology Information (NCBI) databases and a phylogenetic tree was drawn in order to examine the relationship among horse populations in different countries. To determine whether the population has undergone mutation or natural selection, Tajima's neutrality test [22] was conducted.

The positions of the polymorphic nucleotides of Coloured horses, the evolutionary relationship among the horses studied and the neutrality test results were analyzed using the Maximum Composite Likelihood method [23] of the MEGA4 software [24]. All positions, including missing data and spaces, were removed from the data set and all sequences were brought to the same size. Analyses

Table 1. *The position of polymorphic nucleotides*

Horse No	15450	15494	15496	15521	15532	15534	15538	15542	15585	15596	15597	15601	15602	15603	15604	15617	15649	15650	15651	15657	15666	15667	15703	15709	15720	15737	15740	15770	15771	15773	15776	15777	15787	15806	15809	15811	15826	15827	15838	15869	15870	15871
1	C	T	A	G	C	C	A	C	G	A	A	T	T	T	G	C	A	A	G	T	G	A	T	C	A	T	A	C	T	A	T	A	A	T	A	C	A	G	C	C	C
2	.	C	G	.	.	T	.	.	.	.	.	.	.	C	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	T	T
3	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	T	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	T	.
4	.	C	G	.	.	T	.	.	A	.	.	.	.	C	A	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	C	T	.
5	T	C	G	.	.	T	.	.	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	T	T
6	.	.	.	.	.	.	G	.	A	.	.	.	.	.	.	T	.	G	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	G	A	.	C	T	.
7	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	T	.	G	.	.	.	G	C	.	.	.	.	.	.	.	.	G	.	C	G	.	.	A	.	C	.	.
8	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.
9	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
10	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	T	.	G	.	.	.	G	C	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.
11	.	C	G	.	.	T	.	.	A	.	.	.	C	C	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.
12	.	.	.	.	.	.	.	T	A	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
13	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.
14	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
15	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	T	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.
16	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
17	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	T	.	.	.	.	.	G	C	.	.	.	.	.	.	.	.	.	G	C	G	.	.	A	.	C	.	.
18	.	.	.	.	.	.	G	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	C	.	.
19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	A	.	C	.	.
20	.	.	.	.	.	.	G	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	C	.	.
21	.	C	G	.	.	T	.	.	A	.	.	.	C	C	A	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	C	T	.
22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	G	A	.	C	T	.
23	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	C	.	.	G	A	.	C	.	.
24	.	.	.	A	-	.	.	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	T	C	G	.	.	.	C	.	.	.	A	.	C	T	.
25	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	C	.	G	.	.	.	C	.	.	.	.	C	.	.	G	A	.	C	.	.
26	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.
27	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	.	.	.	C	.	.	C	.	.	.	G	.	C	.	.	G	.	C	.	T	.	A	.	C	.	.
28	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	.	.	.

were performed over a total of 519 bases. Haplogroups were determined according to the mtDNA terminology reported by Achilli et al.[8].

The Ethics Committee Approval for the study was obtained from Inönü University Experimental Animals Local Ethics Committee (2017/A-30).

RESULTS

In the study, 519 bp of the mtDNA D-loop region for 28 Coloured horses from Turkey were analysed and 42 polymorphic sites were determined *(Table 1)*.

The haplogroups determined according to the polymorphism regions obtained in individuals are shown in *Table 2*. Ten different haplogroups were identified and the frequency of haplogroup N was observed a high (21.43%) in this study.

The evolutionary history was inferred using the UPGMA method *(Fig. 5)*. The total branch length in the evolutionary relationship was measured as 0.1026. It was observed that the horses were genetically separated from each other, but they were clustered in 4 groups, and 1 horse (sample no. 24) was placed alone in a separate place from the other horses.

The relationship between horses used in the study and the horse populations of different countries was also examined *(Fig. 6)*. It was observed that the horses in the population studied were intertwined with very different horse populations, but a certain number of Coloured horse samples were not mixed with other populations and remained in their own populations.

Estimates of evolutionary differentiation and standard errors between the calculated DNA sequences according to the paired comparison of the base differences in the DNA sequences of Coloured horses studied are shown in *Table 3*. It was determined that the base differences between horses ranged from 0.000 to 0.032. The highest base difference was observed between horses 7, 9, 10, 14 and 28 with 5, and between horses 7 and 10 with 11. It was observed that there was no base difference among horses 9, 14 and 28, horses 13, 23 and 25, and between horses 20 and 18, horse 15 and 3, and horse 21 and 4.

Table 2. *Haplogroups identified*

Haplogroups	N	Frequences (%)
A	3	10.71
C	2	7.14
D	1	3.57
E	2	7.14
I	4	14.29
L	5	17.86
M	1	3.57
N	6	21.43
P	3	10.71
Q	1	3.57

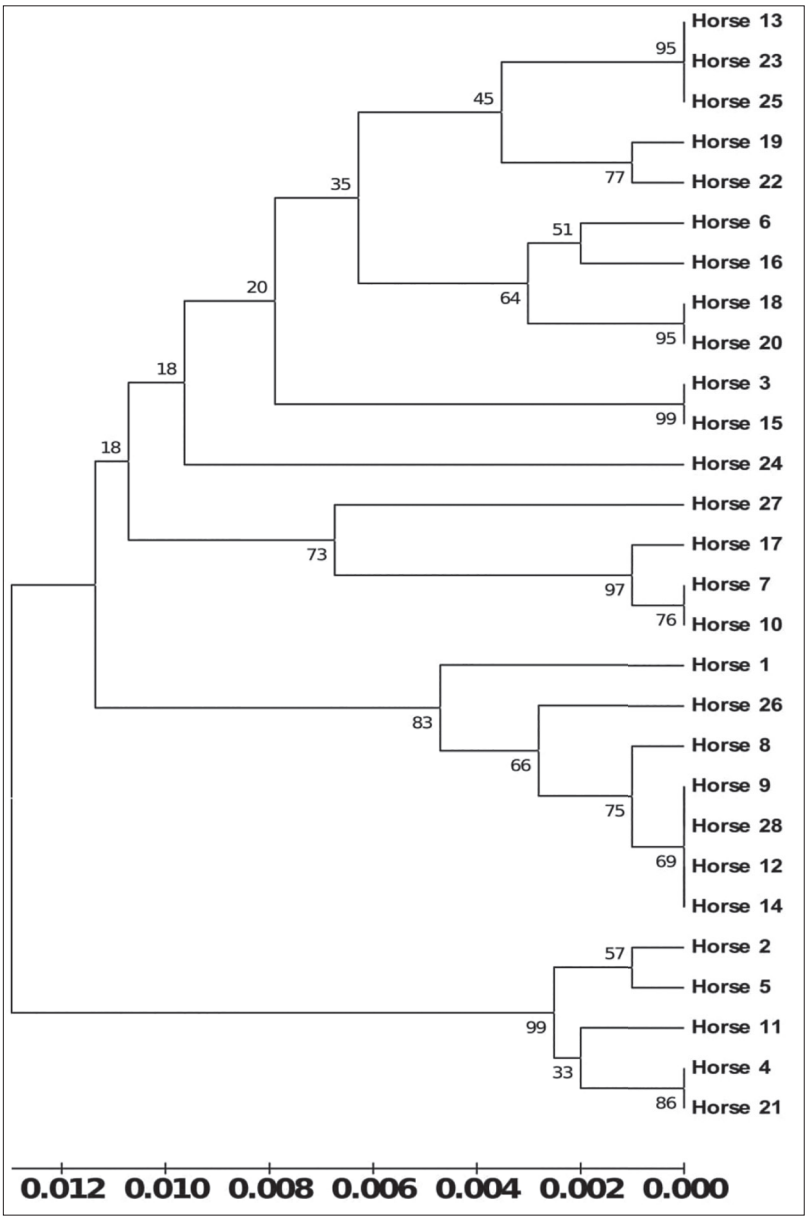

Fig 5. UPGMA dendogram showing the relationships among Coloured horses

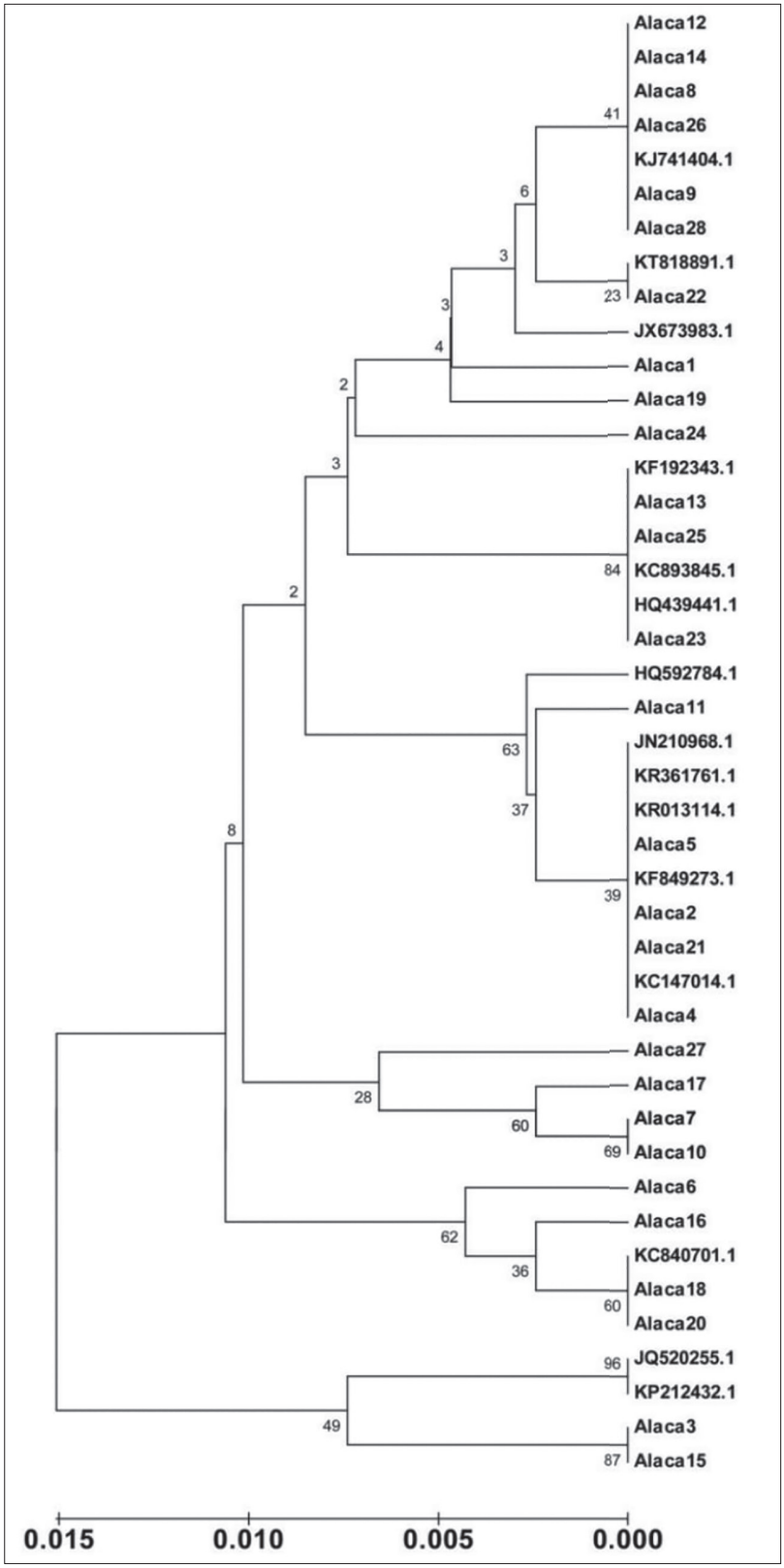

Fig 6. Evolutionary relationships of 45 taxa
Alaca: Coloured Horse; JX673983.1: Ethiopian horse; HQ439441.1: Akal Teke; HQ592784.1: Native Canadian Horse; JN210968.1: Iranian native horse; JQ520255.1: Noriker Horse; KC147014.1: Franches-Montagnes; KF192343.1: Italian Ventasso horse; KC840701.1: Arabian horse; KF849273.1: Vladimir heavy draught; KJ741404.1: Icelandic horse; KC893845.1: Celtic horse; KP212432.1: Draft horse; KR013114.1: Italian Salernitano horse; KR361761.1: Welsh Pony, section A; KT818891.1: Hungarian Gidran horse

Tajima neutrality D test results are shown in *Table 4*. It was determined that the nucleotide diversity was 1.89% and the Tajima D value was -0.31.

DISCUSSION

It is reported that mtDNA represents ancestral genetic diversity in horse populations [4,5]. Studies conducted on native horse breeds reported 23 to 43 different polymorphic regions, 2 to 164 haplotypes and 6 to 14 haplogroups in D-loop region of mtDNA [25-32].

In the studies where samples from many different regions were evaluated together, between 31 to 39 different polymorphic regions and haplogroups ranging from 17 to 68, and 19 to 33 haplotypes were identified [33-35]. In addition, a total of 99 polymorphic regions and 97 haplotypes were found as a result of the entire D-loop of mtDNA sequence analysis of the Arabian horse breed in Middle Eastern countries [36]. Although the number of horses in this study is low unlike other studies, the number of regions showing polymorphism (42) and the number of haplogroups (10) are high and this suggests that these horses may have many ancestral origins.

As a result of mtDNA sequence analysis conducted in 5 native horse breeds of Turkey, 68 polymorphic regions were identified; 54 haplogroups and 151 haplotypes were detected [17]. In this study, although a small number of horses were studied from a single population, similar results were obtained. In the study where many horse breeds of different countries were compared, distance of base differences of Anatolia and Çukurova horse breeds of Turkey was found to be 0.005 [33]. In this study, it was determined that the base differences in the population ranged between 0.000 and 0.032. According to the results obtained, it was observed that the base differences within the same population are larger than the base differences among the different populations. As a result of these findings, it was considered that the Coloured horse population has more different genotypes and may have different ancestral origins.

In a study where 18 different haplogroups were obtained in horses from different continents, haplogroups outside of D and E were reported to be located in the Middle

Table 3. *Estimates of evolutionary differentiation between DNA sequences (vertical triangles) and standard errors (inverse right triangle)*

Horse No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1		0.007	0.006	0.007	0.007	0.006	0.006	0.004	0.004	0.006	0.007	0.005	0.006	0.004	0.006	0.006	0.006	0.005	0.005	0.005	0.007	0.005	0.006	0.007	0.006	0.004	0.006	0.004
2	0.022		0.006	0.002	0.003	0.007	0.008	0.007	0.007	0.008	0.003	0.008	0.007	0.007	0.006	0.006	0.007	0.006	0.006	0.006	0.002	0.005	0.007	0.007	0.007	0.007	0.007	0.007
3	0.018	0.020		0.007	0.007	0.005	0.006	0.006	0.007	0.006	0.007	0.007	0.006	0.007	0.000	0.004	0.006	0.006	0.004	0.006	0.007	0.004	0.006	0.006	0.006	0.006	0.006	0.007
4	0.026	0.004	0.024		0.004	0.006	0.008	0.007	0.007	0.008	0.003	0.008	0.007	0.007	0.007	0.006	0.007	0.006	0.006	0.006	0.000	0.006	0.007	0.007	0.007	0.007	0.007	0.007
5	0.026	0.004	0.024	0.008		0.007	0.008	0.008	0.008	0.008	0.004	0.008	0.007	0.008	0.007	0.007	0.008	0.007	0.007	0.007	0.004	0.006	0.007	0.007	0.007	0.008	0.008	0.008
6	0.022	0.024	0.016	0.024	0.028		0.007	0.006	0.007	0.007	0.007	0.007	0.006	0.007	0.005	0.003	0.007	0.003	0.004	0.003	0.006	0.005	0.006	0.007	0.006	0.006	0.007	0.007
7	0.022	0.028	0.016	0.028	**0.032**	0.023		0.007	0.007	0.000	0.008	0.008	0.006	0.007	0.006	0.006	0.002	0.006	0.006	0.006	0.008	0.006	0.006	0.007	0.006	0.007	0.005	0.007
8	0.008	0.026	0.022	0.026	0.030	0.022	0.026		0.002	0.007	0.007	0.003	0.006	0.002	0.006	0.006	0.007	0.005	0.006	0.005	0.007	0.006	0.006	0.007	0.006	0.003	0.006	0.002
9	0.010	0.028	0.024	0.028	**0.032**	0.024	0.028	0.002		0.007	0.007	0.002	0.006	0.000	0.007	0.007	0.007	0.006	0.006	0.006	0.007	0.006	0.006	0.007	0.006	0.003	0.007	0.000
10	0.022	0.028	0.016	0.028	**0.032**	0.023	0.000	0.026	0.028		0.008	0.008	0.006	0.007	0.006	0.006	0.002	0.006	0.006	0.006	0.008	0.006	0.006	0.007	0.006	0.007	0.005	0.007
11	0.026	0.004	0.024	0.004	0.008	0.024	0.032	0.026	0.028	**0.032**		0.008	0.007	0.007	0.007	0.007	0.008	0.006	0.006	0.006	0.003	0.006	0.007	0.007	0.007	0.007	0.007	0.007
12	0.012	0.030	0.026	0.030	0.030	0.026	0.030	0.004	0.002	0.030	0.030		0.007	0.002	0.007	0.007	0.007	0.006	0.007	0.006	0.008	0.006	0.007	0.008	0.007	0.004	0.007	0.002
13	0.016	0.022	0.018	0.026	0.026	0.018	0.021	0.020	0.022	0.021	0.022	0.024		0.006	0.006	0.005	0.006	0.005	0.004	0.005	0.007	0.003	0.000	0.006	0.000	0.006	0.006	0.006
14	0.010	0.028	0.024	0.028	**0.032**	0.024	0.028	0.002	0.000	0.028	0.028	0.002	0.022		0.007	0.007	0.007	0.006	0.006	0.006	0.007	0.006	0.006	0.007	0.006	0.003	0.007	0.000
15	0.018	0.020	**0.000**	0.024	0.024	0.016	0.016	0.022	0.024	0.016	0.024	0.026	0.018	0.024		0.004	0.006	0.006	0.004	0.006	0.007	0.004	0.006	0.006	0.006	0.006	0.006	0.007
16	0.018	0.020	0.012	0.024	0.024	0.004	0.020	0.022	0.024	0.020	0.024	0.026	0.014	0.024	0.012		0.006	0.003	0.003	0.003	0.006	0.005	0.005	0.006	0.006	0.006	0.006	0.007
17	0.020	0.026	0.018	0.026	0.030	0.025	0.002	0.024	0.026	0.002	0.030	0.028	0.020	0.026	0.018	0.021		0.006	0.006	0.006	0.007	0.006	0.006	0.007	0.006	0.007	0.005	0.007
18	0.016	0.022	0.018	0.022	0.026	0.006	0.021	0.016	0.018	0.021	0.022	0.020	0.012	0.018	0.018	0.006	0.019		0.005	0.000	0.006	0.004	0.005	0.006	0.005	0.005	0.006	0.006
19	0.016	0.018	0.010	0.022	0.022	0.010	0.018	0.020	0.022	0.018	0.022	0.024	0.008	0.022	0.010	0.006	0.020	0.012		0.005	0.006	0.002	0.004	0.005	0.004	0.006	0.006	0.006
20	0.016	0.022	0.018	0.022	0.026	0.006	0.021	0.016	0.018	0.021	0.022	0.020	0.012	0.018	0.018	0.006	0.019	**0.000**	0.012		0.006	0.004	0.005	0.006	0.005	0.005	0.006	0.006
21	0.026	0.004	0.024	**0.000**	0.008	0.024	0.028	0.026	0.028	0.028	0.004	0.030	0.026	0.028	0.024	0.024	0.026	0.022	0.022	0.022		0.006	0.007	0.007	0.007	0.007	0.007	0.007
22	0.014	0.016	0.012	0.020	0.020	0.012	0.020	0.018	0.020	0.020	0.020	0.022	0.006	0.020	0.012	0.008	0.018	0.010	0.002	0.010	0.020		0.003	0.005	0.003	0.006	0.006	0.006
23	0.016	0.022	0.018	0.026	0.026	0.018	0.021	0.020	0.022	0.021	0.022	0.024	0.000	0.022	0.018	0.014	0.020	0.012	0.008	0.012	0.026	0.006		0.006	0.000	0.006	0.006	0.006
24	0.022	0.024	0.020	0.028	0.028	0.024	0.028	0.026	0.028	0.028	0.028	0.030	0.018	0.028	0.020	0.020	0.026	0.022	0.014	0.022	0.028	0.012	0.018		0.006	0.007	0.007	0.007
25	0.016	0.022	0.018	0.026	0.026	0.018	0.021	0.020	0.022	0.021	0.022	0.024	**0.000**	0.022	0.018	0.014	0.020	0.012	0.008	0.012	0.026	0.006	**0.000**	0.018		0.006	0.006	0.006
26	0.008	0.026	0.022	0.026	0.030	0.022	0.026	0.004	0.006	0.026	0.026	0.008	0.020	0.006	0.022	0.022	0.024	0.016	0.020	0.016	0.026	0.018	0.020	0.026	0.020		0.006	0.003
27	0.020	0.026	0.022	0.026	0.030	0.026	0.014	0.024	0.026	0.014	0.030	0.028	0.020	0.026	0.022	0.022	0.012	0.020	0.020	0.020	0.026	0.018	0.020	0.026	0.020	0.024		0.007
28	0.010	0.028	0.024	0.028	**0.032**	0.024	0.028	0.002	**0.000**	0.028	0.028	0.002	0.022	**0.000**	0.024	0.024	0.026	0.018	0.022	0.018	0.028	0.020	0.022	0.028	0.022	0.006	0.026	

Table 4. *Results of Tajima's Neutrality Test*

N	S	P_s	Θ	π	D
28	42	0.080153	0.020597	0.018882	-0.311316

n = Total number of samples; S = Total number of polymorphic regions; p_s = Ratio of bases that show difference; Θ = Rate of mutation in population; π = nucleotide variety; D = Tajima test statistic

East [8]. In the study of mountain horse breeds of Bulgaria, it was reported that mainly Eastern and Western Eurasia and Middle East haplotypes were found [35]. In this study, it was observed that three Coloured horses in D and E haplogroups were found in haplogroups of Northern Europe horses and the horses in other haplogroups were found in haplogroups of Central Asia and the Middle East horses.

There are studies showing that F haplogroup is a haplogroup specific to *E. przewalskii* wild horses [8]. Studies have shown that Arabian horses in the Middle East (Khanshour and Chothran, 2013), Bulgarian horse breeds [35] and native horse breeds in Asia and Caucasia [32,37] do not have F haplogroup. Similar to other studies, this study showed that F haplogroup was not present in Coloured horses in Turkey close to above mention regions. Since horses included in this study are in the haplogroup of horses of Central Asia and the Middle East, similar results were obtained with those horse breeds in these countries.

It was reported that in Kabardey horse breed of North Caucasus, the highest haplogroup rate was observed to be 19% in G haplogroup, and this was followed by L, Q and B haplogroups, approximately 12% [32]. In this study, 24 out of 28 horse samples were collected from Erzurum, Kars and Ardahan provinces under the influence of Caucasus region and there was no G and B haplogroup detected; while L (5 horses, 17.86%) and Q (1 horse, 3.57%) haplogroups were detected. It was considered that because the Coloured horse population in Turkey has the haplogroups of horses of Central Asia and in the Middle East, Coloured horses population may be originated from the Eurasia region centre of domestication.

Phylogenetic trees indicate that populations with similar nucleotide sequences have more recent common ancestry than populations with different nucleotide sequences. As a result of the studies carried out on Asian, European, Middle East and American horse populations, it was detected that while all samples from these countries are divided into 2 groups in phylogenetic tree, they were in mixture with each other [8]. In other studies, it was reported that except one horse breed, the other horse breeds studied were in mixture with each other [25,28,36].

In a study conducted in 5 native horse breeds of Turkey, it was detected that there was no breed specific pylogenetic group and they were in mixture with each other [17]. In this study, although there is only one horse population, the samples were collected from different regions. It was determined that these horses were in mixture with each other and did not show a similar clustering as a single population. It was considered that the native horse breeds in many countries do not form their own groups as in this study, since the domestication regions of horses are different, and that they may have different ancestral origins and uncontrolled mating programs.

When neutrality tests and phylogenetic trees are interpreted, more detailed information can be obtained about the history of populations. In order to determine whether populations have undergone mutation and natural selection, Tajima's neutrality test was used [38,39]. When nucleotide diversity (1.89%) was compared with the number of polymorphic regions (42) in the Coloured horse population used in this study, it was detected that Tajima D has a negative value (-0.31), and that there was a population expansion, albeit small, in the investigated population.

In the studies in which D-loop region of mtDNA was investigated, the nucleotide diversity was found between 1.52 and 2.8% [26,28,31,35,36] and 2.1% in 5 native horse breeds of Turkey [17]. In this study, nucleotide diversity was found to be 1.89%, similar to those of these studies.

In the study, it was found that Korean horse breed was located in the same cluster as the Mongol horse breed, which was reported to be its ancestor [40] and there has been a close genetic relationship between Chinese Mongolian horses and other Mongolian horses [37]. Likewise, it is necessary to carry out additional studies that evaluate these horses and other horse breeds that are likely to be their ancestors and found in the same habitats with the Coloured horse breed.

As a result, according to the study findings, it was understood that the genetic structures of the horses examined are different from each other. Horses originate from different mothers, except a small number of horses. In addition, in this study where the horse population was compared with horse populations from different countries; it was detected that Coloured horses were located at different phylogenetic cluster compared with other horses breeds. This suggests that Coloured horses have had their own genotype characteristics over time. It is necessary to determine whether this horse population is separated from the other native horse breeds of Turkey and horse breeds of nearby countries, and to register them as a different breed.

Competing Interests

The authors declare that they have no conflict of interest.

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Research Article

Combined Use of Laurel Essential Oil and Vacuum Packing to Extend the Shelf-Life of Rainbow Trout *(Oncorhynchus mykiss)* Fillets

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Abstract

In this study, the effect of the combined use of different concentrations of laurel essential oil (1%, 2%) and vacuum packing on the shelf-life of rainbow trout during cold storage was investigated. Laurel essential oil was applied on trout fillets by spraying. In 14 days of storage, the changes in the microbiological (total viable counts, total psychrotrophic bacteria, *Pseudomonas,* lactic acid bacteria, *Enterobacteriaceae,* coliforms), chemical (TVB-N, pH) and sensory quality characteristics were observed. Upon microbiological, chemical and sensory analyses, it was found that 2% laurel essential oil delayed microbial spoilage in vacuum-packed rainbow trout, thereby extending shelf-life for approximately 4 days and enhancing sensory characteristics. Therefore, it has been concluded that the combined use of laurel essential oil and vacuum packing is promising in extending the shelf-life of seafood and may be used in food industry. Further, the use of laurel essential oil as an alternative to synthetic additives may be recommended for providing microbial safety hence extending shelf-life of other meat and meat products.

Keywords: *Rainbow trout, Essential oil, laurel, Shelf-life, vacuum packing*

Gökkuşağı Alabalığı *(Oncorhynchus mykiss)* Filetolarının Raf Ömrünü Uzatmak İçin Defne Esansiyel Yağı ve Vakum Paketlemenin Birlikte Kullanımı

Öz

Bu çalışmada farklı konsantrasyonlarda defne esansiyel yağı (%1, %2) ve vakum paketlemenin kombine kullanımının soğuk muhafaza boyunca gökkuşağı alabalıklarının raf ömrününe etkisi araştırılmıştır. Defne esansiyel yağı, alabalık filetolarına püskürtme şeklinde uygulanmıştır. 14 günlük depolama süresince mikrobiyolojik (toplam bakteri, toplam psikrotrofik bakteri, *Pseudomonas,* laktik asit bakterisi, *Enterobacteriaceae,* koliform), kimyasal (TVB-N, pH) ve duyusal kalite özelliklerindeki değişiklikler izlenmiştir. Yapılan mikrobiyolojik, kimyasal ve duyusal analizler sonucunda %2 defne esansiyel yağının vakum paketlenmiş gökkuşağı alabalığı filetolarında mikrobiyal bozulmayı geciktirerek raf ömrünü yaklaşık olarak 4 gün uzattığı ve duyusal kalite özelliklerini arttırdığı belirlenmiştir. Bu nedenle defne esansiyel yağı ve vakum paketlemenin kombine kullanımının deniz ürünlerinin raf ömrünün uzatılmasında umut verici olduğu ve gıda endüstrisinde kullanılabileceği sonucuna varılmıştır. Ayrıca diğer et ve et ürünlerinin mikrobiyal güvenliğin sağlanması ve dolayısıyla raf ömrünün uzatılması için sentetik katkı maddelerine alternatif olarak defne esansiyel yağının kullanımı önerilebilir.

Anahtar sözcükler: *Gökkuşağı alabalığı, Esansiyel yağ, Defne, Raf ömrü, Vakum paketleme*

INTRODUCTION

Global fish production peaked at about 171 million tonnes in 2016. With relatively static capture fishery production, decreased wastage and increase in aquaculture, 88% of the total fish production was for direct human consumption. This production reached a record level in 2016 with a consumption rate of 20.3 kg per capita [1].

Fish and fish products are globally appreciated throughout the world due to their high nutritional value and delicious taste. Therefore, it is very important in the international fishing industry to ensure the safety of edible fish [2]. However, due to microbiological growth and lipid oxidation, fish and other seafood are among the most rapidly perishable food products. Combined with consumers' interest in natural products that are free of chemical preservatives,

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it is quite difficult to provide safe and high-quality seafood. Therefore, there has been a growing interest in the antioxidant and antimicrobial effects of natural preservatives such as essential oils as an alternative to synthetic additives to enhance the oxidative and microbial stability of food and to increase shelf-life [3].

Most consumers demand food without artificial and harmful chemicals including those used as antimicrobial substances and preservatives. Therefore, there has been a growing interest in more natural and non-synthesized antimicrobial substances as an alternative to antimicrobial substances conventionally used for extending shelf-life of food and struggling against foodborne pathogens. Aromatic plants and their components have been researched as potential preventors of bacterial growth, and the majority of such properties have been attributed to essential oils and other secondary plant metabolites. Essential oils obtained from different sources are commonly recommended due to their potential antimicrobial properties [4].

Bay laurel *(Laurus nobilis)* essential oil (EO) has been acknowledged to have a broad range of potential application areas including health and food, as an antiseptic, antidiarrheal, antimycotic, antimicrobial, antiinflammatory, antioxidant and anticarcinogenic substance [5]. Microbial spoilage is very fast and easy in fresh and slightly processed food products such as fish and meat. There has been a growing demand for natural products as an alternative to synthetic food additives [6]. Preservation methods employing natural products have become a focus of interest for researches, thus many studies have been conducted especially to extend the shelf-life of fish and other seafood [6-11].

Vacuum packing involves placing a product into a film with low permeability, removing the air from the package and applying a hermetic seal. It has been shown that vacuum packing extends the shelf-life of food for six days or longer. On the other hand, although rancidity does not develop due to extended period of storage, undesired smell and taste due to bacterial activity may occur [12]. Therefore, this study investigates the potential use of laurel EO in varying concentrations (1%, 2%) for extending the shelf-life of vacuum-packed rainbow trout.

MATERIAL and METHODS

Plant Material

Laurel leaves were freshly collected at Samandağ district, Hatay, Turkey and dried. The dried laurel leaves were ground in a mechanical grinder for extracting the EO. The laurel oil was obtained by performing water steam distillation on ground leaves in a Clevenger apparatus (Wisd-Wise Therm). For this aim, 50 g grinded sample was placed in a round bottom flask and 500 mL distilled water was added, which was then placed in the Clevenger apparatus. The EO obtained after a 3-h distillation process was preserved in closed dark colored bottles at 4°C until use in the tests.

Determination of Volatile Components

Gas Chromatography-Mass Spectrometer (GC-MS) Analysis was conducted in East Anatolia High Technology Application and Research Center, Atatürk University. The analysis of the EO was performed using a Thermo-Finnigan Trace GC/ Trace DSQ /A1300 (EI quadrapole) (Thermo-Finnigan, San Jose, CA) equipped with an SGE-BPX5 MS capillary column (Scientific Instrument Services Inc., Ringoes, NJ) (30 m x 0.25 mm i.d., 0.25 ím). For GC-MS detection, an electron ionization system with an ionization energy of 70 eV was used. Helium was the carrier gas, at a flow rate of 1 mL/min. Injector and MS transfer line temperatures were set at 220 and 290°C, respectively. The program used was 50-150°C at a rate of 3°C/min, held isothermal for 10 min, and finally raised to 250°C at 10°C/min. Diluted samples (1/100, v/v, in methylene chloride) of 1.0 microliter were injected and in the splitless mode. The components were identified on the basis of the comparison of their relative retention time and mass spectra with those of standards, Wiley7N, TRLIB library data of the GC-MS system, and literature data [13]. The quantitative data were expressed as area percent. The results were also confirmed by comparison of the compounds elution order with their relative retention indices on nonpolar phases reported in the literature [13].

Sampling and Packing

Rainbow trout fillets each weighing approximately 100 g were supplied from a local sales center in Kars. The samples were brought into the laboratory under cold chain conditions and immediately taken into cold storage in a cooled incubator and the packing process was initiated immediately. A total of 3 testing groups were formed in this study. In order to form the control group, the fillets were packed in a vacuum machine (Henkelman, mini Jumbo, Istanbul-Turkey) in polyamide film bags (BC Vakum Ambalaj, Istanbul) without any treatment. For the laurel group, 5 mL laurel EO in concentrations of 1% and 2% in distilled water with 0.2% Tween 80 was applied on the fillets by spraying, which were then vacuum packed in the same way as the control group. The entirety of the samples was stored at 4°C, and the microbiological, chemical and sensory analyses were conducted on days 0, 2, 4, 6, 8, 10, 12, 14 of cold storage.

Microbiological Analysis

For microbiological analyses, 10 g of each sample was collected and weighed under aseptic conditions and transferred into stomacher bags, 90 mL sterile physiologic saline solution was added and homogenized in the stomacher for 2 min. After preparing the decimal dilutions of the samples, the inoculation was made to microorganism-specific media by pour plate and streak plate methods.

For total viable counts, plate count agar (Oxoid CM 325) was incubated at 30°C for 48 h; for total psychrotrophic bacteria, plate count agar (Oxoid CM 325) was incubated at 7°C for 10 days; for *Pseudomonas* spp, pseudomonas agar base (Oxoid CM 559) and C-F-C supplement (Oxoid SR 103) were incubated at 30°C for 48 h; and for lactic acid bacteria (LAB), man rogosa sharpe agar (Oxoid CM 361) was incubated at 30°C for 3-5 days; for *Enterobacteriaceae*, violet red bile glucose agar (Oxoid CM 485) was incubated at 35°C for 48 h; for coliform group bacteria, violet red bile lactose agar (Oxoid CM 107) was incubated at 37°C for 24 h; and for fecal coliform group bacteria, violet red bile lactose agar (Oxoid CM 107) was incubated at 44.5°C for 24-48 h [14].

Chemical Analysis

Determination of Total Volatile Basic Nitrogen (TVB-N): Determination of TVB-N was performed according to the method of Antonacopoulos [15]. For this aim, approximately 10 g homogenized sample was weighed in 0.1 mg sensitive analytical balance and transferred into the tubes of the Kjeldahl apparatus, and approximately 1 g magnesium oxide and 100 mL distilled water were added. In an Erlenmeyer flask, 10 mL 3% boric acid and eight drops of methyl-red were added, then completed with an average of 100 mL distilled water and placed on the distillate collection section of the distillation unit. The distillation was continued until 200 mL liquid accumulated. Then, the distillate collected was titrated with 0.1N hydrochloric acid until color change.

pH Measurement: 10 g fish sample of each group was weighed and homogenized with 100 mL distilled water. The pH value of the homogenizate was measured by a digital pH meter (Thermo-Orion 3 Star).

Sensory Analysis

The sensory assessment of the fish fillets was performed by 4 academics at the Kafkas University, Department of Food Hygiene and Production, and the Department of Food Engineering in the Faculty of Engineering and Architecture. The sensory analysis was performed on days 0, 2, 4, 6, 8, 10, 12 and 14 of storage by modifying *(Table 1)* the Quality Index Method (QIM) recommended by Bonilla et al.[16]. In the sensory analysis table, 0 indicates very fresh fish whereas gradually increasing values indicate spoilage based on the period of storage.

Statistical Analysis

The tests were repeated three times and the averages were calculated and transformed into the $\log_{10}$ base. The data obtained was examined by one-way analysis of variance (ANOVA). In the evaluation of the differences among the groups, the Least Significant Difference (LSD) test was used. The statistical analyses were performed in the SPSS 20 software package.

Table 1. *Quality index method scheme*

Quality Parameter			
Skin	Brightness	Bright, shining	0
		Slightly dull	1
		Dull	2
	Mucus	Thin, transparent	0
		Slightly thick, dull	1
		Thick, yellowish	2
Meat	Texture	Firm	0
		Slightly soft	1
		Very soft	2
	Blood	Bright red, none	0
		Pale red, dull	1
		Clouded, brown	2
	Smell	Fresh	0
		Seaweed	1
		Sour milk	2
		Acetic /Ammonia	3
		Laurel oil smell	Yes/No
	Color	White, greyish	0
		Slightly yellowish, slightly pinkish	1
		Yellow, completely pink	2
	Brightness	Transparent	0
		Dull	1
		Milky	2
	Breakdown status	No breakdown	0
		Slightly broken down but intact	1
		Slightly broken down, not completely intact	2
		Intensely broken down	3

RESULTS

The GC-MS analysis of laurel EO revealed 92.98% of the components. The main components of laurel EO were determined as 1,8-cineole (62.36%), a-terpinyl acetate (10.54%) and sabinene (8.44%). *Table 2* shows the main components of laurel EO as analyzed by GC-MS.

The results of the microbiological analyses conducted at different days of cold storage on control (vacuum-packed group), 1% and 2% laurel EO-treated vacuum-packed samples are shown in *Fig. 1a,b,c,d,e,f*. In the control group samples, the initial total viable count (day 0) was determined as 4.14 log cfu/g, and in the samples involving 1% and 2% laurel oil, it was determined as 4.07 and 3.50 log cfu/g, respectively. On day 14 of cold storage, the total viable count in the control samples and 1% and 2% laurel oil-involving samples were 9.98, 9.93 and 7.83 log cfu/g, respectively. It was determined that there was no

significant difference between the control and 1% laurel oil group (P>0.05), however there was a significant difference with the 2% laurel oil group (P<0.05).

The initial (day 0) total psychrotrophic bacteria count in the control, 1% and 2% laurel oil groups were determined as 4.10, 4.06 and 3.36 log cfu/g, respectively (P>0.05). On day 14 of cold storage, the total psychrotrophic bacteria count of the control, 1% and 2% laurel oil-involving samples was determined to be 9.87, 9.74 and 7.64 log cfu/g, respectively. It was determined that there was no significant difference between the control and 1% laurel oil group (P>0.05), however there was a significant difference with the 2% laurel oil group (P<0.05). The initial (day 0) *Pseudomonas spp* count in control, 1% and 2% laurel oil groups were determined as 4.09, 4.01 and 3.26 log cfu/g, respectively (P>0.05). On day 14 of cold storage, the total *Pseudomonas* bacteria count of the control, 1% and 2% laurel oil-involving samples was determined as 9.04, 8.98 and 7.56 log cfu/g, respectively. A statistically significant difference was determined between the control and the 2% laurel oil group (P<0.05). The initial (day 0) LAB count in the control, 1% and 2% laurel oil groups were determined as 3.35, 3.13 and 2.70 log cfu/g, respectively (P>0.05). On day 14 of cold storage, this number was 6.42 log cfu/g in the control group whereas in the 2% laurel oil group, it was determined as 4.80 log cfu/g. The initial (day 0) *Enterobacteriaceae* count in the control, 1% and 2% laurel oil groups were determined as 1.43, 1.10 and 0.77 log cfu/g, respectively (P>0.05). On day 14 of cold storage, this number was 7.60 log cfu/g in the control group whereas in the 2% laurel oil group, it was determined as 6.37 log cfu/g. It was determined that there was no significant difference between the control and 1% laurel oil groups (P>0.05), however there was a significant difference with the 2% laurel oil group (P<0.05). The initial (day 0) coliform count in the control, 1% and 2% laurel oil groups were determined as 2.50, 1.89 and 0.76 log cfu/g, respectively (P>0.05). On day 14 of cold storage, this number was 7.35

Table 2. *Main components of laurel EO analyzed by GC-MS*

Retention Time	Compounds	%
10.13	a-pinene	2.84
11.83	Sabinene	8.44
12.02	b-pinene	2.25
14.64	1,8-Cineole	62.36
17.84	linalool	3.73
21.54	Terpinen-4-ol	2.82
29.10	a-terpinyl acetate	10.54
	Others	7.02

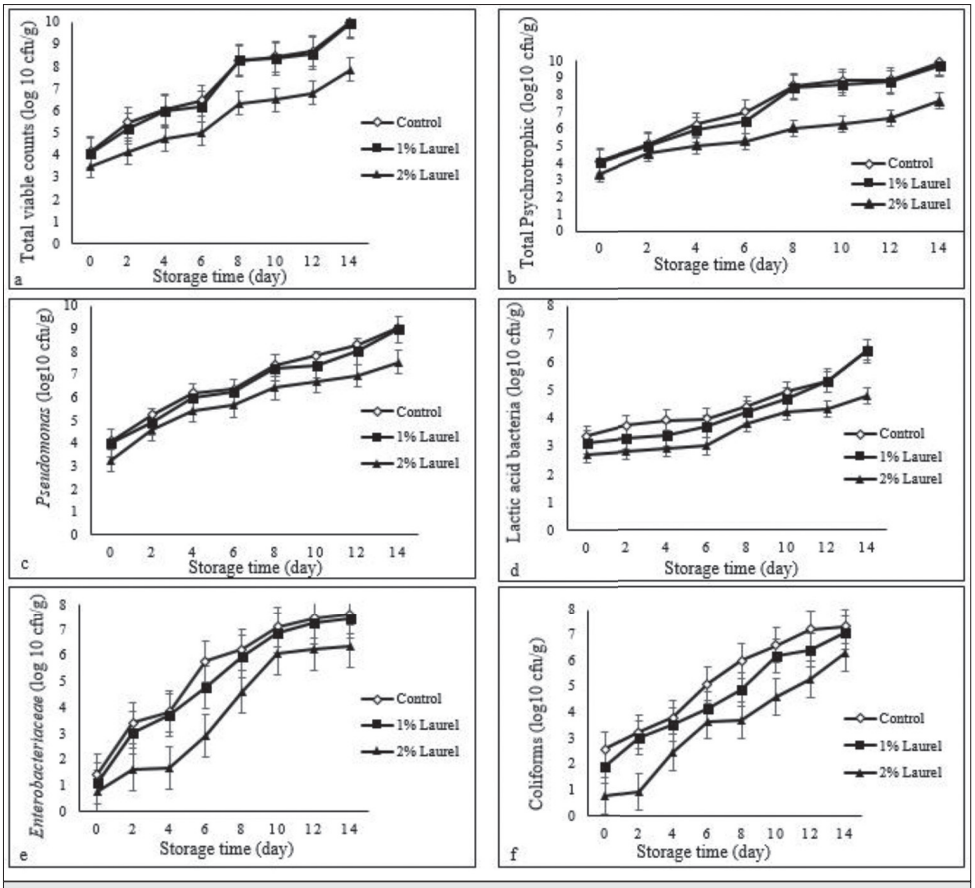

Fig 1. Total viable (a), Total psychrotrophic bacteria (b), *Pseudomonas* (c), Lactic acid bacteria (d), *Enterobacteriaceae* (e), Coliform bacteria (f) counts of rainbow trout fillets treated with various concentrations of laurel EO during cold storage

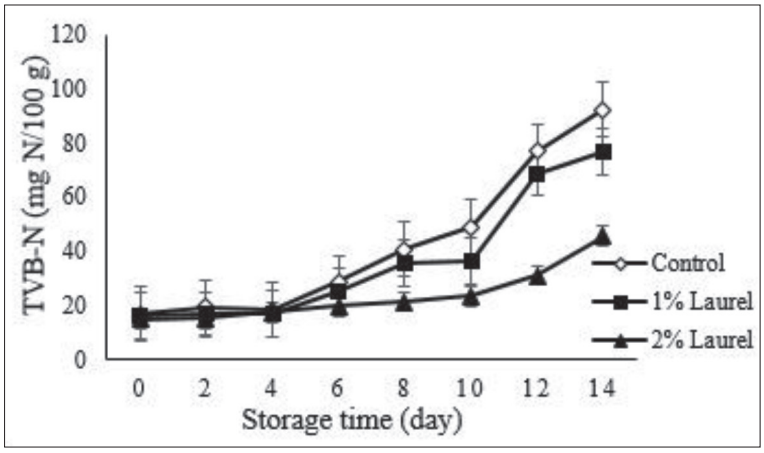

Fig 2. TVB-N values of rainbow trout fillets treated with various concentrations of laurel EO during cold storage

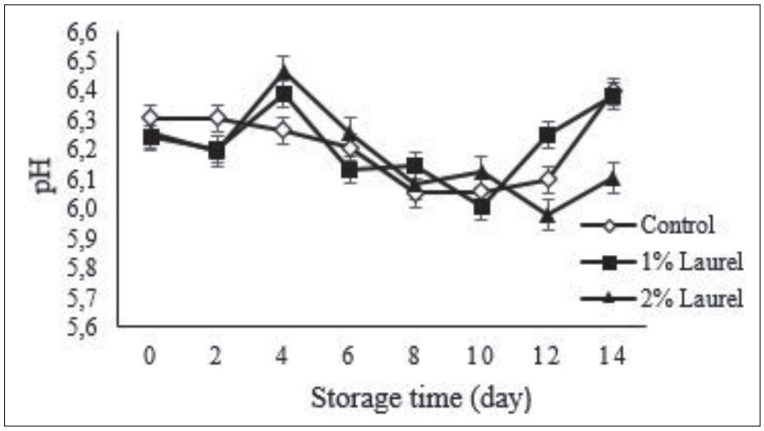

Fig 3. pH values of rainbow trout fillets treated with various concentrations of laurel EO during cold storagege

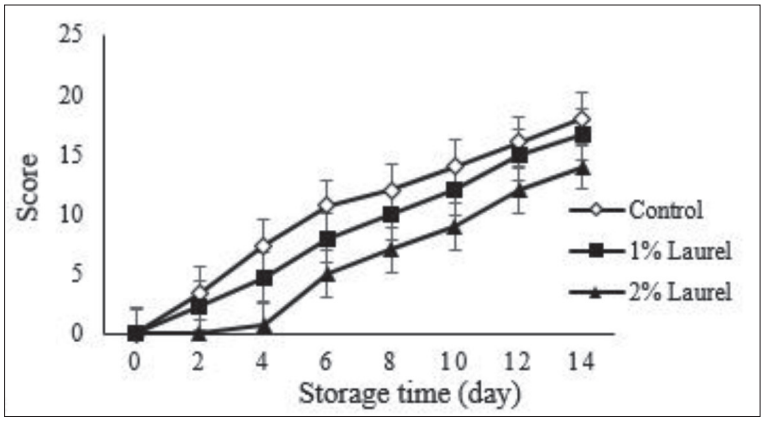

Fig 4. Change in sensory quality scores of rainbow trout fillets treated with various concentrations of laurel EO during cold storage

log cfu/g in the control group whereas in the 2% laurel oil group, it was determined as 6.30 log cfu/g. A statistically significant difference was determined among the groups (P<0.05). In none of the control, 1% or 2% laurel oil groups, fecal coliform group microorganisms were isolated.

It was observed that the TVB-N value increased in the control, 1% and 2% laurel oil groups throughout the period of storage. The initial (day 0) TVB-N value in control, 1% and 2% laurel oil groups were 16.6, 16.3, 14.9 mg/100 g, respectively, and there was no statistically significant difference among the groups (P>0.05). On day 14 of cold storage, this number was 92.1 mg/100 g in the control group whereas in the 2% laurel oil group, it was determined as 45.5 mg/100 g, and a statistically significant difference was detected between these values (P<0.05). *Fig. 2* shows the TVB-N values measured for the samples throughout cold storage.

It was determined that there was no statistically significant difference among the pH values of the control, 1% and 2% laurel oil groups at the initial (day 0), however on day 14 of cold storage, there was a statistically significant difference among the groups (P<0.05). *Fig. 3* shows the pH values of the samples as measured throughout cold storage.

It was determined that the sensory score of the rainbow trout fillets increased depending on the period of cold storage. On day 14 of storage, a statistically significant difference among the groups was determined (P<0.05). *Fig. 4* shows the scores of the change in the sensory quality of the rainbow trout fillets during cold storage.

DISCUSSION

In this study, the effect of various concentrations of laurel EO on the shelf-life of vacuum-packed rainbow trout fillets was investigated. The composition of the EO obtained from laurel leaves was determined by GC-MS. Many factors including the genotype of plant varieties, seasonality, geographical and weather conditions highly affect the composition of an EO [10]. In this study, the main components consisted of 1,8-cineole (62.36%), a-terpinyl acetate (10.54%) and sabinene (8.44%). These results are partially compatible with the results of previous studies [10,17-20].

In rainbow trout fillets, the initial total viable count was 4.14 log cfu/g in the control group whereas this number increased in all groups during storage. Many other researchers previously obtained similar results [10,21-24]. The maximum permissible level for freshwater fish is 7 log cfu/g [25]. In the control and 1% laurel group, this level was surpassed on day 8 of storage and the panelists also indicated that the samples had spoiled in terms of the sensory aspects. As for the 2% laurel group, it was

determined that the limit of 7 log cfu/g was surpassed on day 14. Zhang et al.[23] reported that in vacuum-packed (VP) carp fillets, the limit of 7 log cfu/g was surpassed on day 8. This study also revealed similar findings. The initial psychrotrophic bacteria count was determined as 4.10 log cfu/g in the control group. It was determined that the control and 1% laurel group surpassed the limit of 7 log cfu/g on day 8 of storage whereas in the 2% laurel group, the limit was surpassed on day 14. Özpolat et al.[26] reported that the initial psychrophile count was 3.08 log cfu/g, and there was an increase in the total psychrophile bacteria count based on the period of storage. In this study, the initial psychrotrophic bacteria count was 4.10 log cfu/g and similarly, an increase was determined in the number of bacteria during the period of storage. The initial *Pseudomonas* count was 4.09 log cfu/g in the control group which continuously increased throughout storage. As for the 2% laurel group, it was determined that throughout the period of storage, the *Pseudomonas* count had been significantly lower compared to the control group ($P<0.05$). Zhang et al.[27] reported that in vacuum-packed carp fish samples containing 0.1% cinnamon EO, the control group (vacuum-packed) showed a *Pseudomonas* initial count of 3.5 log cfu/g whereas in the samples containing 0.1% cinnamon EO, it was 2.8 log cfu/g. In this study, the control group had an initial *Pseudomonas* count of 4.09 log cfu/g whereas in the 2% laurel group, it was determined as 3.26 log cfu/g. In line with the aforementioned study, it was observed that the EO enabled a reduction in the initial *Pseudomonas* count. In their study which examined the effect of chitosan, thyme oil or their combinations on the shelf-life of smoked eel fillets stored under vacuum packaging (VP) at 4°C, El-Obeid et al.[8] reported that in the treated samples, the *Pseudomonas* count had been lower throughout the period of storage. This study also produced similar findings. When the control group and the 2% laurel group are compared in terms of the LAB count, it is observed that there had been a significant difference between the groups throughout storage ($P<0.05$). Zhang et al.[27] reported that during the period of storage, there was no significant difference in terms of LAB count between the VP samples and VC samples (0.1% cinnamon EO + vacuum packing). It is considered that this difference may result from factors such as the EO used, the concentration of the EO, initial microbial flora and manner of application. In this study, we have observed a very slow and gradual increase in the LAB count. Zhang et al.[23] reported that in vacuum-packed carp fillets, unlike *Pseudomonas,* the LAB count slowly increased reaching 7.74 log cfu/g after 12 days of storage. The results of this study are compatible with the aforementioned study. The *Enterobacteriaceae* count considered as the hygiene criteria in food, increased throughout the period of cold storage. The control group showed a gradual increase whereas in the 2% group, the *Enterobacteriaceae* count was found to be very low until day 8 of cold storage. Vilela et al.[28] reported that in vacuum-packed samples at 2°C, the *Enterobacteriaceae* count was generally lower in the samples of the group treated with laurel EO. Similarly, this study also determined that laurel EO was quite effective on both the initial and final *Enterobacteriaceae* count. Thus, our results are compatible with the aforementioned study. Özoğul et al.[9] in a study investigating the effect of nanoemulsions containing rosemary, laurel, thyme and sage EO on the quality characteristics of rainbow trout, reported that the initial *Enterobacteriaceae* count was 2.27 log cfu/g which increased depending on the period of storage in all groups and especially in the control group. In another study, Özoğul et al.[7] reported that the initial *Enterobacteriaceae* count was determined as 2.28 log cfu/g in the control group, which reached 5.92 log cfu/g in the group treated with laurel extract at the end of the storage, and that laurel and myrtle extracts reduced microbial development in fish. This study is also in conformity with both of the aforementioned studies. The number of microorganisms in the coliform group also revealed an increase in similarity with the *Enterobacteriaceae* count, based on the period of storage. da Silveira et al.[10] reported that in fresh sausage, the application of laurel leaves EO in concentrations of 0.05 and 0.1 g/100 g significantly reduced the total coliform population and extended the shelf-life of the product for 2 days. This study also determined that 1% and 2% laurel EO enabled a significant reduction in the initial count and that the coliform count had been lower than the control group until the last day of storage.

TVB-N is produced by the degradation of proteins and non-protein nitrogenous compounds as a result of microbial activity and used as a fish spoilage indicator [9,29,30]. The maximum permissible level of TVB-N in fish and fishing products is 35 mg N/100 g [8,31,32]. The initial (day 0) TVB-N value was 16.6 mg/100 g in the control group and it was determined that this value increased in all groups throughout the storage. The researchers also obtained similar results [9,21]. On day 14 of cold storage, the TVB-N value was determined as 45.5 mg/100 g in the 2% laurel group. Özoğul et al.[9] determined the TVB-N value as 44.91 mg/100 g in the laurel group on day 24 of storage, and reported that when the samples were rejected upon microbiological assessment, the TVB-N values had been lower than the limit in all samples. As for the present study, unlike the aforementioned study, it was determined that the TVB-N value had exceeded the limit when the samples surpassed the microbial limits and were rejected by the panelists in terms of sensory aspects.

In rainbow trout fillets, the initial (day 0) pH value was determined as 6.31 in the control group. There were various rates of decrease or increase in the pH values based on the period of storage, however on the final day of storage, all groups showed a slight increase in the pH value. Our findings have been compatible with those of Erkan et al.[30].

According to the results of the sensory analysis, the panelists reported that on day 8 of cold storage, the control

and 1% laurel groups were spoiled whereas the 2% laurel oil group was rejected on day 14 in sensory terms. The panelists informed that at the initial of storage (day 0) the smell of laurel oil was intense which eventually decreased during the storage period and the smell had become pleasant. The control group received the highest score through the period of cold storage whereas the 2% laurel oil group received the lowest score. It was determined that the findings of the microbial, chemical and sensory analyses were parallel. Özoğul et al.[9] reported an increase in the sensory scores of control and treatment groups throughout the period of storage and indicated that the shelf-life of rainbow trout reached 14 days for control and 17 days for treatment groups. In this study, the period was 8 days for the control and 1% laurel groups, whereas in the 2% laurel group, it was 14 days. Erkan et al.[30] reported that 1% thyme and laurel EO addition increased the shelf-life of fresh bluefish for about 3-4 days. Zhang et al.[27] reported that cinnamon EO extended the shelf-life of vacuum-packed carp fillets for approximately 2 days. Vilela et al.[28] reported that laurel EO had a significant effect on the preservation of color and shelf-life of fresh minced meat. In this study, it was determined that 1% laurel EO had a similar effect with that of the control group, hence did not extend shelf-life. On the other hand, 2% laurel EO was quite effective on microbial spoilage and extended shelf-life for approximately 4 days. The differences between these studies are considered to originate from the manner of application of EO.

The results reveal that the combination of vacuum packing and 2% laurel EO delays microbial spoilage in rainbow trout fillets and extends shelf-life for approximately 4 days. It has also been revealed that it enhances sensory quality characteristics, and particularly creates a pleasant smell and is preferable. It has been concluded that laurel EO may be used in extending the shelf-life of seafood due to its positive effects on microbial, chemical and sensory quality. Further, it may constitute an alternative to synthetic food additives for enabling the microbial safety of other meat and meat products as well as stability in sensory properties.

Conflict of Interest

The authors declare no conflict of interest.

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Research Article

Serum Cu, Mn and Zn Levels and Oxidative Stress in Cattle Performing Tongue-playing [1]

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Abstract

This study was aimed at determining serum copper (Cu), manganese (Mn) and zinc (Zn) levels and the occurrence of oxidative stress in cattle performing tongue-playing stereotypies. The study material comprised of 25 healthy cattle and 50 stereotypic cattle performing tongue-playing, which were of a varying age and sex composition. The stereotypic animals were observed to display non-physiological tongue movements either by raising their heads and stretching their tongue out at long or short time intervals, or by contorting, rolling or swinging their tongue and making circular tongue movements in their mouth. These animals were confirmed to perform tongue-playing stereotypies. 10 mL blood samples were taken from each animal included in the study, and the sera extracted were analysed for serum Cu, Mn, Zn, malondialdehyde (MDA), nitric oxide (NO), total oxidant status (TOS) and total antioxidant status (TAS) levels. While the serum Mn levels of the stereotypic animals were lower than those of the healthy animals ($P<0.001$), it was observed that the serum Cu and Zn levels of the stereotypic and healthy animals did not differ ($P>0.05$). While the MDA ($P<0.001$), NO ($P<0.001$) and TOS ($P<0.05$) levels of the stereotypic animals were found to be higher than those of the healthy animals, the TAS ($P>0.05$) levels did not differ between the two groups. In result, it was concluded that Mn deficiency has an important role in the aetiology of tongue-playing stereotypies, and that this behavioural disorder is associated with oxidative damage or inflammation. Oxidative stress in tongue playing disease is thought to be formed due to cellular damage or stress.

Keywords: *Cattle, Copper, Manganese, Zinc, Tongue-playing, Malondialdehyde, Nitric oxide Total oxidant status, Total antioxidant status*

Dil Oynatma Hastalığı Olan Sığırlarda Serum Cu, Mn, Zn Seviyeleri ve Oksidatif Stres

Öz

Bu çalışma dil oynatma hastalığı olan hayvanlarda bakır (Cu), mangan (Mn), çinko (Zn) seviyeleri ve oksidatif stres gelişip gelişmediğinin belirlenmesi amacıyla yapılmıştır. Çalışmanın materyali farklı yaş ve cinsiyetlerde 50 dil oynatma hastalığı ve 25 sağlıklı sığırdan oluşturuldu. Çalışmaya alınan hasta hayvanların başlarını yukarıya kaldırarak uzun veya kısa aralıklarla dillerini ağızlarından çıkararak kıvırma, döndürme, sallama ve halka oluşturma gibi fizyolojik olmayan dil hareketleri yaptıkları görüldü ve klinik olarak dil oynatma hastalığı tanısı konuldu. Çalışmaya dahil edilen tüm hayvanlardan 10 mL kan alınarak serumları ayrıştırıldıktan sonra serum Cu, Mn, Zn, malondialdehit (MDA), nitrik oksit (NO), total oksidan durum (TOS) ve total antioksidan durum (TAS) belirlendi. Hasta hayvanların serum Mn değerleri sağlıklı hayvanlara göre düşük olduğu ($P<0.001$), Zn ve Cu değerlerinde ise fark olmadığı ($P>0.05$) belirlendi. Sağlıklı hayvanların NO ($P<0.001$), MDA ($P<0.001$) ve TOS ($P<0.05$) değerleri sağlıklı hayvanlara göre yüksek bulunurken TAS ($P>0.05$) değerinde fark olmadığı belirlendi. Sonuç olarak dil oynatma hastalığının etiyolojisinde Mn eksikliğinin önemli rol oynadığı, oksidatif hasar veya yangı geliştiği belirlendi. Dil oynatma hastalığında meydana gelen oksidatif stresin hücresel hasar veya strese bağlı olarak şekillendiği düşünüldü.

Anahtar sözcükler: *Sığır, Bakır, Mangan, Çinko, Dil oynatma, Malondialdehit, Nitrik oksit Total oksidan durum, Total antioksidan durum*

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INTRODUCTION

Tongue-playing is a behavioural disorder characterized by the display of non-physiological tongue movements such as the rolling, contorting and swinging out of the mouth of the tongue, in the form of recurrent episodes. Observed particularly in fast-developing high-yield breeds, tongue-playing is encountered in Turkey and many other countries across the world [1].

Although the exact causes of this disorder remain unknown, reports indicate that stress-inducing feeding and management conditions, play and mimicry instincts, genetics, and the deficiency of certain trace elements such as copper (Cu), cobalt (Co) and manganese (Mn) can be involved in its aetiology [1-3]. Cu, Zinc (Zn) and Mn, which are trace minerals, are important for organism [4,5]. Although found at very low levels in the body, trace elements are essential to the continuity of life and several physiological processes, including growth, development and reproduction [1-3].

Normally, in the body, there is an equilibrium between the antioxidant defence system and the generation of lipid peroxidation-inducing free oxygen radicals, which is referred to as the oxidative balance [6-8]. The disrupt of this balance between oxidants and antioxidants in favour of the former is described as oxidative stress [9-11]. On human and animal diseases oxidative stress plays a significant role [12]. In such cases, the antioxidant defence system fails to neutralize oxidative stress, which results in cellular damage and the loss of cell functions [8]. Parameters commonly used for the assessment of oxidative stress associated with disorders and diseases include serum malondialdehyde (MDA), nitric oxide (NO) and total oxidant status (TOS) levels. On the other hand, the antioxidant status is assessed by means of the measurement of total antioxidant status (TAS) levels [13]. To our knowledge there is no report about oxidative stress and steorotypic behaviour disorders.

This study was aimed at determining serum Cu, Mn and Zn levels and the occurrence of oxidative stress in cattle performing tongue-playing stereotypies.

MATERIAL and METHODS

Animal Material

The study was conducted after receiving approval from the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK, Investigation code: 2017/020, No 2017/02). Fifty stereotypic (Group I) and 25 healthy (Group II) cattle, including 11 males and 39 females with an age ranging from 6 months to 7 years, all of which were raised under similar management and feeding conditions, constituted the study material. In Group I, 4 of the male animals and 5 of the female animals were of the Native Black breed, whilst the remainder were of the Simmental breed. Group II included 10 Native Black and 15 Simmental cattle of both sexes, which were 1-5 years old. The tongue-playing disorder was diagnosed on the basis of the clinical symptoms displayed by the stereotypic animals.

Collection of Samples

Ten-mL jugular blood samples were taken from the animals included in Groups I and II. These samples were centrifuged at 3000 rpm for 10 min for the extraction of sera. The serum samples were stored at -20°C until the biochemical analyses were performed.

Biochemical Analyzes

The extracted sera were used for the measurement of total NO levels with a colorimetric method based on the Griess reaction [14], the measurement of MDA levels with a method based on thiobarbituric acid (TBA) reactivity [15]. Serum TAS and TOS levels were detected using a commercial Rel Assay Diagnostics kit (Turkey) according to the manufacturer's instructions.

The serum Cu, Mn and Zn levels were measured with the aid of a Perkin Elmer AAS-800 (USA) atomic absorption analyser. Prior to the analyses, all equipment was treated with 10% HNO_3, washed with ultra-distilled water and dried. Four intermediate standard solutions were prepared from the stock solution (1.000 µg/mL). Four work standard solutions were also prepared from the stock solution. When employing atomic absorption spectrometry (AAS), a hallow cathode lamp was used for each element. Once the current strength and light path of the lamp, the energy, aspiration period, reading period and air type (air, acetylene) were adjusted, the air compressor was started. All other calibration was done such that the confidence interval of the standards was 0.99500-1.00000 and the calibration coefficient (C.V.) was 99.5% [16].

Statistical Analysis

Statistical evaluation of the results was done using SPSS® (SPSS 20, IL, USA) software. The statistical differences between the groups were evaluated with the t-test. In the statistical evaluation, $P<0.05$ was considered to be statistically significant.

RESULTS

It was observed that the animals included in Group I raised their heads, stretched their tongue out at either long or short intervals, and displayed non-physiological tongue movements, including contorting, rolling, swinging and circular movements. The animals displayed these tongue movements for a period ranging from 5 to 45 min. Some animals were reported to display continuous tongue-playing, which resulted in reduced feed consumption and retarded growth. Furthermore, on-farm observations revealed that

Table 1. *Serum Cu, Mn, Zn, NO, MDA, TAS and TOS levels of the stereotypic and healthy animals*

Parameters	Stereotypic Animals X±SE (n=50)	Control Animals X±SE (n=25)	P Value
Cu (ppb)	374.73±31.08	337.9±24.46	P>0.05
Mn (ppb)	0.17±0.01	0.69±0.04	P<0.001
Zn (ppm)	0.76±0.019	0.77±0.025	P>0.05
NO (nmol/mL)	31.85±0.76	17.78±0.83	P<0.001
MDA (µmol/L)	21.37±0.56	9.86±0.59	P<0.001
TAS (mmol Trolox Eqv/L)	1.55±0.27	1.62±0.06	P>0.05
TOS (µmol H_2O_2 Eqv/L)	660.31±10.51	596.40±22.91	P<0.05

1-2 animals in each barn displayed tongue-playing stereotypies.

The results of the biochemical analyses of the serum samples pertaining to the stereotypic and control animals are presented in *Table 1*. The serum Zn, Cu and Mn levels and the NO, MDA, TAS and TOS levels of the two groups were statistically compared. The comparison of the two groups for trace elements showed that, while the serum Mn levels of the stereotypic animals were lower than those of the healthy animals (P<0.001), the serum Zn and Cu levels did not differ between the two groups (P>0.05). Furthermore, the comparison of the groups for the oxidative parameters demonstrated that, while the NO (P<0.001), MDA (P<0.001) and TOS (P<0.05) levels of the stereotypic animals were higher than those of the healthy animals, no difference was observed between the two groups for the TAS levels (P>0.05).

DISCUSSION

Tongue-playing, a common behavioural disorder encountered in Turkey and many other countries across the world, is clinically characterized by non-physiological tongue movements, including contorting, rolling, swinging and circular movements, observed in the form of recurrent episodes. This disorder is generally more common in fast-developing high-yield cattle breeds [1,2]. In the present study, in agreement with the clinical symptoms previously described for the disorder, the stereotypic animals were observed to raise their heads, stretch their tongue out at either long or short time intervals, and contort, roll, swing or circulate their tongue. It was informed that this disorder resulted in reduced feed consumption and retarded growth. While the majority of the stereotypic animals were of the fast-developing Simmental breed, a few Native Black cattle were also observed to display tongue-playing stereotypies.

The exact cause of tongue-playing remains unknown, yet it is suggested that it could be a habitual disorder related to the play and mimicry instincts of animals [1-3] On the other hand, in this study, as only 1 or 2 cases were observed among animals raised in the same barn, the play and mimicry instincts were considered not to be involved in the aetiology of the disorder.

Of trace elements, Cu is known to play a significant role in skin, hair, bone, nervous tissue and myocardial development, as well as in reproduction, growth, hair pigmentation, the immune system, and the oxidation system of tissues [17]. Furthermore, Cu serves as a co-factor of several enzymes, including cytochrome c oxidase, ceruloplasmin, lysyl oxidase, peptidylglycine alpha-monooxygenase, tyrosinase, superoxide dismutase and dopamine beta-mono-oxygenase, and also acts in cell respiration [1,18-20]. Also, it has been reported that Cu deficiency is involved in the aetiology of tongue-playing [1,2]. On the contrary, in the present study, it was determined that the difference between the serum Cu levels of the stereotypic and control animals was statistically insignificant (P>0.05). Accordingly, it was concluded that Cu had no role in the aetiology of tongue-playing.

Manganese is a trace element, which is particularly important for the maturation of the bone tissue matrix as well as for bone structure development in young animals. Mn acts as the activator of several enzymes, including kinases, decarboxylases and hydrases, and is also a component of metalloenzymes. It also contributes to lipid and carbohydrate metabolism, cell functions and the building of the cell membrane. Mn is also known to influence brain functions and the immune system [21-24]. Furthermore, Mn deficiency is reported to play an important role in the aetiology of tongue-playing [1-3]. In agreement with these reports, the present study demonstrated that the serum Mn levels of the stereotypic animals were lower than those of the control animals (P<0.001), thus, it was concluded that Mn deficiency was involved in the aetiology of tongue-playing.

Another trace element, Zn is essential to the body. It is not only found in the structure of several major metalloenzymes, including carbonic anhydrase, alkaline phosphatase, RNA and DNA polymerase, and alcohol dehydrogenase, but is also involved in the functions of these enzymes [24,25]. Therefore, the deficiency of this trace element adversely affects the functions of many internal organs and body systems. A previous study carried out in

cattle performing tongue-playing stereotypies showed that there was no difference between the serum Zn levels of the stereotypic and healthy animals [17]. Similarly, in the present study, it was determined that Zn was not involved in the aetiology of tongue-playing.

Excess production of reactive oxygen species causes oxidative stress [26]. In the event of such an imbalance, the antioxidant system fails to prevent oxidative stress, and cell damage occurs, which hinders cell functions [7,8,13,27]. Plasma MDA level can be used as an indicator of degenerations in cellular membranes which can be used as indicators of oxidative stress [13,28,29]. Two of the most common parameters used for the assessment of oxidative stress associated with diseases and disorders are serum MDA and TOS levels. The most common parameter measured to determine the antioxidant status is TAS [13]. In the present study, the comparison of the serum TAS levels of the stereotypic animals with those of the control animals revealed no statistically significant difference to exist ($P>0.05$), whilst the serum MDA ($P<0.001$) and TOS ($P<0.05$) levels of the stereotypic animals having been found to be higher than those of the healthy animals demonstrated the presence of oxidative stress in the animals displaying tongue-playing stereotypies.

Nitric oxide is the end product of the conversion of arginine into citrulline by nitric oxide synthase, an NADPH-dependent enzyme [30]. NO, which contains an unpaired electron, is a very strong free radical and causes nitrosative damage to many biomolecules. Furthermore, NO also shows effect as a strong vasodilator. In addition, NO also has neurotransmitter, immunomodulatory, and cytotoxic effects, the last being exhibited against substances harmful to the body [31]. The excessive production of NO is claimed to cause neural defects and to affect impulse transmission [32]. At the same time, NO is a free radical and is involved in several physiological and pathological processes. Bacterial endotoxins, protozoa and bacterial antigens lead to NO production by macrophages and show cytotoxic effect. A similar mechanism is also valid for tumour cells. Therefore, NO is considered to be a component of non-specific immunity [30,33-35]. In the present study, the serum NO levels of the stereotypic animals were found to be higher than those of the healthy animals ($P<0.001$). This suggested that the tongue-playing disorder could be associated with the development of inflammation.

In result, it was determined that Mn deficiency has an important role in the aetiology of tongue-playing. Furthermore, the serum TAS levels of the two groups not differing from each other, and the MDA and TOS levels of the stereotypic animals having been determined to be higher than those of the healthy animals demonstrated oxidative stress to have developed in the animals performing tongue-playing stereotypies. On the other hand, the serum NO levels of the stereotypic animals having been observed to be higher than those of the healthy animals suggested inflammation to have also developed in the animals performing tongue-playing stereotypies. On the basis of these results, it was ascertained that tongue-playing was associated with oxidative stress and inflammation. It is considered that further research is required to elucidate the underlying reasons of the cellular damage and stress associated with tongue-playing.

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Research Article

Effect of Sugammadex and Rocuronium Combination on Cranial Neurotoxicity in Rats: Experimental Study

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Abstract

Sugammadex is frequently used in clinical anesthetic practice. In order to determine whether sugammadex has neurotoxic effect, histopathological evaluation and antioxidant status of brain and cerebellum tissues were investigated. Thirty-two adult sprague dawley rats were divided into four groups. Purine Control Group (Group 1, n=8) had no action. The control group (Group 2, n=8) received 16 mg/kg serum physiologic, the sugammadex group (Group 3, n=8) received 16 mg/kg sugammadex and the sugammadex+rocuronium group (Group 4, n=8) received 16 mg/kg sugammadex and 1 mg/kg rocuronium. After drug administration, brain and cerebellum tissues were evaluated histological and biochemically for and oxidative damage. The overall structure of the brain and cerebellum tissues were normal in all groups. Images of the layers and the neurons contained there in were normal. No pathological findings were found. There had no statistically significant difference between the groups in terms of glutathione enzyme activity levels (GSH-Px) and the malondialdehyde (MDA) levels in rat brain. GSH-Px and MDA levels had a slight increase in sugammadex and sugammadex+rocuronium group in cerebellum tissues. These increases were statistically significant. But this increase did not cause any histopathological damage to the cerebellum tissue. The study demonstrates that sugammadex is safe for reversal of rocuronium effects in rats. Sugammadex in the dose ranges used in the studies, did not produce adverse effects in rats brain and cerebellum tissues.

Keywords: *Rat, Neuromuscular Block, Sugammadex, Rocuronium, Brain tissues, Cerebellum tissues, Neurotoxicity*

Ratlarda Sugammadex ve Rocuronium Kombinasyonunun Kraniyal Nörotoksisite Üzerine Etkisi: Deneysel Çalışma

Öz

Sugammadeks klinik anestezi uygulamalarında sıklıkla kullanılır. Sugammadeksin nörotoksik etkiye sahip olup olmadığını belirlemek için, beyin ve serebellum dokularının histopatolojik değerlendirmesi ve antioksidan durumu araştırıldı. Otuz iki erişkin sprague dawley sıçanı dört gruba ayrıldı. Purin Kontrol Grubuna (Grup 1, n=8) işlem yapılmadı. Kontrol grubuna (Grup 2, n=8) 16 mg/kg serum fizyolojik verildi. Sugammadex grubuna (Grup 3, n=8) 16 mg/kg sugammadex ve sugammadex+rocuronium grubuna (Grup 4, n=8) 16 mg/kg sugammadeks ve 1 mg/kg rokuronyum verildi. İlaç uygulamalarından sonra beyin ve beyincik dokuları histolojik ve oksidadif hasar açısından biyokimyasal olarak değerlendirildi. Beyin ve beyincik dokusunun genel yapısı tüm gruplarda normaldi. Katmanların ve içerdiği nöronların görüntüleri normaldi. Patolojik bulgu bulunamadı. Gruplar arasında sıçan beyninde glutatyon enzim aktivite düzeyleri (GSH-Px) ve malondialdehit (MDA) düzeyleri açısından istatistiksel olarak anlamlı bir fark yoktu. Serebellum dokularında GSH-Px ve MDA düzeyleri sugammadeks ve sugammadeks+rocuronium grubunda hafif bir artış gösterdi. Bu artışlar istatistiksel olarak anlamlıydı. Ancak bu artış, beyincik dokusunda histopatolojik hasara neden olmadı. Çalışma, sugammadeksin sıçanlarda rocuronyum etkisinin tersine çevrilmesinde güvenli olduğunu göstermektedir. Çalışmada kullanılan doz aralıklarında sugammadeks sıçan beyni ve beyincik dokularında olumsuz etki oluşturmamıştır.

Anahtar sözcükler: *Rat, Nöromüsküler Blok, Sugammadeks, Rocuronium, Beyin dokusu, Beyincik dokusu, Nörotoksisite*

INTRODUCTION

In general anesthesia, muscle relaxant agents have used to facilitate surgical applications. Muscle relaxants are two types as depolarizing and non-depolarizing. Non-depolarizing muscle relaxants compete with acetylcholine (Ach) to bind to nicotinic receptors at the neuromuscular junction [1]. Rocuronium is the most commonly used steroid-building agent in non-depolarizing muscle relaxants. In order to regain the muscle functions of the patient and prevent residual neuromuscular block, it is necessary to remove the effect of the muscle relaxant agents temporarily [2,3]. Classic

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reversal agents, such as neostigmine, implement their activity by raising the levels of Ach at the neuromuscular junction by competitive inhibition of Ach-esterase. Classic reversal agents may cause side effects owing to their nonselective potentiation of muscarinic Ach receptors [4]. Sugammadex is a recently developed γ-cyclodextrine, prepenced to keep non-depolarising neuromuscular blocking agents. Sugammadex reverses the neuromuscular block of rocuronium. The agent does not bind to plasma proteins and biologically passive. It has no effectiveness on Ach-esterase or any receptor system in the body. Because of its ability to rapidly reverse neuromuscular block, reducing residual block formation and ensuring patient safety, it's usege is increasing day by day [5]. In animal and human studies, it has been proved that sugammadex reverses the muscle relaxant effect of rocuronium. Although the safety and efficacy were reported in the studies, the side effects were not clearly demonstrated [6,7].

In general, oxidative stress is considered to be an early marker of neurological disease, and therefore, early oxidative stress detection is considered to be of great preventive value [8]. Drugs can cause free radical damage with their metabolites. Free oxygen radicals on cell membrane fatty acids initiate lipid peroxidation. Oxidation of polyunsaturated fatty acids result in aldehydes. Malondialdehyde (MDA) is the best known aldehyde. MDA causes cross-linking and polymerization of membrane components. MDA neutralizes receptors and membrane-bound enzymes in membranes. It can also cause serious damage to membrane proteins. The most known glutathione peroxidase (GSH-Px) in live tissues is amongst antioxidants. Antioxidants repaire oxidative damage and neutralize various reactive intermediates. Brain tissue is one of the most susceptible tissues of free radical-induced oxidative damage. MDA and GSH-Px are most commonly used to determine the oxidative damage caused by free radicals in tissues [9]. Although the penetration of sugammadex is minimal in the blood-brain barrier, studies have suggested that sugammadex may be neurotoxic [10]. Studies have shown that sugammadex activates mitochondria-dependent apoptosis. Depletion of neuronal cholesterol levels has been shown as a cause. Cholesterol homeostasis was associated with oxidative stress [11]. Therefore, we planned to study oxidative stress in brain and cerebellum tissue.

In this study it was evaluated neuropathological effects of sugammadex in rats by biochemical and histopathological methods. Additionally, this preclinical study compared the neurotoxic effects of sugammadex with sugammadex and rocuronium combination.

MATERIAL and METHODS

Ethics Approval

The study protocol was approved by the Local Ethics Committee for Animal Experiments of Adiyaman University, Turkey (Approval no: ADYU-HADYEK: 2018-2).

Animals

All experiments were conducted according to our National Institute of Health Guide for the Care and Use of Laboratory Animals [12]. The study was conducted in Experimental Animal Laboratory (Adiyaman, Turkey) in 2018. Thirty-two pieces, adult Sprague-Dawley male rats weighing 300-350 g were fed with standard pellet diet and water *ad libitum*. Room temperature (22-25°C) and humidity (50-55%) were monitored daily. Lighting was provided using cool white fluorescent lamps, with 12-h light and dark cycles (06.00-18.00).

Experimental Groups

The animals were divided into four groups (n=8).

Group 1 (Purine Control Group): The rats were consisted of animals with maintenance conditions in accordance with routine ethical rules that were not performed surgical treatment.

Group 2 (Control Group): The rats were given 16 mg/kg intravenous (IV) 0.9% isotonic saline.

Group 3 (Sugammadex Group): The rats were given 16 mg/kg IV Sugammadex (Bridion®; Schering-Plough Corporation, Oss, Netherlands).

Group 4 (Sugammadex + Rocuronium Group): The rats were given 1 mg/kg IV rocuronium (Esmeron®; Organon, Istanbul, Turkey) and three min later 16 mg/kg IV sugammadex (Bridion®; Schering-Plough Corporation, Oss, Netherlands).

All drugs were administered intravenously over the tail vein. Three days after the appropriate action, the brain and cerebellum tissues under ketamine/xylazine anesthesia were atraumatically removed from the skull by dissection starting from the neck. Brain and cerebellum tissues were divided into interhemispheric incisions for biochemical and histological examinations.

Biochemical Evaluation

The removed brain and cerebellum tissue samples were washed with saline at a temperature of +4°C, were placed in ependorf tubes according to the cold chain principles and stored at -70°C until examined. In tissue samples, tissue homogenates for MDA and GSH-Px measurements were prepared in cold with 0.15 M KCl (10%, w/v) homogenizer.

Malondialdehit; lipid peroxidation is an indirect indicator of the amount of free oxygen radical formed in the tissue. The tissue MDA concentration as a marker of lipid peroxidation is based on the method of Uchiyama [13] the supernatant extracted from the N-butanol phase of the pink colored product as a result of the MDA reacting with thiobarbituric

acid at 95°C was determined by spectrophotometer at 535 and 520 nm. Brain tissue was homogenized in 10% trichloroacetic acid and then centrifuged. After mixing the superficial liquid part with an equal volume of 0.67% thiobutyric acid, it was incubated in boiling water for 15 min at 90°C, then cooled and centrifuged. The tissue MDA concentrations were measured in nmol/g tissue under 532 nm absorbance.

Glutatyon peroksidaz; GSH-Px catalyzes the reduction of harmful peroxides such as lipid peroxide and hydrogen peroxide. During this reduction, the reduced glutathione is converted into oxidized glutathione. GSH-Px analysis was performed according to the method described by Ellman [14] Glutathione in the analysis tube reacted with 5i-dithiobis2-nitrobenzoic acid to give yellow-greenish color and the light intensity of this color was measured by spectrophotometer at a wavelength of 410 nm.

Histological Analysis

Other brain and cerebellum tissues were fixed with 10% formaldehyde at room temperature. Tissues were kept in this solution for ten days. After fixation, the tissues were washed in tap water to remove the fixation solution in the tissue. Then dehydration and polishing were performed according to routine histological tissue follow-up procedure. Finally, the completed tissues were made into paraffin blocks. 10 µm volume was calculated from the paraffin blocks with cavaliere method and 7 µm thick sections were taken for histological examination. For histopathological evaluation, sections were stained with cresyl violet (Merck, Cresyl violet acetate, FN 1107635, Germany). Cresyl violet staining is used in neurosciences to analyze neuron variations in various pathological cases. The images obtained by Carl Zeiss Axiocam ERc5 digital camera attachment microscope were examined and evaluated.

Statistical Analysis

The Statistical Package for the Social Sciences 22.0 program (SPSS Inc., Chicago, IL, USA) was used for the statistical study. The one sample Kolmogorov-Smirnov test was used to determine whether the datas was distributed normally. One Way ANOVA test was used to analyze the MDA and GSH-Px values of the groups. Kruskal-wallis test was used for data not showing normal distribution and Dunnet test for multiple comparisons. Mann-Whitney U test was used to compare the groups. The results were reported as mean±standart deviation (SD). Results were assessed at a 95% confidence interval, and P value <0.05 was accepted as statistically significant.

RESULTS

As a result of the statistical measurements of the rat brain GSH-Px levels: 2022.35±430.02 nmol/g in the purine control group, 2067.22±391.34 nmol/g in the control group, 1917.39±469.98 nmol/g in the sugammadex group, and 2041.58±263.02 nmol/g in the sugammadex + rocuroniun group, were found as wet tissue *(Table 1)*. Compared study data; There was no statistically significant difference between the groups in terms of GSH-Px level (P=0.881). MDA, which is defined as an indicator of free radical damage in tissues, did not show a significant difference with sugammadex application compared to control groups. In the purine control group 995.36±121.03 nmol/g, in the control group 1012.95±119.70 nmol/g, 886.91±145.05 nmol/g in the sugammadex group and 1090.00±307.35 nmol/g in the sugammadex + rocururonium group. No significant difference was found in MDA levels in brain tissue (P=0.223).

As a result of the statistical measurements of the rat brain GSH-Px levels: 1770.76±74.33 nmol/g in the purine control group, 1785.18±157.27 nmol/g in the control group, 1669.80±126.07 nmol/g in the sugammadex group, and

Table 1. *Distribution of GSH-Px and MDA values in brain tissue of groups*

Parameter	Brain Tissue				
	Purine Control	Control	Suggammadex	Suggammadex + Rocuronium	*P value*
GSH-Px (nmol/g)	2022.35±430.02	2067.22±391.34	1917.39±469.98	2041.58±263.02	0.881
MDA (nmol/g)	995.36±121.03	1012.95±119.7	886.91±145.05	1090.00±307.35	0.223
P<0.05					

Table 2. *Distribution of GSH-Px and MDA values in cerebellum tissue of groups*

	Cerebellum Tissue				
Parameter	Purine Control (n=8)	Control (n=8)	Suggammadex (n=8)	Suggammadex+ Rocuronium (n=8)	*P value*
GSH-Px (nmol/g)	1770.76±74.33^{ab}	1785.18±157.27^{ab}	1669.8±126.07^{b}	1844.47±75.28^{a}	*0.037**
MDA (nmol/g)	1086.65±118.9^{ab}	945.95±195.60^{b}	1363.86±375.21^{a}	1342.09±334.49^{a}	*0.013**

** There is no statistically significant difference between the groups with the same letters and there is a statistically significant difference between the groups with different letters (P<0.05)*

1844.47±75.28 nmol/g in the sugammadex+rocuroniun group were found as wet tissue *(Table 2)*. Compared study data; There was statistically significant difference between the groups in terms of GSH-Px level (P=0.037). Increase of GSH-Px is significant in sugammadex and sugammadex+rocuronium group.

Malondialdehyde, which is defined as an indicator of free radical damage in tissues, showed a significant difference with sugammadex application compared to control groups (P=0.013). In the purine control group 1086.65±118.92 nmol/g, in the control group 945.95±195.60 nmol/g, 1363.86±375.21 nmol/g in the sugammadex group and 1342.09±3334.49 nmol/g in the sugammadex + rocururonium group. There was a slight increase in sugammadex and sugammadex rocuronium group. This increase was statistically significant. When examined with histo-pathological data, it was observed that the increases and decreases were at acute level. The free radical increase due to the increase in MDA did not cause any histopathological damage to the cerebellum tissue.

In the examined sections of the groups, the cortex was wrapped with a loose connective tissue piamater. The layers of the cerebral cortex could not be distinguished apart from the lamina molecule *(Fig. 1-group 1a,1b,1c)*. The cells of the nerve tissue were distributed homogeneously to the entire cortex under the lamina molecule. The boundaries of the neurons were regular and the nuclei were prominent. Pyramidal neurons were also found between oval or rounded neurons. Cell extensions were prominent and smooth *(Fig. 1-group 2a,2b,2c)*. The general

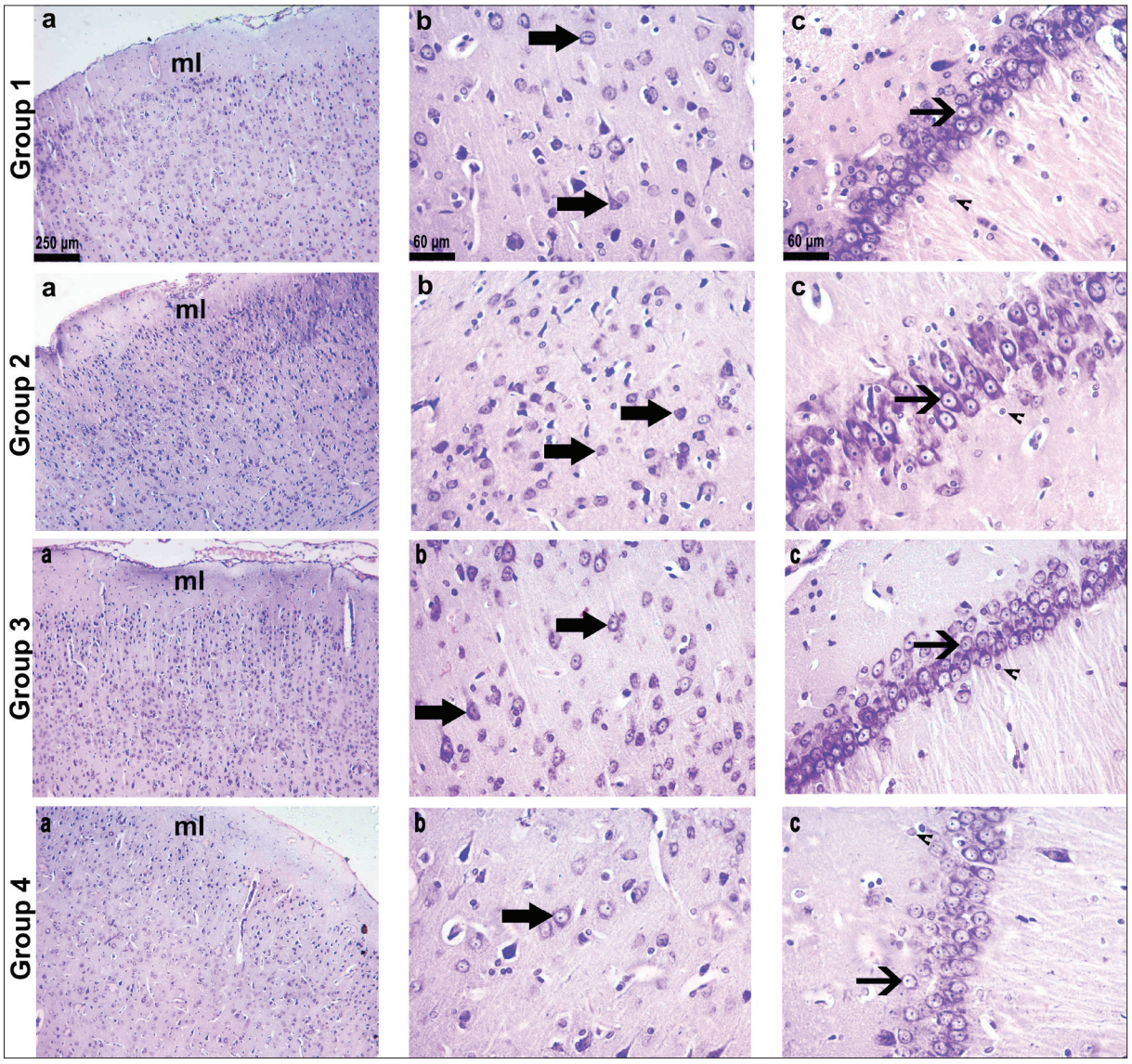

Fig 1. Histopathological evaluation of brain tissues
Light microscopic image of brain tissues belonging to group 1a, group 2a, group 3a and group 4a, respectively (x4, Krezil violet staining); Light microscopic image of group 1b, group 2b, group 3b and group 4b, respectively (x40, Krezil viole staining); Light microscopy of hippocampus belonging to group 1c, group 2c, group 3c and group 4c, respectively (x40, Krezil Violet Coloring); ml; molecular layer; Black thick arrow; pyramidal neurons; Black fine arrow; neurons in the hippocampus; Black arrow head; neuroglial cells

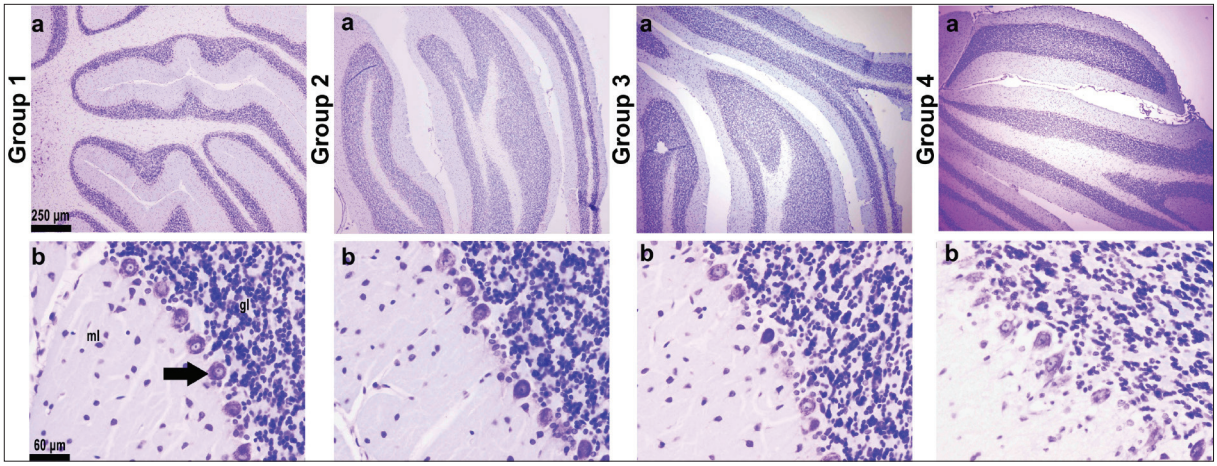

Fig 2. Histopathological evaluation of cerebellum tissues
Light microscopic appearance of cerebellum tissues of group 1a, group 2a, group 3a and group 4a, respectively (x4, Krezil violet staining); Light microscopic image of group 1b, group 2b, group 3b and group 4b, respectively (x40, Krezil viole staining); ml; Lamina moleculeare; gl; granular layer; Black thick arrow; purkinje cell

structure of the hippocampus and neurons were normal. At the light microscopic examination, the pericardies of the neurons were well defined; pramidal. In addition, the nuclei of these cells were echromatic and distinctive nucleolus. Dendrites and axons, the extensions of the neurons, were of normal width. Neuroglia cells were also normal *(Fig. 1-group 3a,3b,3c)*. The overall structure of the brain tissue was normal in all groups *(Fig. 1-group 4a,4b,4c)*. Images of the layers and the neurons contained there were normal. No pathological findings were found.

The sections of the cerebellar cortex could be clearly distinguished in the sections examined. On the outermost side of the piamater-wrapped organ, the cortex was composed of lamina molecule, purkinje cell layer (ganglional layer) and granular layer from the outside *(Fig. 2-group 1a,1b)*. The lamina molecule is a neuron-poor layer and the purkinje cells in the ganglional layer with the largest cells of the cerebellum were normal. In these cells, echromatic nucleus and a prominent nucleolus were seen. The dendrite from the top of the Purkinje cells was branched in the molecular layer. The granular layer was composed of multiple small diameter neurons *(Fig. 2-group 2a,2b)*. The overall structure of the cerebellum was normal in all groups *(Fig. 2-group 3a,3b; group 4a,4b)*. Images of the layers and the neurons contained there were normal. No pathological findings were found.

DISCUSSION

Cyclodextrins are chemical entities of natural origin, which are derived from bacterial. Sugammadex is a modified γ-cyclodextrin molecule. Only sugammadex received license for use in cyclodextrin molecules. Others did not pass drug phase tests. It has been understood that sugammadexin has a chemical interaction mechanism called encapsulation with neuromuscular agents [15]. Cyclodextrin molecules are employed to convert lipophilic agents to hydrophilic forms. Most cyclodextrins affect receptor functions by altering the lipid structure in the cell membrane. In one a study, methyl-β-cyclodextrin has been reported to reduce neuronal excitability in the hippocampal area. Methyl β-cyclodextrin affects cholesterol levels in post-synaptic areas. N-methyl-D-aspartic acid receptor-dependent glutamate transmission may impair the transmission [16]. Neuroprotective effects have been demonstrated in studies with methyl-β-cyclodextrin and 2-hydroxypropyl-b-cyclodextrin [17-19]. A recently study was determined that sugammadex had a neuroprotective effect against transient cerebral ischemia. The study showed that treatment with 16 mg/kg and 100 mg/kg sugammadex had a neuroprotective effect in a transient cerebral ischemia/reperfusion injury rat model [20]. They reported that sugammadex had a protective effect against ischemia-reperfusion injury in brain. But a recent study showed that rocuronium has dose-related deleterious effects on the central nervous system and can produce dose-dependent excitatory effects and seizures [21]. Also it is known that rocuronium can permeate the CSF after intravenous injection [22].

Since sugammaddex has a high molecular weight, it's passage from the blood brain barrier (BBB) and placenta is low. But, in cases where cholesterol plugs are formed in the cerebral arteries and hypoxia occurs due to insufficient blood flow, tissue damage occurs and BBB structure is broken. In addition, in the case neurodegenerative diseases and traumatic brain or spinal cord injury BBB transition is easier [23].

Experimental studies have shown the neuroprotective characteristics of cyclodextrins, however the adverse effect of sugammadex on neuronal cell cultures has led to debate on this topic. The study showed that sugammadex causes apoptosis and neuron death in primary cell cultures. Sugammadex has been reported to induce apoptotic

activation by leading to oxidative stress and cholesterol hemostasis. Sugammadex reduced membrane-related cholesterol levels. They reported that they were sensitive to oxidative stress among neuronal cell types [11]. We took this work as a reference. Although studies have been conducted on the brain, any article in the literature was not directly related to histopathological changes caused by rocuronium or sugammadex in rat cerebellum tissues. We evaluated the MDA and GSH-Px levels as indicators of oxidative stress and free radical injury in the brain and cerebellum tissue. Reversal of neuromuscular blockage induced by steroidal neuromuscular blocking agents such as rocuronium can be achieved using normal dose of sugammadex. Intravenous sugammadex demonstrates linear pharmacokinetic properties over the dose range of 1-16 mg/kg [24]. Therefore, 16 mg/kg sugammadex were given to rats in our study. Isotonic saline was applied to the control group at the same volume. Rats in Group 4 were given sugammadex after rocuronium application. It was determined the effects of sugammadex on the histopathological and biochemical structure of rat brain and cerebellum tissues after neuromuscular blockage. The overall structure of the brain and cerebellum tissues were normal in all groups. No pathological findings were found. There was no statistically significant difference between the groups in terms of glutathione enzyme activity levels and the MDA levels in rat brain. MDA and GSH-Px levels were altered in sugammadex and sugammadex rocuronium group in cerebellum tissue. MDA and GSH-Px levels remained at the acute level and did not show its effect at the pathological level in the tissue. The cerebellum is responsible for the purposeful execution of voluntary movements. The information is transmitted directly to the cerebellum via the proprioceptive receptors in the muscles [25]. Muscle relaxant rocuronium and antagonist sugammadex was effected to oxidative damage in cerebellum. However, the effect was acute and transient because it was not affected histopathologically. To the best of our knowledge although there are studies on the brain, its effect on the cerebellum has not been investigated. We think that this study will shed light on new studies in which the efficacy of the drug will be investigated. This current study, sugammadex and sugammadex-rocuronium applied in rats did not show any neurotoxic effect. Sugammadex in the dose ranges used in the study, did not produce adverse effects in animal study.

For clinicians, it is important to protect the patient's brain during surgery and in intensive care. For this purpose, selection of suitable agents is required. The results obtained in animal experiments provide reference to the use of drugs in humans. The aim of this study was to determine the effect of rocuronium and sugammadex on brain and cerebellum tissues in rats. As a result, rocuronium and sugammadex application had no negative effect on brain and cerebellum tissues. In our study, the neurotoxic effects of the drugs were not observed. For the antagonism of both peripheral and central long-term residual rocuronium effects, it may be advisable to consider sugammadex.

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Conflicts of Interests

The author declare no conflicts of interests.

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Research Article

The Effect of Testosterone Supplementation on Capillar Morphometry and VEGF Expression Level of the Heart in Aged Mice [1]

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Abstract

The aim of this study was to determine the effect of testosterone hormone supplementation on the capillary morphometry and VEGF expression level of the heart of old mice using histological and molecular techniques. A total of 30 old mice were enrolled in 3 groups: sham operation group (Control), gonadectomy group (G) and gonadectomy and testosterone supplementation group (GTS). The capillary number and the inner diameter of larger capillary vessels in the heart were measured by light microscope in terms of angiogenic processes. The levels of angiogenic factor, VEGF (vascular endothelial growth factor), mRNA in the heart tissue were determined by RT-PCR. The capillary densities decreased in female mice, in which G and GTS ($P<0.05$) groups compared to the control group, whereas there was no alteration in male mice. Gonadectomy led to a reduction in VEGF expression. Interestingly, testosterone replacement (in GTS group) caused even more reduction in VEGF expression in male and female ($P<0.05$) compared to the control group. The present results showed that there was a variation affected by gender in the regulation of angiogenesis by testosterone in the heart. However, testosterone replacement was not sufficient to restore the effects of gonadectomy on the heart in old mice.

Keywords: *Angiogenesis, Testosterone, Aged mice, Heart*

Testosteron Takviyesinin Yaşlı Farelerin Kalbinde Kapillar Morfometri ve VEGF Expresyon Seviyesine Etkisi

Öz

Bu çalışmanın amacı yaşlı farelerin kalbinde testosteron hormon takviyesinin anjiyogenik etkisini moleküler ve histolojik olarak tespit etmektir. Toplam 30 fareden 3 çalışma grubu oluşturulmuştur. Bunlar; yalancı operasyon yapılan (kontrol) grup, gonadektomi yapılan (G) grup ve hem gonadektomi hem de testosteron takviyesi yapılan (GTS) gruptur. Kalp dokusunda kapillar sayısı ve geniş kapillar damarların iç çapı ışık mikroskobunda ölçülmüştür. Kalp dokusundaki VEGF (vasküler endoteliyal büyüme faktörü) mRNA seviyesi RT-PCR ile ölçülmüştür. Dişi farelerde; kontrol grubuna göre G ve GTS ($P<0.05$) grup farelerin kalbindeki kapillar yoğunluk azalmış, erkeklerde ise değişmemiştir. Kısırlaştırma farelerde kalpteki VEGF mRNA seviyesinde bir azalmaya yol açmıştır. Testosteron takviyesiyle (GTS grup) kalpteki VEGF mRNA seviyesinde kontrol grubuyla karşılaştırıldığında erkek ve dişilerde ($P<0.05$) daha fazla azalma görülmüştür. Bu çalışmayla testosteronun erkek ve dişilerin kalbinde anjigenik etkisinin farklı olduğu saptanmıştır. Yaşlı erkek farelerde kısırlaştırmanın anjiyogenik olaylarda negatif etkisinin olduğu belirlenmiştir. Bununla birlikte testosteron takviyesi yaşlı farelerin kalbinde gonadekteminin etkilerini düzeltmek için yeterli olmamıştır.

Anahtar sözcükler: *Anjiyogenezis, Testosteron, Yaşlı fare, Kalp*

INTRODUCTION

Angiogenesis, a physiological process for vessel growth and remodeling, is not only a normal process in the regulation of growth and development, but also involved in tumor development. The lack of angiogenesis may cause cardiovascular and cerebrovascular diseases. The angiogenic factors, found in a lot of organs and tissues, are consisted of proteins and they stimulate angiogenesis. Some of the latter factors are vascular endothelial growth

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factor (VEGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) [1].

Vascular endothelial growth factor, a member of subfamily of growth factors, is an essential catalyst promoting endothelial cell proliferation, initiating angiogenic sprouting and creating vascular structures. VEGF-A is the most important one in mediating endothelial cell proliferation. VEGFs interact with a set of cellular membrane tyrosine-kinase receptors. VEGF-A regulated by androgens is a major mediator of androgen actions on endothelial cell proliferation [2]. In addition, many factors can increase the synthesis of angiogenic agents or directly stimulate them. It has been reported that many hormones, especially estrogen, affect these factors [3]. It is well known that estrogen increases the vascularization in some organs (e.g. the uterus, the heart, and the brain) by releasing angiogenic factors and increasing their expressions in female rats and women [4-6]. There are few and limited studies on the effect of testosterone on angiogenesis [7-11]. Testosterone has protective effects on formation of cells in the arterial vessels [12]. Testosterone has different kinetic effects in vessels; in the first stage testosterone has vasodilator effect, in longer-term exposure, testosterone increases the blood pressure and causes renal function abnormalities [13,14].

In addition to the factors mentioned above, angiogenesis is also affected by the age. It is known that aging plays as a major factor for the interruption and the changes in angiogenic processes [15-17]. Aging is one of the main risk factors for the development of atherosclerosis and, therefore, for coronary artery disease. Age associated remodeling of the vascular wall includes luminal enlargement, intimal and medial thickening, and increased vascular stiffness [18].

There is no literature about how aging and testosterone hormone affect the mammalian heart. Therefore, the aim of this study was to determine, the effects of testosterone hormone on angiogenic process in the heart of aged female and male mice.

MATERIAL and METHODS

A total of 15 female and 15 male Swiss albino mice at 12 months of age were included in the experiments. The mice were obtained from the Animal Experimental Unite of Faculty of Veterinary Medicine, Adnan Menderes University, Turkey. The ethical committee approval was taken from Adnan Menderes University (with no: B.30.2.ADÜ.0.00.00.00/050.04/2011/098). Mice were housed in standard cages under normal conditions (20-24°C and 50-60% humidity) and were fed *ad libitum* with unlimited access to the commercial pelleted diet and water. Among the experimental groups, the mice in the first group (control) exposed to the same stress (sham operated) with other experimental groups at 12 months of age (only skin incision and closure). The mice in the second experimental group (G) were gonadectomized at the same age with the control group, but they did not receive testosterone replacement. The mice in the third experimental group (GTS) received both gonadectomy surgery and testosterone replacement once a day for one month after post-operative day 10. Each group consisted of 10 mice (5 males and 5 females). For the animals in GTS group, 0.01 mL testosterone (250 mg/mL Sustanon 250®, Organon) was administered by subcutaneous injection as a single dose [19]. Mice were anesthetized by intraperitoneal administration of ketamine (90 mg/kg)/xylazine (10 mg/kg) combination. Mice were considered as old at 12 months of age (365 days). At 365 days of growth, the gonadectomy surgery was done. After 10 days of healing period, testosterone replacement therapy was applied for 30 days (365 + 10 + 30 = 405 days). All mice were then sacrificed at day 405 after they were born. Whole blood was collected by exsanguination from all animals. Testosterone levels in the blood samples were measured using ELISA kit (for Mouse Testosterone (T), USCN Life Science Inc.® Wuhan) according to the manufacturer's instructions. The hearts were removed from the mice and cut in the middle of transversal plane. Half of the organs were collected for the histological examination and the other half was devoted to the molecular investigations.

For histological examination, the tissue samples were kept in 10% buffered formalin solution and were embedded in paraffin after appropriate tissue tracking. For each tissue from paraffin blocks of 5 µm, three serial sections were taken with an interval of 30 µm. After applying the process deparaffinization, sections were stained with Mason trichrome method [20]. The number of capillaries and inner diameter of larger capillary vessels were measured in the heart tissues. The slices were analyzed and photographed under a light microscope (Leica DMLB) that is equipped with a calibrated digital camera (Leica DC200 CD camera and Q-win Standard imaging analyses programme). Histomorphometric analyses were performed at a magnification of x 40-100. Both capillary number and inner diameter were measured and averaged results of different 15 microscopic fields.

For molecular investigation, total RNA extraction from the heart tissue samples was performed using geneJET RNA Purification kit (Fermentas) according to the manufacturer's instructions. Reverse transcription using 2 µg of total RNA, was done with the revertAid First Strand cDNA Synthesis kit (Fermentas) containing M-MuLV reverse transcriptase enzyme following manufacturer's instructions. The resulting cDNA was used for real time PCR amplification. Primers were designed to be specific for mouse sequence using web-based QuantiProb design software (www.quiagen.com). The forward primer for VEGF was 5'-GGAGATCCTTCG AGGAGCACTT-3' and reverse primer was 5'-GGCGATT TAGCAGCAGATATAAGAA-3'. For RNA extraction and PCR procedures of standardization and control, the house-

keeping transcript (GAPDH) were used. The forward primer for GAPDH was 5′-GAGGGGCCATCCACAGTCTTCT-3′ and reverse primer was 5′-GGAGCCAAACGGGTCATCATCTC-3′. Genes were amplified using QuantiTect SYBR PCR Kit (ABM) as previously described by Shidaifat et al.[7].

Statistical analysis was used in the SPSS 19.00 software package. Distributions of data were analyzed using Shapiro-Wilk test. Nonparametric distribution the data was checked using the Kruskal-Wallis test. Bonferroni- corrected Mann-Whitney U test was applied as post-hoc test. The presence of a correlation between the angiogenic events in the brain tissue and the levels of testosterone was also tested by Spearsman's test. A P-value less than 0.05 were considered significant.

RESULTS

Testosterone Level

Blood testosterone levels are shown in *Table 1, Fig. 1*. Among male mice after castration, testosterone levels decreased ~18.8-fold (P<0.01) and testosterone replacement increased hormone levels ~13.1-fold among castrated mice (P<0.01). In female mice, testosterone level was found to be decreased ~2.8-fold in group G. After hormone supplementation, testosterone level increased ~77.2-fold compared to group G (P<0.001). In female mice after testosterone replacement, the testosterone levels were increased in group GTS when compared to control (P<0.05) and group G (P<0.001) *(Table 1)*.

Capillary Number

No significant change was found in capillary densities after gonadectomy for the male mice *(Table 2, Fig. 2)*. Hormone replacement did not affect the capillary numbers in the heart of castrated mice. In female heart, the maximum capillaries were found in control group animals, and the least in group G followed by group GTS mice *(Table 2, Fig. 2, Fig. 5)*. After ovariectomy, a reduction (~1.15-fold) was found in capillary densities of heart in group G compared to the control group. Testosterone supplementation caused ~1.18-fold decrease (P<0.05) on capillary number in group GTS compared to the control group *(Fig. 5)*. Testosterone supplementation did not increase the capillary density in the heart of the gonadectomized female mice.

Vessel Diameter

Inner diameters of the larger capillary of group G and group

Table 1. *Testosterone levels in male and female groups of mice*

Animal Groups (N)	Testosterone Level nmol/L (Mean±S.D.)	
	Male	Female
Control (10)	1.517±1.173 [a]	0.140±0.101 [c]
G (10)	0.089±0.075 [b]	0.051±0.064 [c]
GTS (10)	1.051±0.478 [a]	3.862±2.838 [d]

*[a,b] In male mice after castration testosterone levels were decreased (P<0.01) in G group of mice. Testosterone replacement increased hormone levels in GTS group of male mice (P<0.01) [c,d]. In female mice after testosterone replacement the testosterone levels were increased in GTS group, to compared to control (P<0.05) and G group (P<0.001); **Control:** sham operated group, **G:** gonadectomized group, **GTS:** gonadectomized and testosterone supplemented group*

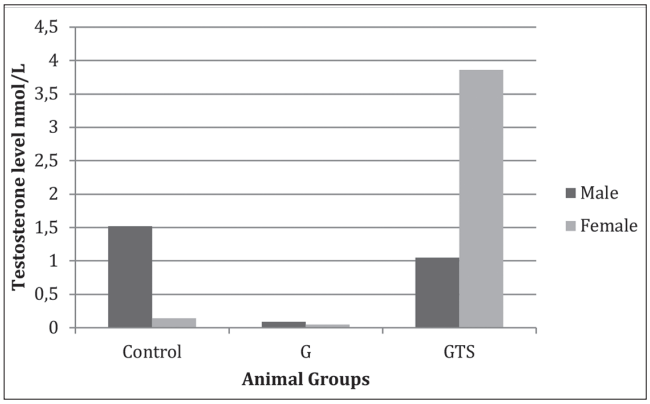

Fig 1. Testosterone levels in male and female groups of mice. Control: sham operated group, G: gonadectomized group, GTS: gonadectomized and testosterone supplemented group

Table 2. *The number of capillaries, inner diameters of larger capillary and VEGF mRNA (ΔCT) values of heart tissues in male and female groups of mice*

Animal Groups (N)		Capillary Number (Mean ± S.D.)	Inner Diameter of Larger Capillary (µm) (Mean ± S.D.)	VEGF mRNA (ΔCT) (Mean ± S.D.)
Male	Control (4)	4.133±0.211	22.228±3.739	4.189±0.929
	G (5)	4.173±0.784	19.023±2.030	2.395±2.154
	GTS (5)	4.306±0.269	18.530±1.868	-0.021±2.492
Female	Control (5)	4.360±0.539 [a]	19.727±9.296	4.357±0.944 [c]
	G (5)	3.773±0.332 [a,b]	17.693±2.829	3.564±0.661 [c,d]
	GTS (5)	3.692 0.102 [b]	15.768±1.712	0.545±2.465 [d]

*[a,b] The number of capillary in female heart significantly decreased (P<0.05) in group GTS compared to the control group; [c,d] The expression level of VEGF mRNA in female heart decreased (P<0.05) in group GTS compared to the control group; **Control:** sham operated group, **G:** gonadectomized group, **GTS:** gonadectomized and testosterone supplemented group*

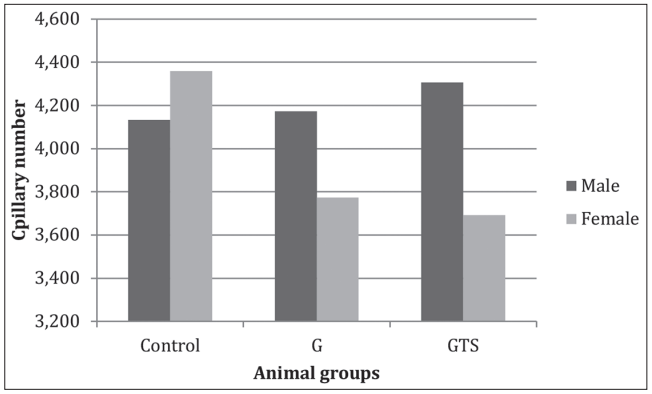

Fig 2. The number of capillaries of heart tissues in male and female groups of mice. Control: sham operated group, G: gonadectomized group, GTS: gonadectomized and testosterone supplemented group

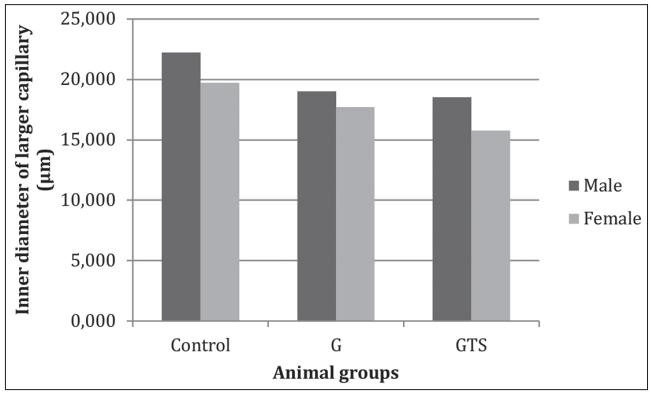

Fig 3. The inner diameters of larger capillary of heart tissues in male and female groups of mice. Control: sham operated group, G: gonadectomized group, GTS: gonadectomized and testosterone supplemented group

GTS were decreased in male heart ~1.17- and ~1.20-fold, respectively, compare to the control group *(Table 2, Fig. 3)*. In female mice, the inner diameter also decreased in group G and group GTS by ~1.11- and ~1.25-fold, respectively, compare to the control group. Testosterone replacement did not affect on the inner diameters of larger capillary of the heart in male and female mice *(Table 2, Fig. 3)*.

Data of RT PCR

It was found that the level of VEGF mRNA (ΔCT) of the male heart was highest in the control group, whereas lowest in group GTS *(Table 2, Fig. 4)*. After castration, VEGF mRNA levels of the heart decreased by ~1.75 fold in group G and testosterone replacement led to a reduction by ~5.21-fold as evidenced by VEGF mRNA levels in group GTS compared to the male controls. VEGF expression decreased by ~3.42-fold in group GTS compared to group G. Similar to that of the male group, the VEGF mRNA levels of the female heart were highest in the control group while were lowest in group GTS *(Table 2, Fig. 4)*. The reduction (~1.22-fold) in VEGF mRNA level of group G was not significant compared to the control group while the reduction (~7.93-fold) in VEGF mRNA levels of group GTS was significant ($P<0.05$). A

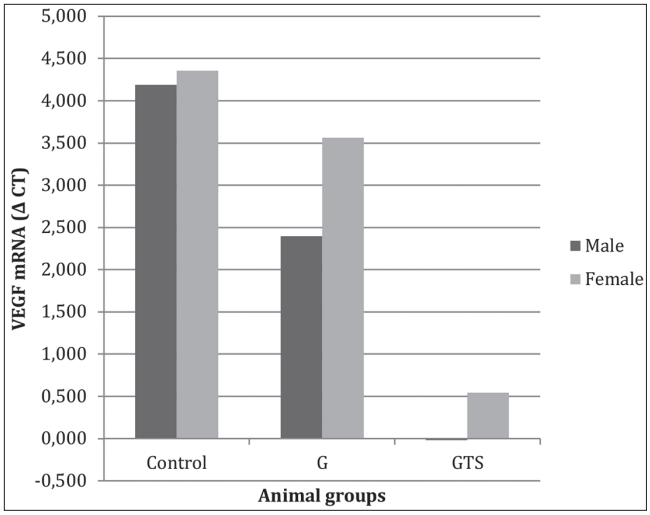

Fig 4. The VEGF mRNA (ΔCT) values of heart tissues in male and female groups of mice. Control: sham operated group, G: gonadectomized group, GTS: gonadectomized and testosterone supplemented group

reduction by ~6.47-fold in VEGF mRNA levels was determined in group GTS compared to group G.

The presence of a correlation between the angiogenic events in the heart tissue and the levels of testosterone was also tested by Spearsman's test. A significant negative correlation (r_s = -0.550, $P<0.05$) was found between inner diameter of larger capillary in the heart tissue and the testosterone levels of female mice, suggesting that increasing testosterone level in female mice leads to a reduction in the inner diameter of larger capillary of the female heart.

DISCUSSION

Limited studies on the effects of androgens on angiogenesis have been found [7,8]. Sieveking et al.[8] reported that androgens stimulate erythropoietin production via VEGF in cell culture and endothelial stem cells. Angiogenesis is also down regulated when the levels of these hormones reduce after castration as stated previously [8]. In addition, an *in vitro* study showed that testosterone affects the development and function of early endothelial progenitor cells, but has no affect on late endothelial progenitor cells [21]. Chen et al.[22] stated that, in the case of experimental myocardial infarcts, estrogen treatment increases in the capillary density by leading bone marrow stem cell mobilization in the heart of ovariectomized rats.

Recent studies showed angiogenic effects of castration and testosterone treatment. Their results showed that there was a reduction in VEGF expression level and capillary density of the heart among castrated rats and testosterone treatment restored the adverse effects. In case of experimental myocardial infarcts among adult rats, capillary density is decreased in castrated rats [23,24]. It was reported that testosterone hormone plays an important

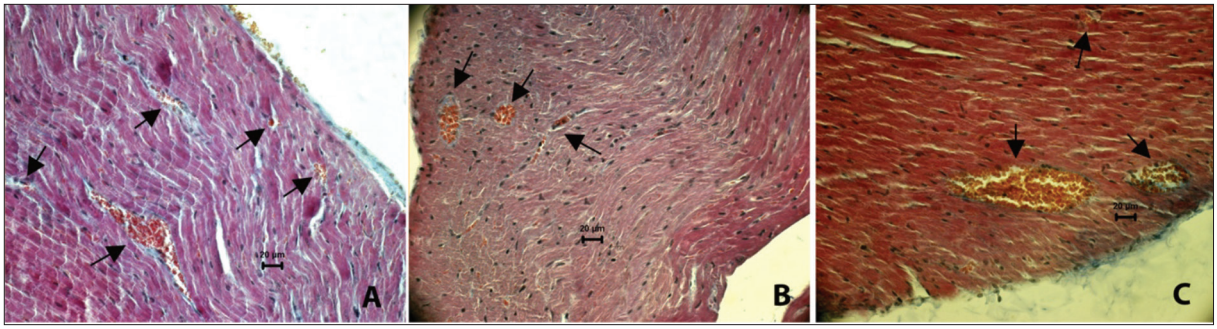

Fig 5. The capillary densities in the heart tissues from female (A, B, C) aged mice. Sections performed triple. A: sham operated group (Control), B: gonadectomized group (G), C: gonadectomized and testosterone supplemented group (GTS). Arrows show capillaries. After gonadectomy operation, capillary densities of the heart decreased in female mice. Testosterone supplementation to gonadectomized aged mice caused a slight decrease in capillary number of the female heart. A significant decrease ($P<0.05$) in group GTS was observed in female heart capillary density compared to the control group

role in the pathophysiology of heart attack in rat and the hormone replacement therapy has positive effects in this regard [25-27].The comparative effects of estrogen and testosterone treatment on castrated rats have been studied and found that the androgen treatment causes an improvement in angiogenesis of the heart muscle more than estrogen treatment does [28]. In our study, castration and hormone supplementation to the ovariectomized mice did not cause significant changes in the heart capillary density and vessel diameter. A slight reduction was found in the inner diameter of larger capillary after castration in male mice. Among female mice, it was found a reduction in the heart capillary density and vessel diameter following ovariectomy. However, a significant difference ($P<0.05$) was observed between capillary number of group GTS and the control group in female mice. Testosterone supplementation did not increase the capillary density and vessel diameter in female heart. The discrepancy among previous studies and our study may be due to the age of the animals. In present study, the aim was to determine the effects of the hormone replacement in elderly mice in contrast to the previous studies, which had been conducted with adult animals.

Previous studies showed that the absence of androgens such as under castration conditions, angiogenesis is down-regulated [8]. In addition, it was reported that the transcription of VEGF increases in cancer cells culture after estrogen and androgen treatments [29] but Sieveking et al.[8] determined an increase in the angiogenesis depending on VEGF in male mice, but not in female mice. Recent studies showed that VEGF can improve recovery from experimental ischemic myocardial injury in adult laboratory animals. VEGF administration was associated with smaller infarct sizes and greater wall thicknesses and greater vascular density in border region of the infarct [30-32]. Chen et al.[22] studied the angiogenic effects of castration and testosterone treatment in the experimental myocardial infarcts. They found that there was a reduction in the expression levels of VEGF and capillary density of the heart tissue in castrated rats. They also found that testosterone treatment restored the adverse effects due to castration [23]. In our study, the amount of VEGF mRNA in old mice (for both sexes) decreased due to gonadectomy. However and unexpectedly, testosterone supplementation caused even more reduction in VEGF expression. The reduction of VEGF mRNA levels of female mice in group GTS was significant ($P<0.05$) compared to the control group.

The effects of androgens on angiogenesis may vary depending on gender. *In vitro* studies showed that the androgens stimulate angiogenic phenomena in males, but not in females. In addition, *in vivo* studies have shown that the endogenous androgens regulate angiogenesis in males, but not in females [8]. Jesmin et al.[5] found a reduction in the vessel density, VEGF levels and VEGF receptors in frontal cortex of the brain in ovariectomized, 44 weeks of age, female rats. They observed a complete normalization in these changes following estrogen treatments [5]. In our study, a reduction was observed in the heart capillary number after gonadectomy in female mice, but not in male mice. Gonadectomy led to a reduction in vessel diameter of the heart tissue in both sexes. Although testosterone replacement of gonadectomized mice caused a mild decrease in vessel diameter of the heart in female mice, male mice were not affected by testosterone replacement. The level of VEGF mRNA decreased after gonadectomy and hormone replacement intensified this effect as the VEGF mRNA levels decreased more after the hormone replacement.

Our results showed that the reduction in testosterone levels in old mice had an important negative effect on VEGF mRNA levels. However, testosterone replacement in male mice was not sufficient to restore this change and to increase angiogenesis. Interestingly, testosterone replacement caused an important reduction in the expression of VEGF in the heart of male mice although previous studies reported increased VEGF expression caused by testosterone supplementation. Further studies are required to show whether the discrepancy between these results is due to the age of the animals because the

response to the androgen treatment may vary depending on the age of the subject.

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Research Article

Evaluation of the Feasibility, Reversibility and Cardiorespiratory Effects of Epidural Dexmedetomidine in Sedated Dogs Undergoing Orchiectomy

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Abstract

The objective of the present study was to evaluate whether epidural dexmedetomidine (DEX) produces sufficient anti-nociception with reversible sedation and cardiorespiratory changes in sedated dogs undergoing orchiectomy. Twelve male dogs weighing 21.7±5.2 kg were used. Dogs received acepromazine (0.025 mg/kg) and morphine (0.25 mg/kg) intramuscularly and a second dose of morphine (0.125 mg/kg) intravenously (IV). DEX (3 µg/kg) was administered epidurally to all dogs (n=12). After confirming complete sensory blockade of the prescrotal region, orchiectomy was performed. If any discomfort was detected during surgery, 2-3 mL lidocaine 1% (maximum two times) was applied into the painful area. Sixty minutes after epidural application, dogs were randomly assigned to receive either treatment of atipamezole (ATP; n=6) or saline (SAL; n=6) IV. None of the dogs required general anesthesia; however, nine out of twelve dogs received lidocaine. The duration of sensory blockade was significantly shorter in ATP than that of SAL. Heart rate, respiratory rate, and rectal temperature showed significantly lower values compared with base after administration of DEX. Administration of atipamezole reversed sedation, sensory blockade and cardiorespiratory changes. In conclusion, epidural DEX did not produce adequate anti-nociception during orchiectomy in sedated dogs. Sedation, sensory blockade and cardiorespiratory changes induced by epidural DEX can be reversed by IV administration of atipamezole.

Keywords: *Atipamezole, Dexmedetomidine, Epidural anesthesia, Orchiectomy*

Orşiektomi Yapılan Sedasyonlu Köpeklerde Epidural Deksmedetomidinin Fizibilite, Reverzibilite ve Kardiyorespiratuvar Etkilerinin Değerlendirilmesi

Öz

Bu çalışmanın amacı; orşiektomi yapılan sedasyonlu köpeklerde epidural deksmedetomidinin (DEX) geri dönüşlü sedasyon ve kardiyo-respiratuvar değişiklikleri ile yeterli anti-nosisepsiyon üretip üretmediğini değerlendirmekti. Çalışmada ortalama ağırlıkları 21.7±5.2 kg olan on iki erkek köpek kullanıldı. Köpeklere intramüsküler yolla asepromazin (0.025 mg/kg) ve morfin (0.25 mg/kg) ardından intravenöz yolla (IV) ikinci bir doz morfin (0.125 mg/kg) uygulandı. Tüm köpeklere (n=12) epidural yolla DEX (3 µg/kg) uygulandı. Prescrotal bölgenin tam duyusal blokajı sağlandıktan sonra orşiektomi operasyonu yapıldı. Ameliyat sırasında herhangi bir rahatsızlık tespit edilirse, ağrılı bölgeye 2-3 mL %1'lik lidokain (en fazla iki kez) uygulandı. Epidural uygulamadan altmış dakika sonra, köpekler IV atipamezol (ATP; n=6) veya salin (SAL; n=6) tedavisi alacak şekilde rastgele gruplandırıldı. Köpeklerin hiçbirinde genel anestezi uygulanması gerekmedi; ancak, on iki köpekten dokuzuna lidokain uygulandı. ATP'de duyusal blokajın süresi, SAL'dan daha kısaydı. Kalp atış hızı, solunum hızı ve rektal sıcaklık, DEX uygulamasından sonraki ölçümler ile karşılaştırıldığında önemli ölçüde düşüş gösterdi. Atipamezol uygulaması, sedasyon, duyusal blokaj ve kardiyorespiratuvar değişiklikleri tersine çevirdi. Sonuç olarak, epidural DEX, sedasyon uygulanan köpeklerde orşiektomi sırasında yeterli anti-nosisepsiyon etki üretmediği belirlendi. Epidural DEX'in neden olduğu sedasyon, duyusal blokaj ve kardiyorespiratuvar değişiklikler IV atipamezol uygulamasıyla tersine çevrilebilir.

Anahtar sözcükler: *Atipamezol, Deksmedetomidin, Epidural anestezi, Orşiektomi*

INTRODUCTION

Orchiectomy, a common procedure performed in domestic species, is considered a painful surgery in veterinary practice [1]. This surgery is performed under general and/or local anesthesia, with local anesthesia being more common in large animals [2-4]. Using local anesthesia would reduce both the costs and complications associated with general

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anesthesia and provide adequate intra- and postoperative analgesia. Recently, there have been some interests in performing various procedures, from minimally invasive surgeries such as orchiectomies to more complicated procedures such as orthopedic operations, by or in combination with local anesthesia in small animals [5-7].

Epidural anesthesia can be employed in a variety of painful conditions to provide anesthesia and analgesia in procedures involving the pelvis, tail, perineum, pelvic limb, and abdomen [8]. Epidural anesthesia could potentially be used alone to provide anesthesia for some surgeries including orthopedic operations and cesarean sections; however, deep sedation is recommended in these situations [9]. Epidural anesthesia could be achieved using different agents and combinations such as local anesthetics, opioids, and/or α_2-agonists [9,10].

Dexmedetomidine (DEX), the active enantiomer of the racemic mixture medetomidine, is the newest available α_2-agonist in small animals and it is considered to be at least two times as potent as medetomidine in dogs [11]. Dexmedetomidine is used as a sedative and an analgesic in small animals [12]. When used as a premedication in dogs, intravenous administration and/or infusion of DEX decreased propofol and inhalation anesthetic requirement in a dose-dependent manner [13-16]. Epidural administration of DEX in isoflurane-anesthetized dogs has also had a dose-related MAC-sparing effect [17].

The ability to antagonize α_2-agonists has made this class of pharmacological agents an appropriate choice in veterinary medicine. Among α_2-adrenergic receptor antagonists, atipamezole is used more frequently in small animal practice. Although atipamezole has been employed for reversing changes induced by epidural xylazine [18,19], its effects on epidurally applied DEX have not yet been evaluated.

The first objective of the present study was to evaluate the feasibility and cardiorespiratory effects of using epidural administration of DEX to perform orchiectomy in sedated dogs. The second objective was to assess the effects of intravenous (IV) administration of atipamezole on sedation, sensory blockade and cardiorespiratory variables after epidural application of DEX. We hypothesized that epidural administration of DEX would provide sufficient anti-nociception during orchiectomy in sedated dogs. Moreover, intravenous atipamezole would reverse sedation, sensory blockade and cardiorespiratory changes produced by epidural application of DEX.

MATERIAL and METHODS

Animals

Twelve adult male mongrel dogs weighing 21.7±5.2 kg and aged 1.5-2.5 years old were used in a randomized design. The animals were transferred to the Veterinary Hospital at least two weeks before the study and maintained under the same conditions in individual cages. Food and water were provided twice per day and *ad libitum*, respectively. Health status was established by a thorough physical examination, complete blood count and total protein. The animals were fasted overnight, but had free access to water until two h prior to the beginning of the procedures. All experiments were started in the morning (09:00-10:00 AM) and finished by 02:00 PM. The Animal Care and Research Committee of Shahid Chamran University of Ahvaz, Ahvaz, Iran, approved all the procedures in the current study [95/3/24/92777].

Study Design

Animals received acepromazine (ACE; 0.025 mg/kg; Alfasan, Woerden, Holland) and morphine (MOR; 0.25 mg/kg; Darou Pakhsh, Iran) into the bulk of hamstring muscles. Thirty min later, a 20 G catheter was placed into the left cephalic vein connected to NaCl 0.9% infusion at a rate of 10 mL/kg/h. After injection of one mL lidocaine 1% (Aburaihan Pharma Co., Iran) subcutaneously, a 20 G catheter was also placed into the right dorsal pedal artery. Forty min after administration of sedatives, a half of the first dose of MOR (0.125 mg/kg) was administered IV, and the animals were positioned onto an operating table in sternal recumbency with legs stretched forward. The lumbosacral area was identified, clipped and prepared aseptically. A 22 G 3.8 cm hypodermic needle with the bevel directed cranially was advanced toward the lumbosacral epidural space until it encountered the floor of the canal. Then, the needle was slightly withdrawn 1-2 mm. The correct positioning of the needle was confirmed by a positive hanging drop test. DEX (Hospira, Inc., USA) was administered epidurally at the dose of 3 µg/kg, over about one min (n=12). The Caution was taken to avoid displacement of the needle as evaluated by the lack of resistance against injection. The final volume of the administered drug was set at 0.22 mL/kg using saline. To provide uniform distribution of the drugs, dogs were maintained in sternal recumbency for at least 10 min. All epidural injections were performed by one investigator (H.I.R).

By ensuring sufficient sensory blockade of the prescrotal area, dogs were positioned in right lateral recumbency. The surgery area was clipped and prepared aseptically. Orchiectomy was performed via the prescrotal technique with a single skin incision. All procedures were performed by a veterinary surgery resident who was not aware of the treatments. Dogs that were not sedated adequately at the beginning of the orchiectomy were excluded from the study. When animals showed any pain-related signs during orchiectomy including any discomfort, grunting and changes in cardiorespiratory parameters, 2-3 mL of lidocaine 1% was splashed or injected into the painful area including the prescrotal skin, subcutaneous tissues, and spermatic cord, and after about 5 min, surgery was continued. If the animals showed pain again, the second

2-3 mL of lidocaine 1% was administered in the same manner. If the dogs showed pain-related responses for the third time, propofol (4 mg/kg; B Braun, Melsungen, Germany) and midazolam (0.2 mg/kg; Exir, Pharmaceutical Co., Iran) prepared previously, were administered IV to continue the procedure under general anesthesia. After accomplishing the surgery, animals were positioned in sternal recumbency and maintained in this position until 60 min had elapsed after epidural administration. At this time, and after administration of ketoprofen (2 mg/kg; Razak, Tehran, Iran), animals received either treatment with atipamezole (ATP; 0.03 mg/kg; Laboratorios Syva, Spain; n=6) or saline (SAL; n=6) IV, brought to the total volume of two mL. Animals received 100% oxygen, after epidural administration until the end of the surgery, via a face mask at the rate of 100 mL/kg/min using a non-rebreathing circuit system. Using a blanket over the animals, it was attempted to maintain rectal temperature above 37°C, during the period of after epidural administration through epidural recovery. At the time of treatment administration, oxygen delivery and fluid therapy were interrupted.

Assessments and Data Collection

Sedation was qualitatively scored at 30 min after administration of ACE-MOR, at the end of surgery, and at the completion of the experiment for each dog, using a numerical scaling system: 1- mild, 2- moderate, 3- deep, and 4- very deep. The onset and duration of sensory blockade were recorded. Sensory blockade was confirmed when responses to superficial and deep pin prick test using a 25 G needle, and pressure applied by a hemostat closed at the second ratchet for 15 s, was negative. Duration of surgery, from draping until last suture knotting, was also recorded.

Heart rate (HR), non-invasive mean arterial blood pressure (MAP), respiratory rate (f_R), and rectal temperature (RT) were measured and recorded at base, at 30 min after administration of ACE-MOR (sedation), and at 5, 10, 20, and 40 min after epidural administration, and 5, 15, 30, and 60 min after treatments administration. HR was counted through hearing by a stethoscope. MAP was measured and recorded by applying an appropriate blood pressure cuff (with the cuff width to metatarsal circumference ratio of at least 40%) over the left dorsal pedal artery, connected to a multi-parameter monitoring system (Burtons, Guardian Industrial Estate, UK). f_R was measured by observation of chest movements, and RT was recorded through a digital thermometer. Lead II electrocardiogram (ECG) was recorded at 5, 20, and 40 min after epidural administration and 5 and 15 min after treatment administration.

Blood samples were taken anaerobically through the dorsal pedal artery at 30 min after administration of ACE-MOR (sedation), and at 5, 20, and 40 min after epidural administration and at 15 and 30 min after administration of treatment. One mL blood from the arterial catheter was removed, a 0.5 mL test sample was collected into a heparinized syringe, and then the first removed sample with 0.5 mL heparinized saline was flushed into the catheter. Using a calibrated gas analyzer (Edan i15, Edan instrument Inc., China), pH, partial arterial pressure of oxygen (PaO_2), partial arterial pressure of carbon dioxide ($PaCO_2$), bicarbonate ion concentration (HCO_3^-) and base excess (BE) of collected samples were measured. All date were measured and/or recorded by one investigator.

Statistical Analysis

Statistical analysis was undertaken by SPSS version 22 (IBM Corporation, NY, USA) for Windows. Sedation scores were compared using the Mann-Whitney U-test. Comparison of body weights, times to onset of sensory blockade and duration of sensory blockade of the scrotal region after administration of treatments, duration of surgical procedure, HR, f_R, RT, MAP, and blood gas parameters, between two treatments, were performed by Independent-Samples t-test. For comparison of HR, f_R, RT, MAP, and blood gas parameters over time in each group, analysis of variance (ANOVA) for repeated measures and Duncan`s test were employed. $P<0.05$ was considered as significant level.

RESULTS

Body weights were not significantly different between the two treatments: 21.5±4.5 for ATP, vs. 21.9±5.8 for SAL. One dog did not show sufficient sedation and therefore was excluded from the study and was replaced. Sedation was assessed as adequate to perform surgery in the other dogs. No difficulty was encountered in locating the epidural site and injection the drugs. Two dogs did not show complete sensory blockade after 30 min of epidural administration of DEX. The procedure was discontinued and the experiment was repeated for these dogs one week later. The remaining dogs tolerated the surgical procedure without any complications and all recovered uneventfully.

None of the dogs needed general anesthesia to perform or complete the surgical procedure; however, only three out of twelve dogs tolerated orchiectomy without any pain-related responses. Six out of twelve dogs received the first 3 mL of 1% lidocaine; and, three out of twelve dogs required the second 3 mL of 1% lidocaine. Lidocaine was administered in four dogs in the prescrotal skin, two dogs in the subcutaneous tissue plus spermatic cord, and three dogs in the spermatic cord.

Sedation score at the completion of the experiment was significantly lower in ATP than those of SAL at 30 min after administration of ACE-MOR and at the end of the surgery ($P<0.01$). The time to onset and duration of sensory blockade of the scrotal region and duration of surgery are showed in *Table 1*. The duration of sensory blockade of the scrotal region after IV administration of treatments was significantly shorter in ATP than that of SAL ($P<0.01$).

Data related to HR, MAP, f_R, RT and blood gas parameters are summarized in *Table 2*. HR decreased significantly after epidural administration when compared with the base (P<0.05); however, it returned to base value after atipamezole administration but not after saline administration. While HR progressively increased after atipamezole administration, it remained relatively stable at lower values compared with the base in the saline group. Comparison of HR between the two treatments showed significantly higher values after atipamezole administration than those of saline (P<0.05). MAP was relatively stable during experiment and a significant difference was not detected after either DEX or treatment administration (P>0.05). Sinus arrhythmia and sinus bradycardia (five dogs in each group) with or without second-degree atrioventricular (AV) block, sinoatrial (SA) block and/or sinus arrests were observed after administration of DEX. After treatment, the aforementioned dysrhythmias were occasionally detected in both groups.

Respiratory rate decreased significantly after ACE-MOR administration and this decrease remained throughout the evaluation period (P<0.05). Respiratory rate after atipamezole administration was significantly higher than that of saline at several time points (P<0.05). A slight decrease in RT was observed over time when compared with the base values. Regardless of treatment, this decrease was significant throughout the evaluation period when compared with base (P<0.05). Although it was not significant, RT was relatively higher after treatments in ATP than SAL.

No significant change was detected in pH over time (P>0.05). Hypoxemia (defined as PO_2<95 mmHg) was observed at the sedation time point, which was resolved by oxygen supplementation after epidural administration. After interruption of oxygenation at treatment administration, hypoxemia was not detected and values were within the normal range. Compared with other time points, significantly lower values of PCO_2 were observed at the sedation time point (P<0.05). No significant changes in HCO_3^- and BE were observed over time (P>0.05). There were no significant differences concerning blood gas values between treatments (P>0.05).

Table 1. *Sedation scores, time to onset and duration of sensory blockade of scrotal region, and duration of surgery in dogs received epidural DEX (n=12) and intravenous atipamezole (ATP; n=6) or saline (SAL; n=6) undergoing orchiectomy*

Variable	ATP	SAL
Sedation 1 (30 min after administration ACE-MOR)	2 (1-2)	
Sedation 2 (at the end of the surgery)	2 (2-4)	
Sedation 3 (at the completion of the experiment)	0 (0-1) *,δ	2 (1-2)
Time to onset of sensory blockade (min)	15.7±6.0	
Duration of sensory blockade (min)	67±7.2 *	89.3±11.0
Duration of surgery (min)	27.9±4.0	

** Significantly different between treatments (P<0.05), **δ** significantly different from Sedation 1 and Sedation 2*

Table 2. *Heart rate (HR), mean arterial blood pressure (MAP), respiratory rate (f_R), rectal temperature (RT) and blood gas parameters (mean ± SD) in dogs received epidural dexmedetomidine (n=12) and intravenous atipamezole (ATP; n=6) or saline (SAL; n=6) undergoing orchiectomy. BE: base excess*

Variable	Base	Sedation	Epidural	5 min	10 min	20 min	40 min	Treatment	Group	5 min	15 min	30 min	60 min
HR (beats/min)	60±9	61±14 †		38±5 †	36±4 †	33±5 †	40±9 †		ATP	48±14 *	48±8 *	49±13 *	59±15 *
									SAL	37±5 †,δ	39±3 †,δ	38±3 †,δ	36±5 †,δ
MAP (mmHg)	78±10	80±9		75±9	69±6	90±11	87±12		ATP	86±8	76±12	85±12	87±14
									SAL	78±12	84±11	85±4	85±15
f_R (breaths/min)	28±9	17±3 †		12±3 †	12±4 †	13±3 †	12±3 †		ATP	15±5 †	17±4 †,*	16±6 †,*	15±1 †
									SAL	13±4 †	12±3 †	11±5 †	12±5 †
RT (°C)	38.8±0.3	38.4±0.3 †		38.1±0.3 †	38.1±0.2 †	38.0±0.3 †	37.8±0.5 †		ATP	37.8±0.5 †	37.7±0.7 †	37.9±0.5 †	37.8±0.2 †
									SAL	37.7±0.1 †	37.5±0.2 †	37.5±0.2 †	37.3±0.2 †
PH	ND	7.35±0.01		7.33±0.01	ND	7.34±0.01	7.33±0.02		ATP	ND	7.34±0.04	7.37±0.01	ND
									SAL	ND	7.32±0.02	7.33±0.04	ND
PO_2 (mmHg)	ND	90±6		447±66 ‡	ND	446±87 ‡	424±68 ‡		ATP	ND	96±3	98±4	ND
									SAL	ND	97±2	97±5	ND
PCO_2 (mmHg)	ND	31±4		40±3 ‡	ND	39±6 ‡	38±6 ‡		ATP	ND	33±7	33±2	ND
									SAL	ND	34±2	35±3	ND
HCO_3^- (mEq/L)	ND	17±2		20±3	ND	21±4	19±2		ATP	ND	20±3	19±2	ND
									SAL	ND	17±2	17±1	ND
BE	ND	-8±3		-5±3	ND	-5±4	-7±3		ATP	ND	-7±3	-8±2	ND
									SAL	ND	-9±2	-9±1	ND

ND: *not determined,* ***†*** *Significantly different from base (P<0.05),* ***δ*** *Significantly different from base in SAL group (P<0.05),* ***‡*** *Significantly different from Sedation (P<0.05), * Significantly different between treatments (P<0.05)*

DISCUSSION

The results of the present study demonstrated that epidural administration of DEX at the dose of 3 µg/kg did not provide sufficient anti-nociception during orchiectomy in sedated dogs. Sedation produced by ACE and MOR increased to moderate to deep levels after DEX application. Epidural DEX decreased HR over time and had no significant effect on MAP. Both f_R and RT showed lower values after epidural administration of DEX compared to those of base. Administration of atipamezole intravenously (0.03 mg/kg), in comparison to saline, decreased the sedation level, reduced the duration of sensory blockade, increased HR, and had no significant effect on MAP. Respiratory rate was higher at several time points, and RT showed non-significant higher values after atipamezole administration when compared with saline. Blood gas changes were not clinically significant in the current study.

Epidural application of α_2-adrenergic agonists such as DEX is of interest as α_2-adrenergic receptors have been identified in the spinal cord and showed to have presynaptic and postsynaptic actions on the modulation of pain [20,21]. These effects appear to be different from the vasoconstrictive action of such drugs [9]. Furthermore, epidural administration of low doses (3.3 µg/kg) of DEX in comparison to IV administration of relatively high doses (10 µg/kg) has been showed that potentiates and prolongs the analgesic properties of DEX in dogs (240 min analgesia compared with 90 min) [22]. Therefore, epidural application of DEX might be associated with lower occurrence and less severe adverse effects including bradycardia and reduction in cardiac output than what would be observed after IV administration [13,22]. The reversibility of α_2-adrenergic agonists is another factor that makes these pharmacologic agents as an appropriate choice in clinical use since this feature could potentially reduce hospitalization and the risks associated with administration of high doses.

In the current study, epidural administration of DEX was employed to provide anti-nociception during orchiectomy in dogs. Although, assessment of sensory blockade in the skin of the surgical site with superficial and deep pin prick test and pressure applied by a hemostat elicited no responses, anti-nociception was not sufficient for pain induced by incision and manipulation of testicles and spermatic cords. Pohl et al.[23] have already showed that epidural application of DEX at a dose of 2 µg/kg with lidocaine could not produce sufficient analgesia during ovariohysterectomy in dogs and all of the animals were finally submitted to isoflurane anesthesia. In an earlier study in dogs, 1.5, 3 and 6 µg/kg epidural DEX yielded isoflurane MAC-sparing effect in a dose-dependent manner [17]. Since the authors of the present investigation were not aware of the effective dose of epidural DEX in conscious dogs, 3 µg/kg DEX was used to avoid undesirable cardiovascular consequences. Considering that this dose was sufficient to prevent pain responses at the time of evaluation, a greater dose might increase the likelihood of successful orchiectomy under epidural anesthesia with DEX. One of the limitations of the current study is that just one dose rate of DEX was examined.

It is common to observe sedation after epidural administration of α_2-agonists as a result of systemic absorption of the drug [9]. Moreover, co-administration of opioids and α_2-agonists might produce more profound and longer lasting sedation [24,25]. In the present study, epidural administration of DEX enhanced previously produced sedation; even though, the probable synergetic effects of MOR and DEX need to be investigated by further studies.

Cardiovascular effects of DEX, as with other α_2-agonists, involve decreased HR, decreased cardiac output and increased systemic vascular resistance [13,26]. In the studies by Camagnol et al.[17] and Pohl et al.[23], HR decreased after epidural administration of DEX in dogs. Similar to previous studies, in this investigation HR decreased after epidural application of DEX. The decrease in HR after administration of α_2-agonists is attributed to increased systemic vascular resistance (in the early phase) and decreased central sympathetic outflow (in the later phase) [26]. Blood pressure, after administration of α_2-agonists, has a biphasic manner in which it increases transiently followed by prolonged hypotension [26,27], however, it has been speculated that α_2-agonists might not be linked to hypotension in dogs [26]. Indeed, in a number of investigations blood pressure remained in an acceptable range after administration of α_2-agonists [11,13,23,28,29], suggesting that the predominant vasoconstriction produced by α_2-agonists likely prevents severe hypotension in dogs [26]. In the current study, MAP remained relatively constant after epidural administration of DEX; however, it should be noted that MAP was at or below the limits of the reported normal range all over the evaluation period. This finding might be explained by using of indigenous dogs with lower normal MAP in the present investigation.

A dose-dependent decrease in f_R and minute volume were reported after α_2-agonists administration in humans [27]. Significant reduction in f_R in dogs given medetomidine (30 µg/kg, IM) has also been reported by Ko et al.[28]. In contrast, epidural (4 µg/kg) and IM (50, 100 and 150 µg/kg) administration of DEX did not change f_R in cats [30,31]. In the current study, f_R remained at lower values in comparison to base after epidural administration of DEX; nevertheless, blood gas values showed no clinical significance. Similar results have been obtained in dogs with clinically used doses of xylazine in which despite observing a decrease in f_R, no changes in pH, PaO_2 and $PaCO_2$ were recorded. The authors of the latter study have attributed the results to concomitant increase in tidal volume with decrease in respiratory rate that consequently resulted in constant minute ventilation [32]. The same mechanism might have been played a role in the present study. Lower values of PaO_2

at the sedation time point in the current study increased after oxygenation which indicated that supplemental oxygenation could be considered as an important aspect in ACE-MOR sedated dogs that received epidural DEX.

A gradual decrease in RT was observed after sedation and this decrease continued after epidural application of DEX. A slight but progressive decrease in RT was also detected after epidural administration of DEX in cats [31]. A decrease in RT was observed in dogs treated with IM DEX; however, this decrease remained on average within clinically acceptable range [33]. Hypothermia induced by α_2-agonists has been attributed to decrease in heat production due to muscle relaxation or because of noradrenergic hypothalamic mechanisms of thermoregulation [34,35]. Based on the results of the current study and other investigations, precise monitoring and maintaining the body temperature are recommended in dogs receiving α_2- agonists.

Intravenous atipamezole was employed to reverse clinical and pharmacologic effects of DEX in dogs. In an earlier study, atipamezole was able to antagonize the sedative and not the analgesic effects of epidural xylazine in dogs [18]. In contrast, epidurally and IV administered atipamezole have reversed both sedation and analgesia in cattle that received epidural xylazine [19]. In a study in dogs, IM atipamezole has completely reversed sedation and analgesia induced by IM DEX; however; HR and f_R did not return to presedation values after atipamezole administration [33]. In the current study, atipamezole when compared with saline, decreased the duration of sensory blockade, reduced sedation and increased HR and f_R. Although HR returned to the presedation values in ATP, f_R did not reach to the base in this group. As with Granholm et al.[33], the inability of atipamezole to reverse changes in f_R could be attributed to the attenuation of sympathetic nervous system activation and lower concentration of stress-related hormones inducing after α_2-agonists administration reported by other investigators [36].

In this study, epidural application of DEX did not provide sufficient anti-nociception during orchiectomy in sedated dogs at the dose rate tested. Epidural DEX produced a degree of sedation in dogs and induced some changes in cardiorespiratory parameters. Intravenous administration of atipamezole decreased the sedation level, reduced the duration of sensory blockade and reversed cardio-respiratory changes.

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Research Article

Effect of Culture Medium Treated with Non-thermal Plasma Energy on the Growth and Viability *In-vitro* of Fibroblast Cells from Asian Elephants *(Elephas maximus)*

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Abstract

Non-thermal plasma (NTP) is being developed for a wide-range of medical applications such as improvement of wound healing, elimination of infective microorganisms, and treatment of cancer. This study investigated the effect of culture medium exposed to NTP on the proliferation in-vitro of skin fibroblasts from Asian elephants. Dulbecco's Modified Eagle's Medium (DMEM) was used as culture medium and was exposed to NTP with three different intensities. The NTP reactive species Nitrite (NO^{2-}) was measured in the treated medium before addition to cells. Fibroblasts were incubated for 24 h with NTP-treated complete medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic/antimycotic. Cell proliferation, the number of cells and viability rate were analysed using flow cytometry 24, 48 and 72 h after the start of the incubation. The proliferation rate of fibroblasts incubated with NTP treated medium was significantly higher ($P<0.05$) than controls and increased in a dose-dependent manner with increasing amount of NTP. Incubation of fibroblasts with NTP did not reduce their viability even at the highest dose of NTP. Culture medium treated with NTP energy may be used to improve healing of skin wounds in elephants. This study successfully shows that the medium treated with NTP was able to stimulate elephant skin fibroblasts proliferation and increase the total cell count but did not reduce cell viability in vitro. Containing buffering agent in culture media might reduce the effect of ROS generated by NTP. This might prevent using high dose of NTP to cause cell apoptosis and induce cell necrosis in this study. Future studies on the skin of living elephant are encouraged to develop more effective and optimum treatment conditions.

Keywords: *Asian elephant, Non-thermal plasma, Culture, Fibroblasts, Skin*

Termal Olmayan Plazma Enerjisi Uygulanan Besiyeri Ortamının Asya Fillerinin *(Elephas maximus)* Fibroblast Hücrelerinin Büyüme ve Canlılığı Üzerine *In-vitro* Etkisi

Öz

Termal olmayan plazma (NTP), yara iyileşmesinin hızlandırılması, enfektif mikroorganizmaların eliminasyonu ve kanser tedavisi gibi çeşitli tıbbi uygulamalar için geliştirilmektedir. Bu çalışmada NTP'ye maruz burakılan besiyeri ortamının Asya fillerinden elde edilen deri fibroblastlarının in vitro proliferasyonu üzerindeki etkisi araştırıldı. Bu amaçla Dulbecco'nun Modifiye Eagle's Medium'u (DMEM) besiyeri olarak kullanıldı ve üç farklı yoğunlukta NTP'ye maruz bırakıldı. NTP reaktif türleri Nitrit (NO^{2-}) hücrelere eklenmeden önce uygulandığı besiyerinde ölçüldü. Fibroblastlar, 24 saat boyunca, %10 Fetal Sığır Serumu (FBS) ve %1 antibiyotik/antimikotik içeren NTP ile muamele edilmiş besiyerinde inkübe edildi. İnkübasyonun başlamasından 24, 48 ve 72 saat sonra hücre proliferasyonu, hücre sayısı ve canlılık oranı akış sitometrisi kullanılarak analiz edildi. NTP ile muamele edilmiş besiyerinde inkübe edilen fibroblastların proliferasyon hızı, kontrol grubundan anlamlı olarak daha yüksek bulundu ($P<0.05$) ve artan NTP uygulaması ile birlikte doz bağımlı olarak arttığı belirlendi. Fibroblastların NTP ile inkübasyonu, en yüksek NTP dozunda bile canlılık oranını azaltmadı. Fillerde cilt yaralarının iyileşmesini hızlandırmak için NTP enerjisi uygulanan besiyeri kullanılabilir. Bu çalışma, NTP uygulanan besiyerinin fil derisi fibroblastlarının proliferasyonunu uyarabildiğini ve toplam hücre sayısını arttırdığını, bununla birlikte *in-vitro* olarak hücre canlılığını azaltmadığını göstermiştir. Besiyerinin tampon madde içermesi, NTP tarafından üretilen Reaktif Oksijen Türlerinin etkisini azaltabilir. Mevcut çalışmada bu durum hücre apoptozisine neden olmak ve hücre nekrozunu indüklemek için yüksek NTP dozlarının kullanılmasını engelleyebilir. Daha etkili ve optimum tedavi koşullarının geliştirilmesi için canlı fillerin derisinde yapılacak çalışmalar teşvik edilmelidir.

Anahtar sözcükler: *Asya fili, Termal olmayan plazma, Besiyeri, Fibroblastlar, Deri*

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INTRODUCTION

Wounds to the skin of Asian elephants *(Elephas maximus)*'s are a common health and welfare problem [1]. Physical damage to the skin of elephants frequently results in wounds. Other causes of skin wounds may include sunburn and nutritional imbalances. Elephant's skin wounds are particularly susceptible to infection and heal slowly because the skin is thick (2.0-2.5 cm) and without sebaceous glands. Compared with other mammals the skin of elephants is dry and takes longer to heal [1]. Clinical treatment of elephant skin wounds leads to a higher risk of drug resistance and a high cost for effective drugs. Consequently, there is on-going pressure to find improved treatment for elephant skin wounds.

By definition, non-thermal plasma (NTP) is partially ionized gas where the energy is stored mostly in the free electrons and the overall temperature remains low. The use of NTP has been developed as a medical option to treat wounds in humans [2]. NTP generates reactive oxygen species (ROS), reactive nitrogen species (RNS), UV-radiation, and electric fields [3]. Most of NTP's effects on the biological system are related to reactive species including H_2O_2, O_3, O_2^-, NO, NO_2, N_2^-, and OH which may enhance wound healing [4-6]. It is known that reactive species, free radicals and some ground state molecules, are produced by mammalian immune system cells, macrophages and neutrophils, and can defend against bacteria and viruses, and also regulate cellular functions [7,8]. Furthermore, reactive species are involved in the regulation of signalling pathways such as growth factors and cytokine receptors [9]. In physiological processes, reactive species play a role in the regulation of vascular contraction, blood coagulation, angiogenesis, inflammation, immune system response, and nerve impulse transmission. Also at cellular level, reactive species regulate cell differentiation, division, migration, and apoptosis by controlling cell-to-cell adhesion, biosynthesis of growth factors, and collagen production [9].

Non-thermal plasma has been used effectively in other medical fields such as to remove dental biofilms, to eliminate oral pathogens, to induce apoptosis of malignant cells [10], inhibit the growth of cancer cells [11] and to stop bleeding [12-14]. NTP may promote wound healing and tissue regeneration by increasing fibroblast proliferation by the release of growth factors such as fibroblast growth factor-7 [15]. In recent years, NTP has been described as an effective wound therapy because it has antimicrobial activity [12,16], it reduces inflammation [12,17], promotes wound healing [16] and does not kill eukaryotic cells [18,19]. There have been many studies of the effects of NTP on wounds in humans [16,20-29], and animals such as rats [30,31], mice [32-34] and pigs [35], but no record of application in elephants. The direct use of NTP for the treatment of wounds in elephants is difficult in field practice because of the difficulty of moving and restraining such large animals. Alternatively, using media treated with high energy NTP to treat skin wounds in elephants may be safer and more convenient for veterinary professionals and animal handlers. For experiment on living elephants might be difficult in terms of collecting optimal data from uncontrollable of wound size, location and type on each elephant. Therefore, it is hard to acquire elephant wounds that have similar cause, size, location as well as type of wound. Creating wound for the study in elephant is hardly possible especially in captive elephant. Apart from that, controlling wound hygiene in elephants is rather difficult due to the elephant behaviour to play sand or dirt.

In recent studies, NTP treatment has successfully been used to decontaminate water samples containing biological and chemical agents [36-39]. Furthermore, NTP treated culture media had an effect on cell growth and morphology in cell culture model [40]. When liquid is exposed to NTP, radical species from NTP outflows into liquid and induce chemical change which causes the treated liquid to then have NTP effects which are almost similar to those caused by direct exposure [41]. Our hypothesis is that culture medium treated with NTP will generate ROS that will enhance the proliferation of skin fibroblasts from Asian elephants.

MATERIAL and METHODS

The reagents, the main components of reagents and the medium formulations together with their vendors and concentrations used in this study are listed in *Table 1*.

Isolation and Culture Elephant's Primary Skin Fibroblast Cells

Skin fibroblast cells were obtained from the ear skin of three Asian elephant carcasses (age range 35 to 55 years old) within 24 h of death. Skin samples, size 3×3 cm^2, were brought to the laboratory in transport medium at 0-4°C within 3 h after collection. Ear skin fibroblasts were prepared using the protocol described by Siengdee et al.[42]. In brief, skin tissue samples were cleaned in phosphate buffered saline (PBS) and chopped into small pieces. Precipitated skin tissues were digested with collagenase type II solution and cultured with explant medium containing 20% FBS at 37°C and 5% CO_2. From the 3rd passage onwards fibroblasts were cultured in culture medium containing 10% FBS and 1% antibiotic/antimycotic at 37°C and 5% CO_2 and routinely trypsinized with 0.25% trypsin/EDTA. Fibroblasts at 2nd-4th passage were frozen in liquid nitrogen for long-term storage and passages 2nd to 6th were used for this study *(Fig. 1)*.

NTP Treatment

Non-thermal plasma experiments were performed using atmospheric pressure NTP dielectric barrier discharge (DBD) TS200 (Engineering Production Equipment Medical, Firenze, Italy) with a DBD helium jet direct plasma probe with an overall 22 mm diameter containing an internal

Table 1. *Components of reagent and medium formulations*

Name	Components	Vendor and Stock Concentration
Culture medium (1.000 mL)	dH_2O + DMEM powder + 4.767% Hepes (w/v) + 3.75% Sodium bicarbonate (w/v) + 1 × antibiotic/antimycotic	• Dulbecco's modified Eagle's medium; DMEM (Gibco; Thermo Fisher Scientific) • Antibiotic/antimycotic (100X) (GibcoTM; Thermo Fisher Scientific, Waltham, MA, USA) stock concentration contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of amphotericin B • Heat-inactivated fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria) • Collagenase type II (Sigma- Aldrich, St. Louis, Mo, USA) stock concentration contains 1 mg/mL of collagenase II in DMEM w/o serum • Trypsin-EDTA (0.5%), no phenol red (Gibco; Thermo Fisher Scientific) • Phosphate-buffered saline (PBS) (10×) (Gibco; Thermo Fisher Scientific) • Gentamicin (50 mg/mL) (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) • HEPES (Sigma-Aldrich, St. Louis, Mo, USA) • Sodium bicarbonate (Sigma-Aldrich, St. Louis, Mo, USA) • MTT (Bio Basic Inc, Markham, ON, Canada) • DMSO (Sigma-Aldrich, St. Louis, Mo, USA) • N-(1-Naphthyl)ethylenediamine dihydrochloride; NAD 2HCl (Fisher Scientific, Loughborough, Leicestershire, UK) • Phosphoric acid (Carlo Erba Reagents, Barcelona, Spain)
Complete medium	Culture medium + 10% FBS + antibiotic/antimycotics w/o NTP treatment	
NTP-treated complete medium	Culture medium + 10% FBS+antibiotic/antimycotics w/NTP treatment	
Explant medium containing	Culture medium + 20% FBS + 1 × antibiotic/antimycotic	
Transportation medium	Culture medium + 10×antibiotic/antimycotic	
Collagenase solution	Culture medium + 10% collagenase type II (v/v) + 10 × antibiotic/antimycotic	
Washing with PBS	PBS + 10×antibiotic/antimycotic + 50 µg/mL gentamicin	
Routine antibiotic dose	1 × antibiotic/antimycotic (containing 100 units/mL of streptomycin, 100 units/mL of penicillin and 0.25 µg/mL of amphotericin B)	
MTT solution	Culture medium + 0.5% MTT (w/v)	
0.25 % trypsin/EDTA	PBS + 0.25% trypsin/EDTA	
Colour reagent solution	dH_2O + 10% 85% H_3PO_4 (v/v) + 0.1% NAD 2HCl (w/v)	

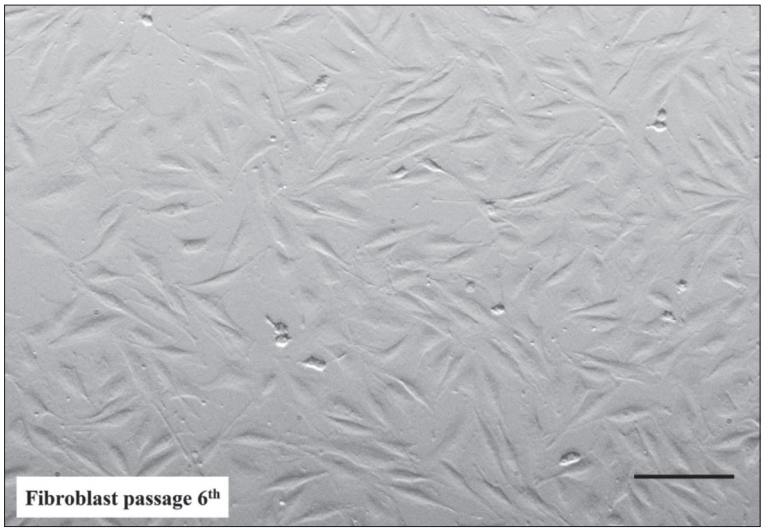

Fig 1. Elephant's skin fibroblast cells used for this study. Scale bar = 20 mm

glass diameter of 10 mm which had an inner core of copper with a diameter of 8 mm. The plasma machine operated at a voltage between 220-240 volts with a frequency of 1.750 KHz. A helium gas flow rate of 1 L/min was used. Six mL of culture medium without FBS and antibiotic/antimycotics was added to a glass container of 40 mm diameter (50 mL beaker washed with deionized water 3 times and sterilized before use) which was placed on a stainless steel table due to its good electrical conductivity *(Fig. 2)*. NTP was generated 0.5 cm above the liquid surface and exposed for 60 sec. Three different treatment conditions were used; low dose (3.24 J/cm^2), medium dose (9.12 J/cm^2) and high dose (15.72 J/cm^2). The FBS and antibiotic/antimycotic were added after NTP treatment in order to prevent denaturation during exposure to plasma. NTP treated culture medium was then supplemented with FBS and antibiotic/antimycotics and is called the complete medium. The NTP treated complete medium was used to treat fibroblast cells immediately. The pH of cell culture was measured before and after exposure to plasma using a Consort C380 pH meter (Consort, Turnhout, Belgium).

Detection of Reactive Species in the Gas Phase

NTP species generated above the liquid cause a complex interaction with the culture medium and change its composition. Transmission of NTP components into the medium leads to the generation of secondary reactive radicals such as nitrate, nitrite and hydrogen peroxide [37-39,43]. The radical species in the NTP above the liquid surface and in the liquid medium were measured. Nitric oxide (NO) and Ozone (O_3) are stable molecules emitted by the gas NTP and result in generation of Nitrites (NO_2^-) and Nitrates (NO_3^-) in NTP treated medium. For the measurement of NO in the NTP, a gas detector (Shenzhen YuanTe Technology, Model SKY2000) was used with a precision of 1 ppb in the range of 0.05 to 100 ppm. For the measurement of O_3, a gas detector (Shenzhen YuanTe Technology model SKY2000) was used with a precision of 2 ppb ranging from 0.05 to 250 ppm. Each measurement was performed in triplicate.

Detection of Reactive Species in Culture Medium Treated With NTP

Comparison between three different NTP treatment doses: In the NTP treated culture medium nitrite (NO_2^-) is one of the reactive species products closely related to Nitric oxide (NO). Nitrite was used to indicate the presence of other

reactive species generated in the medium by the NTP. Nitrite was measured by the presence of the reddish-purple azo dye, formed by the coupling of diazotized sulfanilamide (diazonium salt) with N-(1-naphthyl)-ethylene-diamine dihydrochloride (NED dihydrochloride). The concentration range for this spectrophotometric measurement was 10 to 1.000 µg NO_2^--N/L. Higher concentrations of NO_2^- were determined by sample dilution [44]. Photometric measurements were made at 543 nm using a DU 730 UV-Vis Spectrophotometer (Beckman Coulter, Inc.; USA).

To determine nitrite generated in NTP treated cell culture medium, a standard curve of nitrite in culture medium without serum was created by plotting absorbance of standard against NO_2^--N concentration. Culture medium exposed to NTP was computed for the concentration of nitrite directly from the standard curve. The concentration of nitrite in NTP complete medium was measured to make a comparison between each NTP treatment instantly after treatment (0 h) and 30 min after treatment.

To compare the NO_2^- concentration created by 3 different NTP intensities, culture media without serum were exposed to a low dose (3.24 J/cm^2), a medium dose (9.12 J/cm^2) or a high dose (15.72 J/cm^2), each performed in triplicate. Colour reagent solution (240 µL) was added and well mixed. The solution was left for 30 min at room temperature before determining the absorbance.

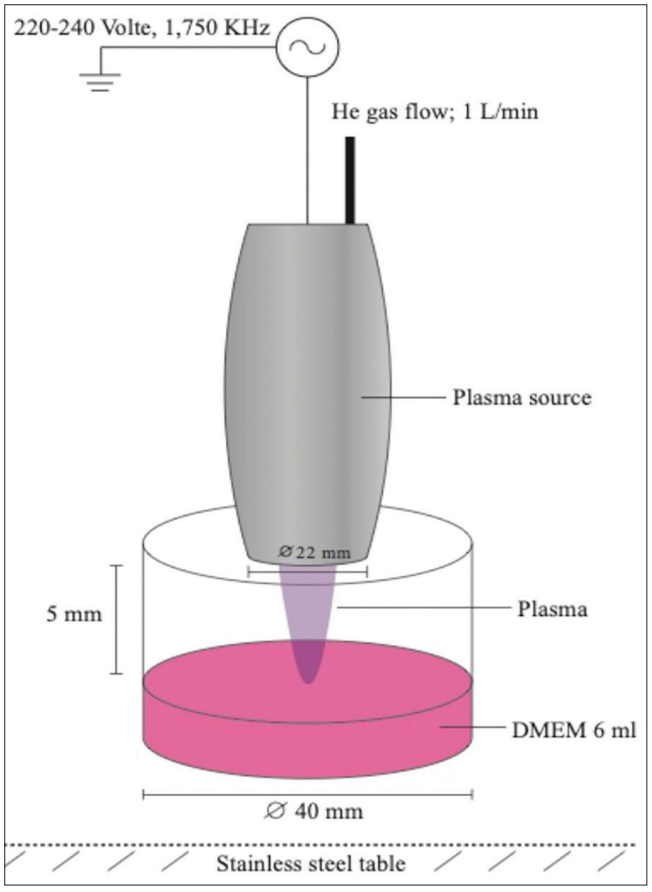

Fig 2. DBD helium jet exposed 6 mL culture medium without serum for 60 sec which contained in 50 ml beaker placed on stainless steel table

Monitoring NO_2^- in treated cell culture media for 24 h: To monitor NO_2^- created by NTP in cell culture medium for 24 h after exposure to NTP at low dose condition (3.24 J/cm^2), the concentration of NO_2^- was measured every 4 h in triplicate for 24 h. At 0 h condition, 240 µL colour reagent solution was added and mixed immediately after being exposed with NTP. The solution was left for 30 min at room temperature before determining the colour absorbance. For the samples at 4, 8, 12, 16, 20 and 24 h conditions, after being exposed to NTP, treated culture medium were left in incubator at 37°C, at 5% CO_2 condition, and measured for the absorbance with calculations to determine the concentration.

Determination of the Period of NTP Incubation Using MTT Assay

The viability of fibroblasts grown in cell medium treated with NTP was determined after different incubation periods. Fibroblasts were grown at a density of 10.000 cells/well in 96 well plates and cultured in complete medium with 10% FBS and 1% antibiotic/antimycotic 200 µL/well for 24 h at 37°C, 5% CO_2. The culture medium (before addition of FBS and antimicrobial addition) was treated with one of three NTP intensities: low dose (3.24 J/cm^2), medium dose (9.12 J/cm^2) and high dose (15.72 J/cm^2). Each medium was treated with NTP for a period of 60 sec. The NTP generating condition was the same as the condition for comparing NO_2 created by 3 different NTP intensities (see above). After seeding for 24 h the incubation medium was replaced with 200 µL NTP-treated complete medium and incubated for a further 24 h. After every 4 h the incubation medium was replaced with new complete medium. For measurement of viability cells were washed once with sterile PBS and then 200 µL MTT solution was added and incubation continued at 37°C, 5% CO_2 for 4 h (cells incubated in culture medium without MTT were used as blank controls). Dimethyl sulfoxide (DMSO) 100 µL was then added to each well and shaken for 5 min. The absorbance at 540 nm was then measured using a microplate reader. The viability of cells incubated in medium treated with NTP was expressed as a percentage of the viability of cells incubated in medium that had not been treated with NTP.

Measurement of the Total Cell Count, Viability and Proliferation of Elephant Skin Fibroblasts Incubated in Medium Treated With NTP

Fibroblasts were grown at a density of 100.000 cells/well in 6-well plates and cultured in completed medium for 24 h. Fibroblasts were incubated in medium that had been treated with zero, low, medium and high intensities of NTP. Consequently 2 mL of NTP-treated completed medium was replaced in each well, for each group of wells in triplicate. Similar to the control group, complete medium without

NTP treatment was continuously replaced and incubated at 37 C, 5% CO_2 condition for 24, 48 and 72 h with changed new complete medium 24 h after incubated with NTP-treated completed medium. Cells were investigated for total cells count, viability rate and proliferation rate using Muse™ cell Analyzer (Merck KGaA; Darmstadt, Germany) at 24, 48 and 72 h.

Total cell count and viability rate were measured by the Muse™ Count & Viability reagent (Merck KGaA; Darmstadt, Germany) which was fluorescent dye-based analysis. Dead and dying cells would lose their membrane integrity and allow the dye to stain the nucleus while live cells did not. This parameter was used to differentiate viable from non-viable cells.

For measuring the cell proliferation rate, the Muse® Ki67 proliferation assay (Merck KGaA; Darmstadt, Germany) was used to determine the percentage of cell's proliferation based on nuclear antigen ki67, which is expressed by proliferating cells in active cell cycle phase (G1, S, G2 and M phase) but absent in the resting phase (G0 phase). The Ki67 proliferation assay utilized ki67 expression to identify proliferating cells and distinguish them from non-proliferating cells or cells stained with IgG1.

Statistical Analysis

The results in this study are shown as a mean value ± standard deviation (SD) of data from three experiments. Measurement of the total cell count, viability and proliferation were performed as three experiments from the three different elephants and each experiment was replicated three times. Statistical significance was estimated by one-way analysis of variance followed by Dunnett T3 post hoc test. A P value <0.05 was considered statistically significant. All calculations were performed using the SPPS version 14.0 for Windows (SPSS, USA).

RESULTS

NO and O_3 Concentrations in the Gas Phase Generated by NTP Above the Liquid

Gas detector was placed at NTP emission point to measure the concentration of reactive radicals generated by NTP. As the NTP energy increased, the concentration of both reactive radicals, NO and O_3, also relatively increased *(Table 2)*. It was observed that using 100% Helium (He) gas flow rate 1 L/min, the concentration of NO was higher than O_3 at each of the three NTP intensities.

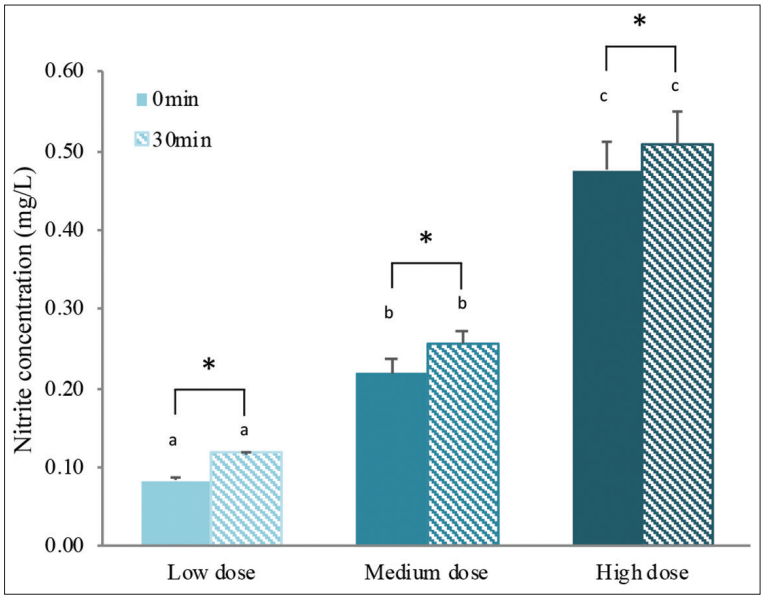

Fig 3. Comparison of nitrite concentration (mg/L) generated in culture medium between three different NTP treatment doses measured immediately and 30 min after exposure to NTP. Results are presented as mean ± standard deviations, each performed in triplicate. * show a significant difference (P<0.05) between measurements at 0 min and 30 min; ^{a,b,c} shows a significant difference between three NTP conditions at the same time

Nitrite (NO_2^-) Concentration in Culture Medium Treated With three NTP Intensities

The concentration of NO_2^- in media at 30 min after exposure with NTP was significantly higher than observed immediately after exposure (P<0.05) for all NTP intensities *(Fig. 3)*. The concentration of NO_2^- in media significantly increased with the NTP energy delivered *(Fig. 3)*.

Nitrite (NO_2^-) Concentration and pH in Culture Medium in the 24 h After Treatment With NTP

The concentration (mg/L) of NO_2^- detected in NTP-treated culture medium exposed to the low intensity condition (3.24 J/cm²) is shown in *Fig 4*. The concentration of NO_2^- 4 h after treatment was significantly higher than immediately after treatment (0 h) and thereafter remained approximately constant for 24 h. This ensured

Table 2. *Emission of NTP reactive radical volume in gas phase*

Parameters	Low Dose (3.24 J/cm²)	Medium Dose (9.12 J/cm²)	High Dose (15.72 J/cm²)
Nitric oxide (NO) (ppm)	3.32±0.13[a]	5.50±0.19[b]	7.48±0.23[c]
Ozone (O_3) (ppm)	0.43±0.6[a]	1.53±0.6[b]	2.67±0.32[c]

He gas rate 1 L/min, Relative humidity [45] 56%, Temperature 25°C. Superscript [a, b, c] show a significantly different (P<0.05) between treatments groups

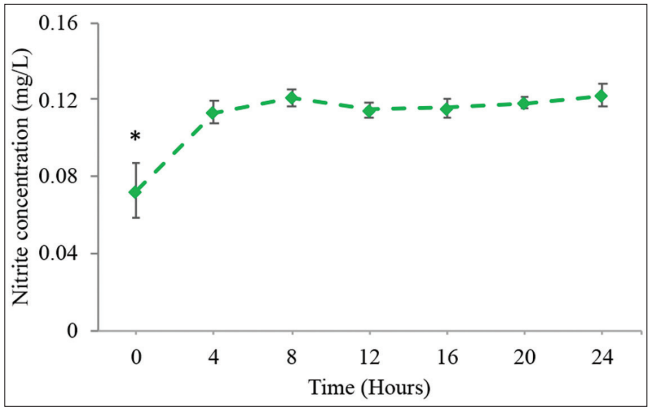

Fig 4. Measurement of the concentration (mg/L) of Nitrite generated in culture medium after being exposed with low intensity condition (3.24 J/cm2) for 60 sec. Results are presented as mean ± standard deviations, each performed in triplication. * show a significantly different (P<0.05)

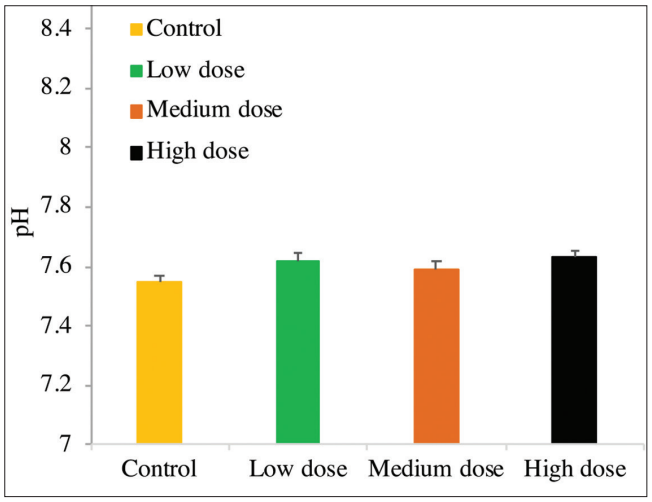

Fig 5. The pH of the cell culture medium after NTP treatment

that reactive species still remained for 24 in cultured medium for 24 h after treatment with NTP.

The pH of the cell culture medium was not influenced by treatment with low, medium or high NTP intensities *(Fig. 5)*.

Cell Viability at Different İncubation Times Measured Using the MTT Assay

The viability of fibroblasts was determined by MTT assay during 24 h of incubation in media treated with low, medium and high NTP intensities *(Fig. 6)*. The percentage of viable cells was higher than 90 percent, except for one time point (12 h) and one NTP intensity (medium).

Effect of NTP Treatment Conditions on Elephant Skin Fibroblasts

At the culture periods of 24, 48 and 72 h, the proliferation rate of fibroblasts in medium treated with NTP was significantly higher (P<0.05) than in cells grown in control medium (untreated with NTP) *(Table 3)*. The proliferation

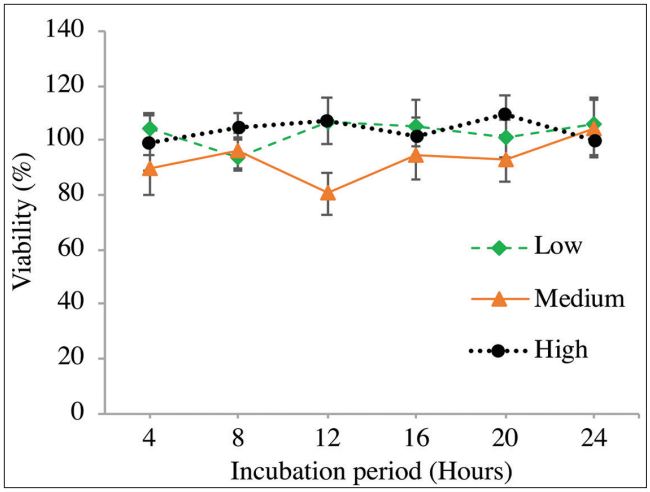

Fig 6. Viability of elephant skin fibroblasts after serial incubation with three different NTP treated complete medium conditions (low dose (3.24 J/cm²), medium dose (9.12 J/cm²) and high dose (15.72 J/cm²)); Cell viability (%) of fibroblasts was evaluated by MTT assay and data are presented as mean ± standard deviations, each performed in triplication

rate increased with increased intensity of NTP exposure from low to high *(Table 3)* (P<0.05). At 24 h, proliferation rate of the treated fibroblasts with low, medium and high dose were 2.54, 2.70 and 3.11 times higher than the untreated cells, respectively *(Table 4)*. At 72 h, the rate of the cell proliferation dropped which might be caused by the cells reaching confluence.

The number of cells in both the NTP treated and the untreated (control) groups approximately doubled in every 24 h *(Table 3)*. The average total cell count of all treatment groups was significantly higher than that of untreated group (P<0.05). The viability of cells at all time points was not significantly influenced by incubation in NTP treated medium *(Table 3)*.

DISCUSSION

Results from the current study has shown that the proliferation rate and total number of elephant skin fibroblasts grown *in vitro* was significantly higher (2-3 fold) when cultured in medium exposed to a NTP. Furthermore, proliferation rate and total cell count of treated fibroblasts was increased according to the NTP dose delivered. The study from Kalghatgi, Friedman [4] that used a low level (4 J/cm²) of NTP from dielectric barrier discharge (DBD) suggested that the reactive species generated interacted with cell membranes and caused sub-lethal cell membrane damage. The treatment of higher level NTP (8 J/cm³) lead to cell apoptosis and induced cell necrosis (or non-specific cell death) and inflammation. The different outcomes between our study and the findings of Kalghatgi, Friedman [4] may be caused by the use of different culture media [40,46] and NTP equipment.

The choice of culture medium may change the nature and

Table 3. *Effect of NTP treatment conditions on elephant skin fibroblasts*

Treatment Conditions		Cell Culture Period (h)		
		24 h	48 h	72 h
Proliferation rate (%)	Control	0.78±0.13[a],[i]	0.94±0.15[a],[ii]	0.84±0.11[a],[i,ii]
	Low dose	1.97±0.34[b],[i,ii]	2.05±0.27[b],[i]	1.74±0.25[b],[ii]
	Medium dose	2.10±0.28[b],[i]	2.45±0.32[c],[ii]	1.70±0.25[b],[iii]
	High dose	2.41±0.33[c],[i]	2.80±0.41[d],[ii]	1.92±0.24[b],[iii]
	Mean±SD	1.79±0.70	2.20±0.74	1.49±0.50
Total cells count ($x10^4$)	Control	238.362±19.479[a],[i]	411.209±5.973[a],[ii]	806.729±48.923[a],[iii]
	Low dose	252.632±12.731[a],[i]	473.822±44.505[b],[ii]	950.263±101.477[b],[iii]
	Medium dose	259.910±15.818[b],[i]	509.208±45,400[b],[ii]	1.037.017±76.506[b],[iii]
	High dose	285.991±24.234[c],[i]	512.399±37.057[b],[ii]	1.058.791±67.946 [c],[iii]
	Mean±SD	258.675±25.550	479.114±56.475	960.185±127.272
Viability rate (%)	Control	84.29±3.86	83.88±6.94	87.09±7.06
	Low dose	87.30±4.01	84.84±3.88	86.62±7.94
	Medium dose	87.63±6.45	87.57±6.88	90.57±5.50
	High dose	84.25±4.65	85.44±3.94	89.50±6.08
	Mean±SD	85.89±4.96	85.47±5.74	88.42±6.71

Superscript [a, b, c] show a significantly different (P<0.05) between treatments groups. Superscript [i, ii, iii] show a significantly different (P<0.05) between times within same treatment group

Table 4. *Proliferation rate related to control group*

Treatment (n=3)	Cell Culture Period (h)		
	24 h	48 h	72 h
Low dose	2.54	2.19	2.07
Medium dose	2.70	2.62	2.03
High dose	3.11	2.99	2.29

number of reactive species generated by NTP treatment. HEPES, a buffering agent for maintaining physiological pH of cell culture medium has a high ability to scavenge reactive species [41,46]. Furthermore, serum and antibiotics also have a minor radical scavenging ability [41,46]. In this study, fibroblasts that had been exposed with high dose NTP-treated complete medium with HEPES, serum and antibiotics showed the highest proliferation rate, compared to other treatment groups. Radical scavenger in culture medium was capable to scavenge reactive species which formed in culture medium after NTP treatment, and resulted in reducing oxidative effects inside the cells by decreasing of intracellular reactive species concentration [46]. This may the reason of using high dose of NTP treatment did not damage cells to death and reduced cell proliferation rate in this study.

NTP induced cell proliferation through intracellular reactive species formation [5], which bound to cell surface receptors and some can induce and generate new reactive species by interaction with cells components and consequently stimulated intracellular signalling pathways [4,41]. Increasing of intracellular reactive species also induced lipid peroxidation, changing of gene expression and cell membrane damage causing sub-lethal damage conditions which lead growth factors and cytokines release [4]. These intracellular mechanism resulted in a change of cell viability, proliferation, alter cell migration or induction of apoptosis which were dependent on NTP doses, treatment times together with cell types [41].

Among the consequences of generating NTP, radio-active emissions and UV-radiation potentially have the most effect on cells [41]. With the exposure of cells to NTP-treated culture media, the direct effects of UV-radiation can be excluded [47]. While direct treatment with NTP would allow cells to be exposed with all NTP components [41], some NTP components will be transferred into the treated liquid at the area of fluid exposed with NTP and will lead to reactive species production in the bulk liquid or liquid-gas interface [38,47]. NO, reactive nitrogen species, and O_3, reactive oxygen species, are one of the main radical species generated by NTP [48]. The amount and proportion of various reactive radicals generated in gas phase depend on plasma gas source [49,50]. In this study which uses Helium plasma, the ratio of NO radical emitted was higher than O_3. As with other studies, it was found that oxygen plasma generated a large number of singlet oxygen and OH radical. Whereas for air plasma, OH radical was less produced. However, the concentration of nitrate and nitrite in the liquid is higher than that produced by other gas plasma. This might be because air plasma generated a large number of NO and NO_2 [49]. Moreover, this study showed that concentrations of NO and O_3 were increased in the gas above by NTP in a dose-dependent manner. In

the study of Kim et al.[48], increasing of plasma treatment time also affects the increase in the amount of oxygen radicals.

After NTP generated plasma species above liquid surface, radical species were delivered as gas phase and then transferred, or some formed secondary generation of reactive radicals at the plasma-liquid interface, leading to chemical changes in the exposed liquid. For instance, nitrate and nitrite are the result of the reaction of plasma generated RNS such as NO and NO_2 and liquid [49]. This study showed that nitrite concentration (mg/L) generated in culture medium increased according to the energy dose delivered and remained in the NTP-treated complete medium for an extended period of time (up to 24 h in this study). This is because these generated radicals are long-lived, so species that can cause these reactions can spread to liquid without losing [47]. However, exposure to plasma with different energy will cause different chemical changes in the exposed liquid, depending on the characteristics and composition of the liquid [40,47].

Plasma radicals which dissolved in treated liquid were the cause of changing of pH by reacting with solution and also generating of secondary relative radicals. However, being buffer solution of culture medium resulted in almost no change of pH after plasma treatment. Consistent with the study of Trizio et al.[40], after plasma treatment, pH of distilled water was altered while culture medium and PBS did not noticeably change. Differing from other fluids e.g. water and PBS, culture medium solution containing amino acids, protein, serum and others organic components interact with reactive species generated by NTP and produced long-lived reactive species in treated culture medium [51]. In addition, there are other factors that influence the amount of ROS generated; the type of NTP machine, the NTP type, the gas generator, the gas flow volume and the materials of the table, and other environmental factors such as temperature and humidity.

Although this study successfully showed that medium treated with NTP was able to stimulate elephant skin fibroblasts *in vitro*, there were some limitations of the study. A monolayer cell culture does not resemble the wound on the skin of an elephant. There are many factors involved in the healing process, such as the interaction of various cell types, cytokines, wound conditions and overall animal health, which might affect the performance of NTP liquid treatment. Further studies using NTP treated liquids on elephant skin wounds are encouraged to develop the most effective and optimum treatment conditions.

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Research Article

Comparison of the Efficacy of Amniotic Membrane Transplantation, Topical Water-Based Propolis Extract, Corticosteroid and Antibiotic Use in Different Combinations on Subacute Corneal Alkali Burns in Rabbits [1][2]

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Abstract

Investigation of the superiority of the usage of propolis extract, amniotic membrane transplantation, corticosteroid and antibiotic both separately and in different combinations was aimed. A total of 40 (20 male, 20 female) New Zealand breed rabbits weighing 2.5-3.0 kg were used in the study. The rabbits were divided into 5 groups on day 7 of alkali burn injury. Amniotic membrane transplantation was applied to 3 groups and each one of these 3 groups had different local medications as 1% water-based propolis extract (AMN+PRP), dexamethasone and tobramycin (AMN+AC) and only tobramycin (AMN+A). The other two groups, which were not performed amniotic membran transplantation, were either treated with propolis (PRP) or left as a control. The defect area results obtained from AMN+PRP group were lower than AMN+A group, and both AMN+PRP and control group at the day 9 and day 14, respectively (P≤0.05). In the measurements made after the second week, AMN+PRP group showed significantly better results in all weeks in terms of defect area (P≤0.05). Corneal thickness was found to be lower in all groups treated with amniotic membrane than in the other groups (P<0.05). This study aimed to find treatment effectiveness of combining the amniotic membrane with propolis, especially as an anti-inflammatory agent comparing to corticosteroids, and it was decided the outcomes can be improved on the further studies..

Keywords: *Rabbit, Cornea, Chemical corneal burn, Amniotic membrane, Propolis*

Tavşanlarda Korneanın Subakut Alkali Yanıklarında Amniyotik Membran Transplantasyonu, Topikal Su Bazlı Propolis Ekstraktı, Kortikosteriod ve Antibiyotiğin Farklı Kombinasyonlarda Kullanımının Etkinliğinin Karşılaştırılması

Öz

Propolis ekstraktı, amniotik membran transplantasyonu, kortikosteroid ve antibiyotiğin ayrı ayrı ve farklı kombinasyonlarda kullanımının üstünlüğünün araştırılması amaçlandı. Çalışmada, 2.5-3.0 kg ağırlıkta toplam 40 Yeni Zelanda ırkı tavşan kullanıldı. Tavşanlar alkali yanık oluşturulmasının 7. gününde 5 gruba ayrıldı. Üç gruba amniyotik membran tranplantasyonu yapıldı ve bu gruplardan birine %1 su bazlı propolis ekstraktı (AMN+PRP), birine deksametazon ile tobramisin (AMN+AK), diğerine de sadece tobramisin (AMN+A) lokal olarak uygulandı. Diğer iki gruba amniyotik memran transplantasyonu yapılmadı, propolis ile tedavi edildi (PRP) veya kontrol olarak bırakıldı. AMN+PRP grubundan elde edilen defekt alanı sonuçları 9. günde AMN+A grubundan 14. günde hem AMN+A hem de kontrol grubundan daha düşüktü (P≤0.05). İkinci haftadan sonraki ölçümlerde AMN+PRP grubu defekt alanı bakımından tüm haftalarda daha iyi sonuç verdi (P≤0.05). Korneal kalınlık sonuçları ise amniotik membran ile tedavi gören tüm gruplarda düşüktü (P<0.05). Bu çalışmada, amniotik membranın propolisle kombine edilmesinin, özellikle kortikosteroidlerle karşılaştırıldığında antienflamatuar bir ajan olarak, tedavi etkinliğinin belirlenmesi amaçlandı ve sonuçların ileriki çalışmalarda geliştirebileceği kanısına varıldı.

Anahtar sözcükler: *Tavşan, Kornea, Kimyasal korneal yanık, Amniotik membran, Propolis*

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INTRODUCTION

Cornea is a transparent and avascular barrier between the eye and environment that allows the light coming into inside. However, corneal respond to the pathologies usually comes out as corneal edema, opacity and neovascularization; and adversely affects vision quality. Protecting the transparency of the cornea following pathologies is one of the most important goals of corneal treatment [1]. Many studies have been done, and many methods have been tried for many years to keep the vision clarity. These methods include medical treatment options for superficial defects and surgical methods such as corneal and conjunctival graft, third eyelid flap application, tissue adhesives, contact lenses, corneal-scleral transposition, autogenous/homologous corneal graft, use of synthetic stitch materials, trans-plantation of amniotic membrane or other organic materials for deeper defects [1-4].

Amniotic membrane is the inner layer of placenta that provides hemostasis [5,6]. It has avascular nature including anti-angiogenic factors and inhibiting proteinase activity, and so it reduces neovascularization and fibrosis, and induces epithelization when it is transplanted to the cornea [2,5,7-10].

Propolis has been used in various parts of the world for treatments because of its antibacterial, antifungal, antiviral, anti-inflammatory, antitumoral, immunostimulatory and regenerative effects on tissues in recent years [11-16]. Local application of the propolis extract has been proposed as an effective treatment options for corneal neovascularization because it suppress the angiogenesis [17].

Using the amniotic membrane for repairing corneal defects has long been known. However, its effectiveness is still controversial. This study aimed to compare the clinical efficiency of amniotic membrane combined with topical propolis extract, topical antibiotic and corticosteroid. Also, investigation of anti-inflammatory effect of the propolis was the priority for the study to find an alternative to corticosteroid usage, because of threatening complications.

MATERIAL and METHODS

Ethical Approval

The study design was approved by Adnan Menderes University Animal Experiments Local Ethics Committee (HADYEK) on 9.10.2014 and numbered 64583101/2014/172.

Animal Studied

A total of 40 (20 male, 20 female) New Zealand breed rabbits weighing 2.5-3.0 kg were used in the study. During the test period, animals were housed in transparent polycarbonate cages with a temperature of 22°C, 50-70% humidity and 12/12 h of light/dark conditions and feeded *ad libitum*. Before the trial, 15 days of acclimatization and standard clinical and ophthalmological examinations executed.

Experimental Design

All rabbits were anesthetized with 2.5 mg/kg xylazine HCl and 20 mg/kg ketamine HCl, and topical anesthesia of the eyeball was achieved by using 0.5% proparacaine HCl. The 6 mm wide filter paper which was soaked in 1 N NaOH, was held on the cornea of the right eye for 1 min to form the alkali burn and immediately after the eye was washed with a sterile eye wash solution. Before the trial groups assignation, 7 days was waited for the burn lesion become subacute form. Waiting period to create subacute lesion was decided based on the references [18-20].

To prevent possible effect of gender, 4 males 4 females rabbit randomly assigned to the groups as following *Table 1*;

Amniotic Membrane Preparation and Transplantation

A placenta from healthy dog cleaned flushed with saline solution containing 50 µg penicillin (1000 IU)/mL, 20 µg/mL streptomycin, 100 µg/mL neomycin, and 2.5 mg/mL amphotericin B. Amniotic membrane was then separated and placed on nitrocellulose paper of 5x5 cm pieces with epithelial surface up, and stored in 99% glycerin at room temperature (as described before [3]). Fifteen days after the amniotic membrane obtained, it was transplanted to the rabbits.

Clinical Ophthalmological Examinations

Reflex tests (pupillary, threat, dazzle and palpebral were scored as positive, weak and negative), corneal defect area measurement (using Photoshop software following calibration of the pictures), corneal thickness measurement (POCKET II Pachymeter, Quantel Medical, USA), corneal opacity scoring (scored between 0-3 as indicated by Lee et al.[18]), corneal neovascularization scoring, conjunctival examination, fluorescein staining and Shirmer I tear test were performed on postoperative day 1, 3, 5, 7, 9 and 14 days and then once a week until the end of 12th week.

Histopathological Examination

Rabbits were euthanized with high dose isoflurane on the 14th days of post-alkali burn (two animals from each group)

Table 1. *Study groups*

Group	Procedure
Control	Saline
AMN+A	Amniotic membrane transplantation + Topical* antibiotic[1]
AMN+AC	Amniotic membrane transplantation + Topical* antibiotic[1] + Topical* corticosteroid[2]
AMN+PRP	Amniotic membrane transplantation + Topical* water-based propolis extract, 1%
PRP	Topical* water-based propolis extract, 1%[3]

** All topical applications were administered 2 drops 3 times a day until the end of the trial; [1] Tobrased® Eye Drop (tobramycin 0.3%, Bilim Drug Company); [2] Dekort® Eye Drop (dexamethasone 0.5%, Deva Drug Company); [3] Eğriçayır® Water-Based Propolis 33% (Diluted to 1% when using)*

and at the end of experiment (six animals from each group). For histopathological examination, the eyeballs were extirpated and fixed in Bouin's solution for 24-48 h. After fixation, the tissues were embedded in paraffin, sectioned in 4 µm thickness and stained with hematoxylin-eosin (H-E). The tissue sections were examined under light microscopy.

Statistical Analysis

Statistical analysis of the data was performed using the SPSS 22 statistical package program (Inc., Chicago, Il, USA). Statistical analysis of STT, defect area, fluorescein and corneal thickness determined by measurement; In order the dimensional differences of the corneal defects would not affect the results, all the data were initially accepted as 100 and the proportional recovery level was determined. The normal distribution of these parameters was investigated by Kolmogorov-Smirnov test and the differences between the group means of these parameters were compared using the one-way ANOVA test. The significance control of the differences between the groups was done by Duncan multiple comparison test. The differences between the mean scores of the parameters determined by scoring (conjunctival examination, corneal opacity, neovascularization) were compared using the Kruskal-Wallis test. Bilateral comparisons were made with Mann-Whitney U test. Reflexes were compared between groups by using Chi-square test.

RESULTS

Since the defects covered a small portion of the cornea and was not sized to precisely prevent vision, reflexes were not disappeared in any rabbit during the trial, and no difference was found associated with recovery or treatment. Also, there was no statistically significant difference in terms of opacity and neovascularization scores.

Fluorescein staining could not be obtained properly due to the placed membrane in groups where amniotic membrane was placed. No statistical difference was found in the analysis of the data obtained from the control group and the PRP group in any stages of the study. Therefore, visible size of corneal defect without staining were evaluated.

For the parameters determined by the measurement, since the other results obtained from the measurements on the intermediate days were not very different, the statistical results of the first two weeks on the 0, 5, 9 and 14 days were mentioned *(Fig. 1)*.

There was no difference between the groups in the

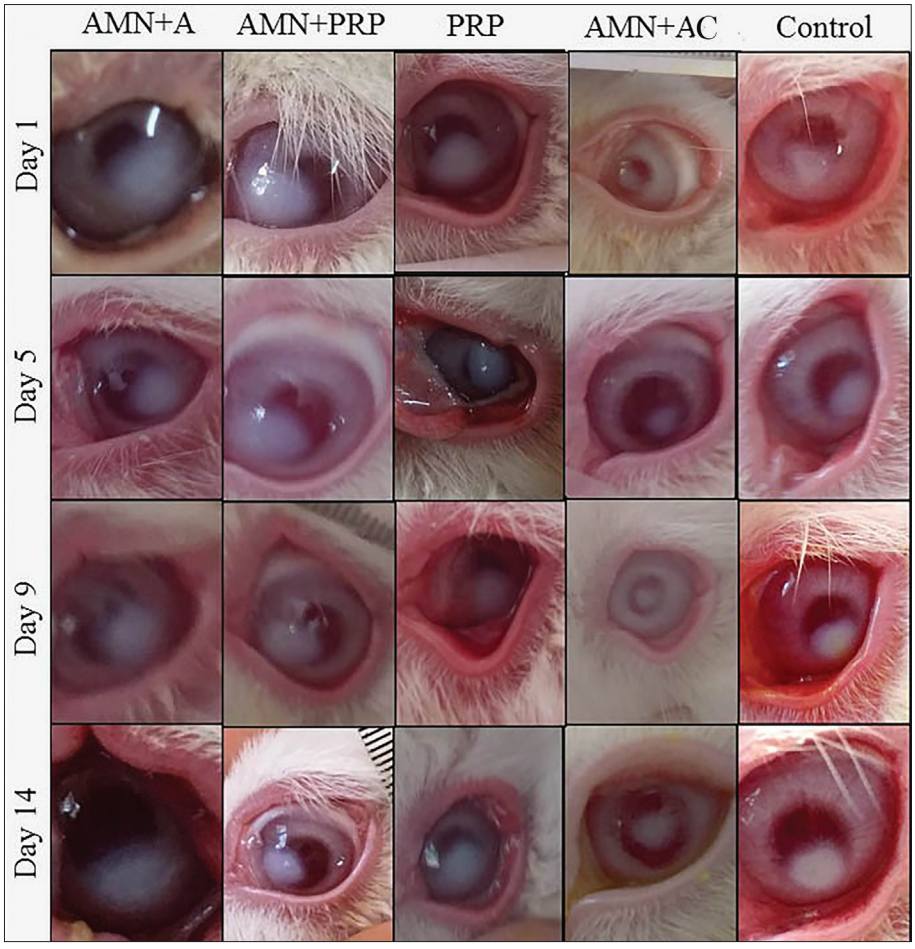

Fig 1. Images of the first 2 weeks of the corneal lesion of rabbits randomly selected from different groups

Table 2. *Shirmer tear test results until the 14th day*

Groups	Day 0		Day 5		Day 9		Day 14	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	100±0	8	160.14±44.22	8	196.27±64.99	8	174.59±46.98
PRP	8	100±0	8	123.26±16.99	8	152.84±19.64	8	118.44±14.04
AMN+PRP	7	100±0	7	106.10±7.13	7	105.20±26.28	7	85.46±12.49
AMN+A	7	100±0	7	104.13±10.48	7	98.49±11.86	7	91.39±7.94
AMN+AC	7	100±0	7	109.03±11.8	7	84.82±16.98	7	98.02±23.43
P	-		-		-		-	

** Differences between the mean indicated by different letters in the same column are significant (P<0.05)*

Table 3. *Defect area measurement results until the 14th day*

Groups	Day 0		Day 5		Day 9		Day 14	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	100±0	8	93.21±2.28	8	89.17±2.45^{ab}	8	87.65±2.93^{a}
PRP	8	100±0	8	95.49±0.97	8	88.82±2.31^{ab}	8	84.08±2.96^{ab}
AMN+PRP	7	100±0	7	91.81±1.60	7	82.06±2.81^{b}	7	77.43±3.20^{b}
AMN+A	7	100±0	7	96.76±1.27	7	92.63±1.62^{a}	7	89.73±1.99^{a}
AMN+AC	7	100±0	7	95.15±1.23	7	87.45±2.01^{ab}	7	82.90±2.89^{ab}
P	*		-		*		*	

** Differences between the mean indicated by different letters in the same column are significant (P≤0.05)*

Table 4. *Corneal thickness measurement results until the 14th day*

Groups	Day 0		Day 5		Day 9		Day 14	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	100±0	8	121.56±5.77^{a}	8	128.02±6.24^{a}	8	124.52±5.89
PRP	8	100±0	8	111.76±7.42^{ab}	8	114.05±10.25^{ab}	8	112.97±10.98
AMN+PRP	7	100±0	7	104.98±2.59^{b}	7	110.80±5.31^{ab}	7	110.27±5.40
AMN+A	7	100±0	7	99.59±1.75^{b}	7	100.19±2.20^{b}	7	99.48±2.14
AMN+AC	7	100±0	7	101.64±1.21^{b}	7	100.47±1.38^{b}	7	99.33±2.09
P	-		*		*		-	

** Differences between the mean indicated by different letters in the same column are significant (P<0.05)*

evaluation of the data obtained from Shirmer tear test until the 14th day *(Table 2)*.

As shown in *Table 3*, the results of AMN + PRP were significantly lower than AMN + A on 9th day, and control and AMN + A group on 14th day (P<0.05).

As shown in *Table 4*, the results of corneal thickness measurement from all of AMN groups at 14th day were found to be significantly better on the 5th and 9th day than the control group (P<0.05).

Histopathologically, at 2nd week post-alkali burn, diffuse corneal edema was observed in all groups. Collagen lamellae were separated into a fine feltwork of pale-staining fibrils. It was noted that the corneal epithelium desquamated and ulcers were formed in the defect area. There was an accumulation of neutrophil leukocytes around the limbal vessels in the control group. In AMN + A, AMN + PRP and PRP groups, there was also seen neovascularization and mild neutrophil leukocyte infiltrations in the superficial 1/3 part of the stroma. In AMN + AC groups, neovascularization accompanied by moderate neutrophil leukocyte infiltrations was observed in the superficial 1/2 part of the stroma.

Reflex examinations were scored normal in almost all rabbits and no difference was found between the treatment groups. In terms of opacity and neovascularization, opacity scores showed statistically significant difference. AMN + PRP were significantly better than the other groups after 8 weeks. In the twelfth week, the results obtained from the

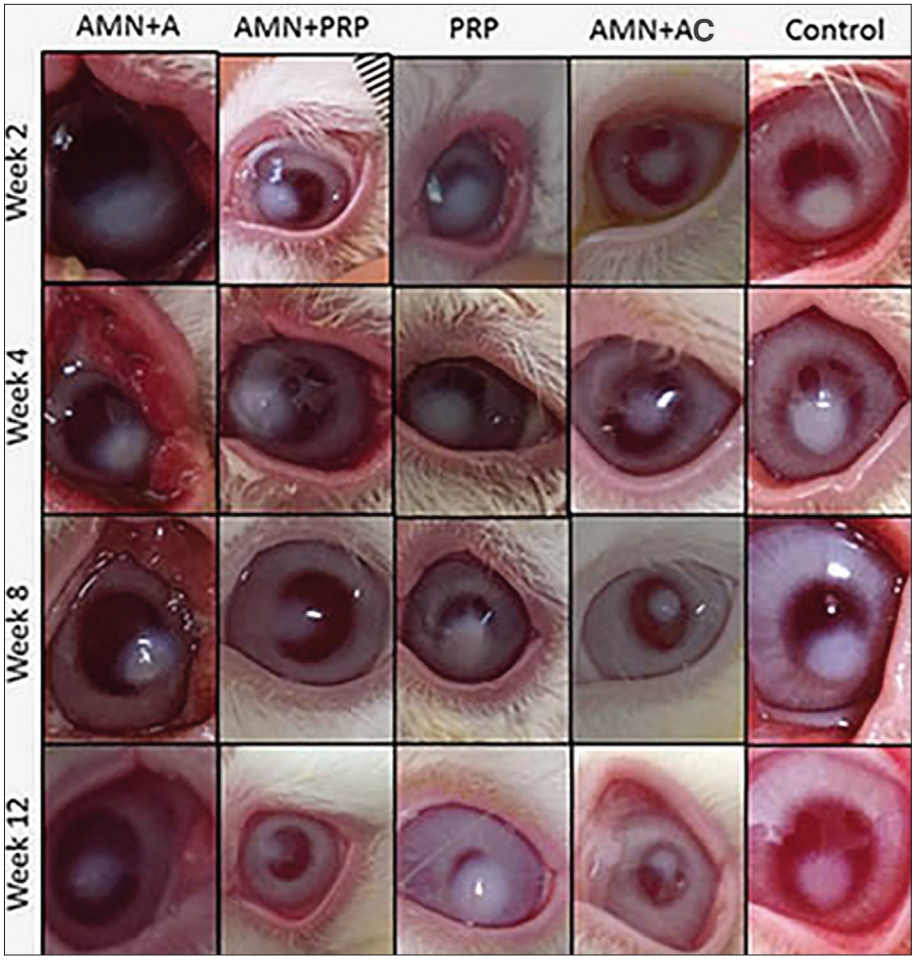

Fig 2. Images of between Week 2 and 12 of the corneal lesion of rabbits randomly selected from different groups

Table 5. *Shirmer tear test results between week 2 and 12*

Groups	Week 2		Week 4		Week 8		Week 12	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	174.59±46.98	6	171.79±37.09[a]	6	122.32±46.13	6	105.83±48.92
PRP	8	118.44±14.04	6	89.80±13.92[b]	6	82.88±12.22	6	52.98±6.69
AMN+PRP	7	85.46±12.49	5	75.76±12.12[b]	5	85.40±23.85	5	60.36±20.53
AMN+A	7	91.39±7.95	5	80.34±6.95[b]	5	65.86±7.95	5	74.38±8.73
AMN+AC	7	98.01±13.43	5	71.42±11.02[b]	5	79.92±12.81	5	82.32±4.18
P	-		*		-		-	

** Differences between the mean indicated by different letters in the same column are significant (P<0.05)*

control and the AMN + AK group were found to be similar to the AMN + PRP *(Fig. 2)*.

As shown in the following table *(Table 5)*, the statistical difference of the data obtained from Schirmer tear test on the 4th week was interpreted as unrelated to treatment (P<0.5).

Defect area results *(Table 6)* was significantly better in AMN + PRP the group than other groups for all weeks (P<0.5).

As shown in corneal thickness measurements *(Table 7)*, higher results were obtained in the PRP group from the 4th week onwards. The results from all of AMN groups was found that was lower than the other groups.

To evaluate the effects of treatments on wound healing, the eye sections were examined histopathologically at 12th-week post-alkali burn. In the control group, corneal ulcer extending into the outer third of the stroma was observed in the defect area. The ulcer area was filled with fibroblast proliferation and fine collagen fibers *(Fig. 3)*. Scattered polymorphonuclear leukocytes infiltration between connective tissue cells was seen. At the base of the ulcer, there were new vessel formation and moderate

Table 6. *Defect area measurement results between week 2 and 12*

Groups	Week 2		Week 4		Week 8		Week 12	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	87.64±2.93^{a}	6	75.48±4.05^{a}	6	63.75±4.42^{bc}	6	55.43±4.62^{bc}
PRP	8	84.08±2.97^{ab}	6	78.68±3.64^{ab}	6	68.20±4.80^{ab}	6	59.84±4.82^{ab}
AMN+PRP	7	77.43±3.20^{b}	5	67.06±4.65^{b}	5	51.20±7.14^{c}	5	43.58±7.53^{c}
AMN+A	7	89.73±1.99^{a}	5	87.72±3.13^{ab}	5	79.89±2.93^{a}	5	72.43±2.84^{a}
AMN+AC	7	82.90±2.89^{ab}	5	76.55±4.03^{ab}	5	62.29±3.19^{bc}	5	53.00±2.45^{bc}
P	*		*		**		**	

** Differences between the mean indicated by different letters in the same column are significant (P≤0.05)*

Table 7. *Corneal thickness measurement results between week 2 and 12*

Groups	Week 2		Week 4		Week 8		Week 12	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	124.52±5.89	6	121.69±7.51^{ab}	6	102.30±5.17^{ab}	6	91.49±3.99^{ab}
PRP	8	112.97±10.98	6	139.19±13.59^{a}	6	112.19±12.93^{a}	6	101.52±10.99^{a}
AMN+PRP	7	110.27±5.40	5	100.61±5.31^{bc}	5	85.84±5.53^{b}	5	72.00±3.17^{bc}
AMN+A	7	99.48±2.14	5	93.44±3.729^{c}	5	86.75±1.02^{b}	5	76.28±1.71^{bc}
AMN+AC	7	99.33±2.09	5	93.87±3.15^{c}	5	81.08±3.86^{b}	5	70.52±1.34^{c}
P	-		**		*		**	

** Differences between the mean indicated by different letters in the same column are significant (P<0.05)*

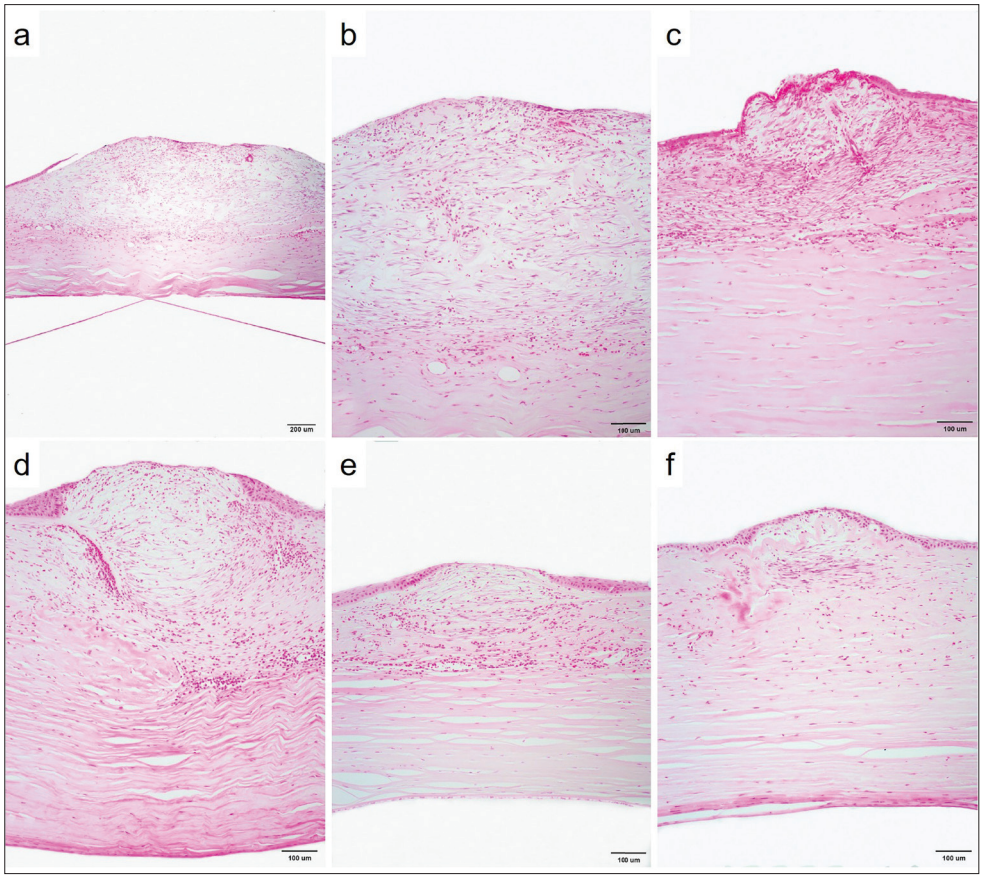

Fig 3. The effects of treatments on alkali burn-induced histopathological changes. Representative photomicrographs of eye sections stained with H-E from each treatment group (12th week post-alkali burn). **A-B.** Control group, **B.** PRP group, **C.** AMN + A group, **D.** AMN + PRP group, **E.** AMN + AC group

polymorphonuclear leukocytes infiltration extending from limbus toward to the defect area.

Treatments with AMN + A, AMN + PRP, AMN + AC or PRP was determined to accelerate the wound healing process. The majority of the defect area was filled with mature granulation tissue. In the defect area, immature connective tissue cell (fibroblast) proliferation and new vessel formations were also seen. The corneal epithelium at the edges of the defect was hyperplastic and the ulcer surface was covered with a single layer of squamous epithelium. There was no difference in the severity of inflammatory changes between AMN + A, AMN + PRP, PRP, and control group. In AMN + AC group, mild polymorphonuclear leukocyte infiltrations were observed in the defect area and the severity of inflammatory changes was lower than that in the control group. It was detected that treatments with AMN + AC and AMN + PRP were diminished the size of the defect area compared to the control group. There was no difference in the size of the defect area between AMN+A, PRP, and the control group.

DISCUSSION

Effectiveness of corneal graft surgery has been widely proven [19-24]. According to literature information, the amniotic membrane promotes epithelialization, reduces inflammation, neovascularization, fibrosis and pain, acts as a substrate for cell growth and biological bandage and, shows antimicrobial effects [5,9-10,25]. Considering all of these, to compare anti-inflammatory and anti-microbial effect of topical propolis treatment following amniotic membrane transplantation with topical antibiotic and corticosteroids usage without any debridement in the subacute alkali burns of the cornea was aimed in this study.

Some clinical data suggests that the amniotic membrane shows anti-angiogenic effects on the cornea [20], and neovascularization [26,27] and an effective treatment option of corneal lesions of the horse [2,28], dog [29], cat [3], rabbit [7] and humans [10,30-32]. In the present study, no statistically significant difference was found between the first two weeks in terms of opacity and neovascularization scores, but after the second week opacity significantly decreased from 8th to 12th weeks in AMN + PRP group. On the 12th week, opacification in the control group and AMN + AC group was similar to the AMN + PRP group. In PRP group and AMN + A group, opacity scoring was high in all weeks compared to the other groups. These findings were assessed as that usage of AMN + A or PRP would not provide efficient contribution to the regression of opacity. And when looked dissenting investigations, some researchers stated that the amniotic membrane transplantation is ineffective, even exacerbating, in reducing inflammation, opacity and neovascularization especially in the early phases of the corneal alkali burn lesion [33]. Our results might be interpreted as that combining the amniotic membrane with propolis might accelerate the reducing of the opacity but might not affect the final outcome.

Because of the controversial usage of the amniotic membrane [32,34-36], most researchers have investigated an adjuvant therapy which can be used with it. Corticosteroids are powerful tools to prevent scarring and to preserve transparency in some forms of keratitis, uveitis, conjunctivitis, scleritis/episcleritis [37]. There are many studies using topical or systemic steroids after amniotic membrane transplantation [30,31,38,39]. However, there are some researchers reporting that corneal ulcers could be treated only with adjuvant systemic immunosuppressive therapy [30].

Propolis is a substance that has been suggested which has regenerative, antibacterial, antifungal, antiviral, anti-inflammatory, antitumoral, immunostimulatory effects on tissues [11-16]. In the present study, almost no signs of severe inflammation and chemosis were observed in the eye and surrounding tissues. The results of Shirmer tear test obtained from the control group at the 4th week were found to be significantly higher however; this difference was not found determinative in terms of healing process. Also, since the defect area did not have enough space to block the view, the reflexes were not completely lost in any rabbit during the study. In addition, the results of the reflex examinations of all groups were almost identical. In the initial measurements, the reflexes scored as week improved in a short time by regressing of schemosis; and were not associated with anti-inflammatory treatment or healing.

Some researchers have suggested that propolis extract would be effective when applied locally for the treatment of corneal neovascularization due to its angiogenesis suppressive effect [17]. Hepşen et al.[40] confirmed this hypothesis and reported that topical application of water-based extract of propolis in the rabbits with corneal defect of the-silver nitrate had an inhibitory effect on corneal neovascularization. Also some researchers stated that in rabbits with chemical corneal injuries, topical application of propolis [14] or, caffeic acid phenethyl ester which is the active ingredient of propolis [41], has equal effect with topical dexamethasone usage in terms of suppressing corneal neovascularization.

In this study, the results of defect area from AMN + PRP group were significantly lower than AMN + A on 9th day, and control and AMN + A group on 14th day. In the following weeks, the AMN PRP group showed significantly better results. The control group showed slow improvement than AMN + PRP group until 4 weeks; in the following weeks the recovery in the AMN + A group was found to be significantly weak. However, some studies suggested that amniotic membrane has little effect on wound healing, neovascularization and inflammation following acute ocular surface burns, as well as on improvement of vision [34,42-45]. There are some investigators suggesting

that the efficiency of the amniotic membrane equal to umbilical cord serum [36]. Another study stated that chondrocyte derived extracellular matrix in corneal alkaline burns gave better results than human amnionic membrane on corneal neo-vascularization, opacity and stromal changes [46]. However, when the present study findings were evaluated, it was observed that co-administration of the amnion membrane with corticosteroids or propolis yielded positive results in terms of clinical improvement in the defect area. In the study performed by Zheng et al.[47], conjunctival coverage combined with amnion liquid supernatant eyedrop showed the better therapeutic effect than conjunctival coverage individually. Pessolato et al.[48] evaluated the amniotic membrane and propolis separately in the reepithelization of second degree dermal burns in rats and reported that both provide beneficial results. In addition, they suggested that they can be used together considering reepithelization and anti-inflammatory effect of amniotic membrane and, debridement and collagen tissue production stimulation and scar formation suppression effect of propolis. Although there was no significant difference between the groups in terms of neovascularization in the present study, it was determined that PRP application was less effective compared to the other treatment methods in terms of corneal thickness and all of AMN groups yielded more successful results.

In conclusion, the amniotic membrane when applied with other treatment methods was successful in the treatment of subacute alkaline burns of the cornea; especially when combined with propolis, faster regression of the defect area was determined. Local application of only propolis were not found very effective. When these data are evaluated, it is thought that the amniotic membrane application in combination with propolis can be improved by planning further studies.

Declaration of Conflicting Interest

The authors declare no financial or other conflicts related to this report.

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Research Article

The Effects of *Androctonus crassicauda* Venom on Pregnant Rats and Their Offspring

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Abstract

Incidents of scorpion stings are common in Turkey. These cases can cause severe envenomation and so represent an important public health problem. In Turkey, the most venomous species are *Leiurus abdullahbayrami* and *Androctonus crassicauda.* There has been no study on the effects of *A. crassicauda* stings on pregnant rats. Consequently, we investigated the effects of the scorpion venom on pregnant rats and their offspring. The supernatant of the *A. crassicauda* venom obtained after the venom extraction was dissolved in 7 mL of sterile saline before the experiment, in this way the injection volume was standardized as 1 mL/rat. Pregnant rats were randomly divided into two groups with six animals in each. *A. crassicauda* venom in 1 mL physiological saline solution (NaCl 0.9%) was subcutaneously injected in rats of the experimental group (EG), while sterile saline solution (1 mL) was subcutaneously administered to the rats of the control group (CG). All injections were applied to each group from the 7th to the 13th days of pregnancy, which correspond to the critical organogenesis period. Based on these results, it was shown that the scorpion venom affects the body weight of pregnant rats, the weights of placental tissues and fetuses in the rat model during pregnancy. *A. crassicauda* venom can induce abortion and cause restrictions in placenta and fetal growth. Therefore, medical professionals should be informed about possible adverse effects and risks in pregnancy.

Keywords: *Scorpion, Androctonus crassicauda, Venom, Pregnancy, Fetal abnormalities*

Androctonus crassicauda Zehirinin Gebe Sıçanlar ve Yavruları Üzerine Etkileri

Öz

Akrep sokması vakaları Türkiye'de yaygın olarak görülmektedir. Bu olgular ciddi zehirlenmeye neden olarak önemli bir halk sağlığı sorununu oluşturur. Türkiye'de en zehirli akrep türlerinin *Leiurus abdullahbayrami* ve *Androctonus crassicauda* olduğu bilinir. *A. crassicauda* sokmalarının gebeler üzerindeki etkileri konusunda bir çalışma yapılmamıştır. Bu nedenle, akrep zehirinin gebe sıçanlar ve yavruları üzerindeki etkilerini araştırdık. Zehir ekstraksiyonundan sonra elde edilen *A. crassicauda* zehrinin süpernatanı, deneyden önce 7 mL fizyolojik tuzlu su (%0.9 NaCl) içinde eritildi, bu şekilde enjeksiyon hacmi, 1 mL/sıçan olarak standart hale getirildi. Gebe sıçanlar rastgele olarak her birinde altı hayvan bulunan iki gruba ayrıldı. 1 mL *A. crassicauda* zehiri, deney grubundaki sıçanlara (EG) subkutan olarak uygulanırken, kontrol grubundaki sıçanlara (CG) steril serum fizyolojik çözeltisi (1 mL) subkutan yolla verildi. Tüm enjeksiyonlar kritik organogenezis dönemine karşılık gelen gebeliğin 7. gününden 13. gününe kadar uygulandı. Bu sonuçlara dayanarak, akrep zehirinin gebelik sırasında sıçan modelinde gebe sıçanların vücut ağırlığını, plasental dokuların ve fetüslerin ağırlığını etkilediği gösterilmiştir. *A. crassicauda* zehri abortu uyarabilir, plasenta ve fetal büyüme geriliğine neden olabilir. Bu nedenle, tıp uzmanları gebelikte olası olumsuz etkiler ve riskler hakkında bilgilendirilmelidir.

Anahtar sözcükler: *Akrep, Androctonus crassicauda, Zehir, Gebe, Fetal anormaliteler*

INTRODUCTION

Scorpions are terrestrial invertebrate animals classified as Arachnida whose existence dates back 430 million years to the Silurian era [1]. As a result, they can be considered living fossils because morphologically they have changed so little [2]. In spite of developing medical approaches and other measures, in many tropical and subtropical countries scorpion envenomation cases still constitute a serious public health problem that can result in death, especially in

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children [3]. Among scorpion species, *Androctonus crassicauda* is known as the deadly scorpion and so is of great concern to medicine [4].

In addition to Turkey, the same scorpion can be found in Jordan, Syria, Iran, Iraq and Saudi Arabia and is medically important in these countries as well [5]. In Turkey, *A. crassicauda* is the most significant scorpion species responsible for scorpion sting cases and for antivenom production. In addition, depending on the structure of the culture and socio-economic characteristics, many women work outdoors, particularly in the southeastern region of Turkey, thus the probability of scorpion stings increases among females. Indeed, many demographic reports in the country confirm that over 50% of scorpion sting cases occur in females [5-7]. As a result, it should be remembered that pregnant women are potential victims.

Some studies have emphasized that the venom of the *A. amoreuxi, Tityus serrulatus* and *T. bahiensis* scorpion species can lead to congenital abnormalities and fetal resorption in pregnant animal models after injection. In addition, *Buthus minax* and *Leiurus quinquestriatus* scorpion venom has been shown to induce abortion in pregnant women. Scorpion stings in pregnant women can result in acute conditions such as vaginal bleeding, fetal loss, preterm delivery and placental and fetal abnormalities [3,8-11].

Although the toxicity of *A. crassicauda* venom is well documented, there is little research about fetal effects on pregnancy. In Turkey, this is the first study specifically designed to determine if repetitive maternal exposure to *A. crassicauda* venom during organogenesis has deleterious effects on pregnant rats and their offspring in the fetal and obstetric outcome. Therefore, the aim of this work in the rat experimental model of pregnancy was to investigate the effects of *A. crassicauda* scorpion venom on pregnant rats and their offspring.

MATERIAL and METHODS

Scorpions and Venom Extraction

The scorpions were captured in Sanliurfa province in the southeastern region of Turkey and kept in plastic cages with free access to food and water. Venom was obtained from mature *A. crassicauda* scorpions by electrical stimulation of the telson as described by Ozkan and Filazi [2]. The venom was diluted with sterile double distilled water and centrifuged at 15.000 rpm for 20 min at 4°C [12]. Then, the supernatant was taken and stored at -20°C until use at Cankiri Karatekin University. The protein content of the venom was also determined according to the Bradford method [13] before use.

Bioethics and Animals

The study was approved by the Experimental Animals Ethics Committee of Gazi University (G.Ü.ET-18.088). Furthermore, all experimental procedures were conducted in accordance with the ethical guidelines of the Ethics Commission on Animal Use (CEUA) and the Guide for the Care and Use of Laboratory Animals of NIH. Adult female (n=12) and male (n=3) Wistar Albino rats weighing 200-250 g were used for experimental procedures. The animals were housed in polysulfone cages at 21-24°C, 40-45% humidity and under light-controlled conditions (12 h light/12 h dark) at the Laboratory Animals Breeding and Experimental Research Center, Faculty of Pharmacy, Gazi University. Tap water and commercial pellet food were available *ad libitum*.

Mating of Rats and Pregnancy Diagnosis

Following one week of acclimatization, female rats were allowed to mate with mature males (one male per four females) from 4.00 p.m. to 8.00 a.m. Pregnancy was determined by the presence of a vaginal plug *(Fig. 1A)*, which showed evidence of mating, and then these females were diagnosed as pregnant [14]. This was considered as pregnancy day 0. The pregnant rats were maintained in individual cages.

Experimental Design

The whole venom of the *A. crassicauda* scorpion was dissolved in 7 mL of physiological saline solution (PSS: NaCl 0.9%) solution and the injection volume was standardized as 1 mL/rat. The subcutaneous injection route in rats was chosen to mimic natural scorpion stings. The pregnant rats were randomly divided into two groups with six animals in each. The dose of 1 mL of the venom was subcutaneously injected in rats of the experimental group (EG), while the saline solution (1 mL) was subcutaneously administered to the rats of the control group (CG). All injections were applied to each group from the 7th to the 13th days of pregnancy [15], which correspond to the critical organogenesis period [16].

Maternal Effects

The female rats were determined for weight gain on 0, 7, 13 and 21 days of pregnancy, the weight gain in both groups were recorded. Also noted were symptoms of the venom injection such as erythema, local pain on the injection site, respiratory perturbation, lacrimation and salivation increase and possible pregnancy complications were recorded after each injection.

Termination of the Experimental Procedure

On the last day (21st) of pregnancy, surgery was aseptically performed on the animals under general anesthesia with xylazine hydrochloride 2% (10 mg/kg; XylazinBio®, Intermed, Ankara, Turkey) and ketamine hydrochloride 10% (100 mg/kg; Ketasol®, Interhas, Ankara, Turkey). The fetuses were removed and an ovariohysterectomy was performed [15]. The uterine horns were cut immediately and the fetuses and their placenta were extracted and weighed to determine the effect of venom on reproductive

organs. Ovaries and uterine tissues of each rat were weighed by precision scale. In addition, they were carefully examined in terms of the gross morphological abnormal findings. The uterine tissues were reweighed after removal of the ovaries. The vitality of offspring removed from the uterus was confirmed with the existence of fetal movement after tactile massage. The numbers of dead and live newborns were recorded. For morphometric analysis, occipitonasal lengths, tail lengths and chest width of all offspring were measured using a micrometer. In addition, the offspring were examined for assessment of any dysmorphology.

Postoperative Treatment and Care

The rats were monitored daily by a veterinarian. The surgical site of each rat was treated with povidone-iodine twice daily. Furthermore, the survival time of the offspring was recorded until the end of weaning. Maternal rats were sacrificed for the next analysis at the end of the lactation period and their internal organs were removed.

Statistical Analysis

The data were evaluated using the SPSS 23.0 program. The results were expressed as mean ± SD or percentage of total number. Mann Whitney U test was used to determine the significance of differences between groups and these were considered significant when P≤0.05.

RESULTS

Within a few minutes of each dose of *A. crassicauda* venom injection, systemic symptoms such as local pain and erythema at the injection site, tachypnea, deep dyspnea, increases in salivary, eye and nasal secretions and jumping, shaking and rigidity in hind limbs began and persisted for approximately 30 min and then the rats returned to their normal state within approximately two hours. There was a significant increase *(Fig. 1)* in the body weight of rats in the EG at the 7th day of pregnancy (P<0.05). In all the measurements after the 10th day of pregnancy, the body weight of pregnant rats in the EG was significantly weaker than the CG (P<0.05) *(Table 1)*. Also, it was observed that the weights of fetuses and placental tissues in the EG were lower than in the CG (P<0.01) *(Table 2)*.

Eighty newborns were detected in rats of the CG and 67 in the EG. There was no fetus in the uterus of one rat in the EG and this rat was evaluated as if aborted *(Fig. 1F)*. A significant decrease (P<0.01) was detected between the newborn weight of venom-treated rats and the control group *(Table 3)*. Although all newborns of venom-treated rats died within one hour of birth, there were no newborn deaths in the CG in this time period. A significant decrease (P<0.05) was observed for occipitonasal and tail lengths and chest width in repetitively envenomed rats in comparison to control rats *(Table 3)*.

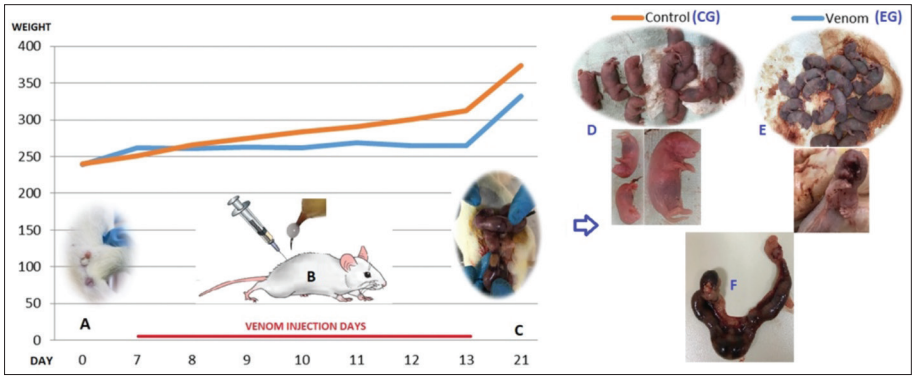

Fig 1. Effect of *Androctonus crassicauda* in pregnant rat and offspings. Pregnancy was determined by the presence of a vaginal plug that considered as day 0 **(A)**, The body weight (g) of rats following administration of *A. crassicauda* venom or saline according to pregnancy days **(B)**, Ovariohysterectomy on 21 days of pregnancy **(C)**, cerebral hemorrhage **(D)** and mouth abnormality **(E)** in newborn of EG, Abortifacient **(F)**

Table 1. *Comparison of body weight (g) measurements of rats between groups*

Pregnancy Days	CG (n=6)	EG (n=5*)	P
0	240.3±9.3	238.9±16.0	0.686
7th	250.5±8.3	261.5±14.3	0.045
8th	266.0±10.9	260.5±15.5	0.261
9th	274.7±8.8	262.5±13.2	0.150
10th	283.8±6.7	262.0±12.1	0.010
11th	290.5±5.6	268.7±12.1	0.006
12th	301.0±12.5	265.3±9.8	0.004
13rd	312.5±17.2	264.4±12.3	0.004
21st	374.0±14.0	332.5±41.7	0.030

*Mean weight ± SD * An abortion*

Table 2. *Comparing weight measurements (g) of internal genital organs with fetuses and placenta between control group and experimental groups*

Parameters	CG (n=6)	EG (n=5)	*P*
Total weight of uterus, ovaries and fetuses	103.3±9.2	67.0±35.3	0.004
Total weight of uterus, ovaries and placenta	82.1±6.4	47.9±27.8	0.004
Weight of ovaries	0.89±0.18	0.83±0.08	0.194
Placental weight	0.83±0.28	0.54±0.08	0.004

Table 3. *The evaluation of some morphometric parameters of offspring between groups*

Parameters	Newborn Rats of CG (n=80)	Newborn Rats of EG (n=67)	*P*
Weight of newborn rats (g)	5.48±0.04	3.07±1.50	0.003
Occipitonasal - tail lengths (cm)	4.0±0.0	2.3±1.1	0.002
Chest width (cm)	2.0±0.0	0.7±0.3	0.002
Tail length (cm)	2.0±0.0	1.1±0.5	0.001
Cerebral hemorrhage-ratio (%)	0 (0%)	67/67 (100%)	0.001
Cleft lip-ratio (%)	0 (0%)	4/67 (5.97%)	0.019

When we examined the macroscopic findings of newborn rats, we observed cerebral hemorrhage macroscopically in all newborns of the EG and none in the CG, and the presence of this pathology was significantly different between the two groups. Also, we defined cleft lips in four newborns of venom-treated rats and none in the CG, and there were significant differences in comparisons between the groups (P<0.05) *(Table 3)*.

DISCUSSION

Scorpion envenomation remains a serious public health problem, especially in the Southeastern Anatolia region of Turkey. In 2008, Ozkan et al.[17] reported 24.261 scorpion sting cases in Turkey, of which 22.6% occurred in Sanliurfa province. Pregnant women are among the possible victims given the high incidence of scorpion sting cases in Turkey. *A. crassicauda* is one of the most venomous scorpions in the world, and that includes Turkey [4-7]. In this research, we chose repetitive doses of the venom because there have been reports of more than one scorpion sting of a pregnant woman in Turkey. As a result, the single scorpion venom was divided equally into seven portions one of which was injected daily together with 1mL of saline solution.

In rats, blastocyst implantation in the uterus occurs between days four and five of pregnancy and then organogenesis continues until the 16th day and fetal development proceeds until birth [14]. In addition, this is a critical stage during which, on the 10th day of pregnancy, the formation of several organs such as the eyes, brain and heart and aortic arches, axial skeleton, palate and urogenital system occur in the organogenesis period. Materials introduced during the organogenesis period may have teratogenic effects [18]. In the assessment of maternal weight, the body weight of rats in the venom group decreased significantly from days 7 to 13 of pregnancy when compared to the control. Contrary to this, Barao et al.[19] reported a significantly greater weight increase from days 16 to 21 of pregnancy after the evaluation of maternal weight in rats injected with *T. serrulatus* scorpion venom on the 10th day of pregnancy.

Both human and experimental animal studies show that the venom of the scorpion induces alterations in the maternal reproductive system [10,15,20,21]. In this sense, *L. quinquestriatus* scorpion venom increases the release of kinins that cause uterine contractions, and this effect caused abortions in rats [21,22]. In addition to *B. occitanus* and *T. serrulatus*, other scorpion venoms had similar results [8,23]. Another *A. amoreuxi* scorpion species caused fetal resorption, ossification defects and weight loss in survivor rats after the venom [24]. Some studies on pregnant rats conducted with *T. serrulatust, T. bahiensis* and *T. discrepans* scorpion venom showed that single or repetitive injections on different days of pregnancy caused pregnancy losses without changes to maternal weight [8-10]. In the present study, it was found that the weights of offspring, placenta and uterine and ovarian tissues decreased in the EG. In particular, it was thought that the reduced weight of placental tissues caused fetal growth retardation. The weights and morphometric parameters of all offspring decreased in the EG and they died within one hour following delivery. It is known that the placenta is a temporary endocrine organ that provides the relationship between offspring and uterine tissue. The various physiological functions of fetuses take place by means of the placenta [16]. It has been suggested that the venom of *A. crassicauda* may cause damage to the placenta and fetal growth restriction.

In many previous clinical cases described by Langley [25], Guler et al.[26], Kamalak and Kosus [27], Kaplanoglu and Helvaci [28], Bozkurt et al.[29], Sarı et al.[30] and Ozturk and

Koca [31], no adverse effect was reported in mothers or their fetuses after being stung by scorpion in different periods of pregnancy. Similarly, in a recent retrospective case series and literature review study, 24 pregnant women were admitted to the emergency department at Suruc (Sanliurfa) State Hospital due to scorpion stings and no important effects were reported on the fetus or the mother according to their medical records [11]. On the other hand, Zengin et al.[32] stated that a woman at 32 weeks of pregnancy developed lethargy and intense pelvic pain as the result of a scorpion sting. The patient was diagnosed with eclampsia as a complication of scorpion sting after medical examination. In addition, Duman et al.[33] reported vaginal bleeding in a woman. Erdogan et al.[34] reported that a pregnant woman stung by a scorpion developed an early membrane rupture and the newborn was followed up with the diagnosis of growth retardation, patent ductus arteriosus and bronchopulmonary dysplasia. Similarly, in a retrospective work of 14 years in Tunisia by Ben Nasr et al.[15] although twenty pregnant women and their fetuses showed no complications, scorpion stings caused intense pelvic pain in two patients and vaginal bleeding in one. In another study, Ismail et al.[24] noted abortion cases in pregnant woman following an *A. amoreuxi* scorpion sting. Finally, in India there are many reports of pregnant women who died because of the red scorpion sting [35]. In the current study, rat dams of the EG survived during the experimental procedure. Eventhough in only one rat abortion was observed and none in the control group, it could refer to the venoms possible abortive effect.

In conclusion, no adverse effects were reported in mothers or their fetuses after being stung by a scorpion in some previous clinical pregnant cases in Turkey. On the other hand, the findings of the current study reveal that the venom of *A. crassicauda* can cause abortion as well as restrictions in placental and fetal growth. It has been shown that scorpion venom affects the development of offspring in the rat model during pregnancy. Therefore, medical professionals should be informed about possible adverse effects and risks in pregnancy. However, further studies are needed in this area to determine effects on the development of offspring.

Conflict of Interests

The authors explicitly state that there are no conflicts of interest in connection with this article.

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Research Article

Genotoxic and Toxicopathological Effect of Aflatoxin B_1 in Grass Carp *(Ctenopharyngodon idella)*

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Abstract

The current study was designed to evaluate genotoxic and toxicopathological effect of aflatoxin B_1 (AFB_1) in fresh water fish also known as Grass carp *(Ctenopharyngodon idella)*. For the *in vitro* production of AFB_1, *Aspergillus flavus* spores were grown on potato dextrose agar (PDA) and then rice was used as a substrate. The quantity of AFB_1 was found 50ppb/g of rice using high performance liquid chromatography (HPLC) technique. A total of n=150 Grass carp (42±5 g) were divided into 5 groups (A, B, C, D, E). Group A (kept as a control), while groups B, C, D and E were exposed to 25 ppb, 50 ppb, 75 ppb and 100 ppb AFB1 per kg of diet, respectively, for 49 days. The average weight gain (WG) and specific growth rate (SGR) of fish was significantly higher in group A (WG-15.35 g and SGR-0.63%) compared to group E (WG-6.55 g and SGR-0.28%). The Feed Conversion Ratio (FCR) was significantly ($P<0.05$) different between control group and groups D and E. No mortality was notice in any group of fish because of AFB_1. However, increasing concentrations of AFB_1 significantly lowered the red blood cells (RBC) count, hematocrit, hemoglobin concentration, mean corpuscular volume (MCV), white blood cells (WBC) count and lymphocytes as compared to the control group. Additionally, significant increase of aspartate amino transferase (AST), alanine amino transferase (ALT), glucose, urea and creatinine was determined in AFB_1 treated groups by serum biochemistry. Total serum proteins and albumin level was found significantly higher in control group (6.05 and 4.2 g/dL), compared to AFB_1 exposed groups including; group B (5.8 and 3.9 g/dL), group C (5.4 and 3.7 g/dL), group D (4.2 and 2.7 g/dL) and group E (3.8 and 2.06 g/dL). The genotoxicity of AFB_1 was only recorded in fish of group D and E exhibiting micronuclei frequency percentage of 0.85 and 2.15% respectively. The histopathological study revealed that higher concentrations of AFB_1 were causing pathological changes in liver, kidney, intestine and gills tissue. The present study concluded that AFB1 was responsible for reduction in fish production performance, alteration in fish hematological and serum biochemical profiles, structural and functional alteration in tissues and DNA of tissues of fish.

Keywords: *Toxicology, Aflatoxin B_1, Grass carp, Ctenopharyngodon Idella, Hematology, Histopathology, Genotoxicity*

Ot Sazanı *(Ctenopharyngodon idella)*'nda Aflatoksin B_1'in Genotoksik ve Toksikopatolojik Etkileri

Öz

Bu çalışma, aflatoksin B_1'in (AFB_1) ot sazanı *(Ctenopharyngodon idella)* olarak da bilinen tatlı su balıklarında genotoksik ve toksikopatolojik etkilerini değerlendirmek amacıyla planlandı. AFB_1'in *in vitro* üretimi için, *Aspergillus flavus* sporları patates dekstroz agar (PDA) üzerinde büyütüldü ve daha sonra substrat olarak pirinç kullanıldı. Yüksek performanslı likit kromatografi (HPLC) tekniği kullanılarak yapılan analizlerde, pirinçteki AFB_1 miktarı 50 ppb/g bulundu. Toplam 150 adet Ot sazanı (42±5 g) 5 gruba (A, B, C, D, E) ayrıldı. A (kontrol), B, C, D ve E grupları 49 gün boyunca sırasıyla kg başına 25 ppb, 50 ppb, 75 ppb ve 100 ppb AFB1'e maruz bırakıldılar. Balıkların ortalama ağırlık artışı (WG) ve spesifik büyüme hızı (SGR), A grubunda (WG-15.35 g ve SGR-0.63), E grubuna kıyasla (WG-6.55 g ve SGR-% 0.28) anlamlı olarak daha yüksekti. Yemden yararlanma oranı (FCR), kontrol grubu ile D ve E grupları arasında anlamlı derecede farklıydı ($P<0.05$). Balık gruplarının hiçbirinde AFB1 nedeniyle ölüm gözlenmedi. Bununla birlikte, artan AFB_1 konsantrasyonları, kırmızı kan hücreleri (RBC) sayısını, hematokrit, hemoglobin konsantrasyonu, ortalama eritrosit hacmi (MCV), beyaz kan hücreleri (WBC) sayısı ve lenfositleri kontrol grubuna göre önemli ölçüde düşürdü. Ek olarak, serum biyokimyasal analizlerinde AFB_1'e maruz kalan gruplarda aspartat amino transferaz (AST), alanin amino transferaz (ALT), glukoz, üre ve kreatinin düzeylerinde anlamlı artış tespit edildi. Toplam serum proteinleri ve albümin seviyesi kontrol grubunda (6.05 ve 4.2 g/dL) AFB_1'e maruz kalan gruplara göre anlamlı derecede yüksek bulundu; B grubu (5.8 ve 3.9 g/dL), C grubu (5.4 ve 3.7 g/dL), D grubu (4.2 ve 2.7 g/dL) ve E grubu (3.8 ve 2.06 g/dL). AFB1'in genotoksisitesi sadece D ve E grubundaki balıklarda kaydedildi ve mikronükleus frekans yüzdesi sırasıyla %0.85 ve 2.15 olarak belirlendi. Histopatolojik bulgular, yüksek AFB_1 konsantrasyonlarının karaciğer, böbrek, bağırsak ve solungaç dokusunda patolojik değişikliklere neden olduğunu ortaya koydu. Bu çalışmada AFB_1'in balık üretim performansında düşüş, hematolojik ve serum biyokimyasal profillerinde farklılaşma, dokularda ve DNA'da yapısal ve fonksiyonel değişikliklerden sorumlu olduğu sonucuna varıldı.

Anahtar sözcükler: *Toksikoloji, Aflatoksin B_1, Ot sazanı, Ctenopharyngodon Idella, Hematoloji, Histopatoloji, Genotoksisite*

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INTRODUCTION

An *Aspergillus flavus*, an opportunistic fungus, is one of the major cause of mycotoxins contamination in crop such as cotton, maize and peanuts around the world [1]. The important mycotoxin is aflatoxin in which AFB_1 is the most toxic, teratogenic, carcinogenic and mutagenic [2]. A high dose of AFB_1 could cause mortality from aflatoxicosis [3].

Aflatoxin contamination in feed has adverse effects on health in fish such as reduced growth rate and presence of gross and microscopic lesions which leads to economic losses due to low production, mortalities and poor quality of fish and fish products [4]. Importantly, AFB_1 tends to induce oxidative stress in the host which may leads to functional and structural alteration in the living body including AFB_1-induced cell injury, lipid peroxidation, misfolded protein formation and DNA damage, which may ultimately lead to neoplasia [5]. The toxic and metabolic effects of AFB_1 and AFM_1 are principally observed in the liver [6]. Evidences have been accumulated that chromosomal damage could occurs due to free radicals formed under the influence of AFB_1 [7]. The reactive oxygen species may be partly involved in the carcinogenic activity of AFB_1 [8]. The minimum permitted levels of aflatoxin recognized by many countries ranges from 1-25 µg/kg of total aflatoxin. The minimum permissive level of aflatoxin for food commodities in global trade is 15 µg/kg [9]. In freshwater aquatic species aflatoxicosis has been studied mainly in rainbow trout [10], American channel catfish [11], Nile tilapia [12], Indian carp [13].

The first aflatoxicosis case in aquaculture was reported in May 1960, when an epidemic of hepatomas occurs in *Oncorhynchus mykiss* hatcheries farm in Idaho, United State. The epidemic of hepatomas was mainly characterized by the presence of carcinoma of primary hepatocytes, with multinodular hepatomegaly by postmortem examination [10].

The present study was designed keeping in view the potential of AFB_1 in the generation of genotoxic and toxico-pathological effects directly in the animal tissues. Therefore we evaluated growth performance, hematological, serological, histopathological and genotoxic effects of various concentrations of AFB_1 in grass carp.

MATERIAL and METHODS

The experiments were carried out according to the Rules and Regulations of the Animal Ethics Committee FAHVS, The University of Agriculture Peshawar. Healthy grass carp (n=150) of similar age (<1 year), weight (40 g-45 g), length (10-12 cm) and of ether sexes were obtained from Government Fish Hatchery, Mardan and were divided into five groups each having three replicates. Group A receiving no treatment while group B, C, D and E were exposed to different concentrations of AFB_1 25 ppb, 50 ppb, 75 ppb and 100 ppb/kg of diet respectively for 7 weeks. Fish were acclimatized for one week in glass aquaria before starting the experiment.

Aflatoxin Production, Extraction and Quantification

Aspergillus flavus spores were cultured on rice for the production of AFB_1 [14]. HPLC and fluorescent detection method was used for the quantification of AFB_1 [15].

Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR)

All fish in each tank were weighed and counted on 7th week. The SGR was calculated as follows:

Specific growth rate (%/day) = ln (final weight) - ln (initial weight) × 100/t

Where t = Number of days. Feed conversion ratio = Total feed intake/weight gain

Hematological and Blood Biochemical Profile

At the end of the experiment the fish were euthanized using clove oil 3-4 drops per liter of water. The blood from the caudal vein of each fish was collected for hematological parameters including RBCs count, Hb, MCV, MCHC, MCH, PCV, TLC and DLC using hematology analyzer (Celly 70). Liver and kidney function tests were determined using the serum. These tests includes glucose, AST, ALT, total protein, albumin, globulin, urea and creatinine estimation using semi-automatic chemistry analyzer (Convergys 100).

Genotoxicity

The genotoxic potential of the AFB_1 was measured by micronucleus (MN) [16]. For micronucleus assay, a thin smear of the freshly collected blood was made on the pre-cleaned slide. Slides were fixed with methanol for 20 min and then allowed to air dry. The slides were stained with Giemsa solution for 25 min. From each slide a total of 2.000 RBCs were examined at 1000X magnification under couple charged device (CCD) attached microscope (Olympus CX41). Micronucleus was scored as ovoid or circular, non-refractive, small chromatin body displaying the same staining and focusing pattern as the normal nucleus. The percentage of micronuclei was calculated by the following formula.

$$\text{Micronucleus \%} = \frac{\text{Number of erythrocytes containing micronucleus}}{\text{Total number of erythrocytes scored}} \times 100$$

Histopathology

The tissue samples of kidney, gills, liver and intestine were collected from freshly euthanized fish and directly put in 10% formalin for fixation to avoid tissue deterioration. The samples were then subjected to histopathology as reported elsewhere [17]. Briefly, these samples were washed overnight with running tape water in order to remove formalin. Then tissues were dehydrated gradually with 30, 50, 70, 80, 90, 100% alcohol respectively. Fish tissues were then cleared from alcohol with xylene. Infiltration of tissues was done with liquid paraffin. After impregnation of tissues the blocks were prepared by using automatic tissue embedding

assembly (Tissue-Tek® TEC™ Sakura). Blocks were made by pouring carefully melted paraffin over the placed tissue in plastic cassettes. Blocks were then shifted and placed in cold chamber of Tissue Tek® and were allowed to solidify. Paraffin tissue blocks were sectioned with thickness of 5 µm by using microtome (Accu-Cut® SRM™ 200 Sakura, Japan). The cut fine sections were placed in water bath (M-1450 Sakura) at 56°C so that it floats over the surface of water and folds were removed. For proper sticking of sections albumin was applied on clean glass slides. Section was mounted over the slide and placed on slide drying hot plate (Mod. 1452, Sakura) for 30-40 min for drying followed by placing in hot air oven (Mod. LDO-060E, Daihan Lab Tech. Co. Ltd, Korea) for 2-3 h for drying and removal of extra paraffin. Slides with sections of tissue were placed for staining after final drying. For staining of slide section, Hematoxylin (H) and Eosin (E) stain was used. Automatic slide staining machine (Tissue-Tek® DRS™ 2000 Sakura, Japan) was used for staining process [17].

Histopathological Lesion Scoring

A semi quantitative lesion scoring method *(Table 1)* was used which involved the application of severity grades and it is a defined numerical severity score of specific lesions [18]. Severity grading relies on estimates of severity rather than actual measurements [19].

Statistical Analysis

The data obtain was compiled in Microsoft Excel and was analyzed through Statistix 8.1 software using one way ANOVA (CRD).

Table 1. *The illustration of severity grading*

Numerical Score	Description	Definition
0	Normal/Minimal 0-25%	The tissue considered being normal, under the conditions of study and considering sex, strain and age strain of the animal. Changes may be present which, under other circumstances, would be considered abnormal
1	Mild 26-50%	The lesion is easily recognized but of limited severity
2	Moderate 51-75%	The lesion is prominent but there is significant potential for increased severity
3	Severe 76-100%	The degree of change is as complete as possible (Most part of the organ is involved)

RESULTS

Our analyses revealed that average weight gain and SGR was significantly affected (P<0.05) by increasing concentrations of AFB_1 in the feed. The higher weight gain and SGR was recorded in control group followed by group B, C, D and E *(Table 2)*.

There was no significant difference in FCR of control group and group B and C (P>0.05) but FCR of control group was significantly high (P<0.05) than group D and E. No mortalities were recorded in all groups and the survival rate was 100% *(Table 2)*.

The total RBC count of group E was significantly (P<0.05) decreased as compared to control group but there was no significant difference between control group and group B and C. For PCV there was no significant difference (P>0.05) in group A, B and C while the PCV of group A was significantly different (P<0.05) from group D and group E. The hemoglobin concentration of control group was significantly different from group C, group D and group E but no significant difference (P>0.05) from group B. The MCV in group A, B, C and D has no significant difference (P>0.05) but the MCV of group A was significantly decreased (P<0.05) from the group receiving higher dose of AFB_1 in the diet. Among all the groups the concentration of MCH and MCHC show no significant difference (P>0.05). On the basis of erythrocyte indices the anemia was classified as normocytic normochromic in group A, B, C and D respectively while microcytic normochromic anemia was found in group E *(Table 3)*.

The TLC of all the groups significantly (P<0.05) decreases with the increase in concentration of AFB_1. The lymphocyte percentage of group A was significantly higher as compared to the groups fed with higher concentration of AFB_1 in the diet but there was no significant difference (P>0.05) between group A and B. There was significant increase (P<0.05) in monocyte and neutrophil in group E as compared to control group. The percentage of basophil and eosinophil have no significant difference *(Table 4)*.

The ALT and AST showing significant increase (P<0.05) in

Table 2. *The average weight gain, specific growth rate, FCR, survival rate and micronuclei of Grass carp fed with different concentrations of AFB_1 added diet for 7 weeks*

Group	Average Final Weight (g)	Average Initial Weight (g)	Average Weight Gain (g)	SGR (%)	FCR	Survival Rate (%)	Micronucleus Frequency (%)
A	57.86	42.51	15.35±0.10a	0.63±0.07a	2.09±0.05c	100	0
B	53.12	43.28	9.84±0.04b	0.42±0.07b	2.14±0.03bc	100	0
C	52.14	43.36	8.78±0.03b	0.36±0.09c	2.18±0.03bc	100	0
D	49.69	42.86	6.83±0.05bc	0.30±0.05d	2.23±0.04b	100	0.85^{a}±0.02
E	48.73	42.18	6.55±0.09c	0.28 ±0.08d	2.34±0.04a	100	2.15^{b}±0.01

Values (Mean±SE) in the column shown by different letters are significantly different (P≤0.05)

Table 3. *Hematological parameters of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group	RBC*10/µL	PCV (%)	Hb (gm/dL)	MCV (fL)	MCH (pg)	MCHC (gm/dL)
A	1.60±0.01a	32.6±0.88a	8.2±0.14a	203.28[a]±0.34a	51.2±0.11a	25.2±0.64b
B	1.50±0.03ab	32.0±0.52a	8.0±0.14ab	201.14±0.8a	50.5±0.94a	25.1±0.73b
C	1.53±0.02bc	30.3±0.66ab	7.6±0.11c	198.23±0.59a	49.7± 0.49a	25.0±0.92b
D	1.47±0.01c	28.6±0.66b	7.6±0.17bc	194.09±0.98ab	51.9± 0.77a	26.7±0.21ab
E	1.40±0.12d	25.6±0.33c	7.0±0.06d	182.45± 0.35b	50.2± 0.31a	27.5±0.3a

Values (Mean±SE) in the column shown by different letters are significantly different (P≤0.05)

Table 4. *TLC and DLC of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group	WBC*10^3/µL	Lymphocyte (%)	Monocyte (%)	Neutrophil (%)	Basophil (%)	Eosinophil (%)
A	12.4±0.55a	71.3±0.88a	1.6±0.33b	24.0±0.15c	1.3 ±0.33a	1.6±0.66a
B	12.7±0.2a	69.0±0.15a	1.6±0.66b	25.3±0.66bc	1.6±0.33a	2.3±0.33a
C	10.5±0.37b	64.6±0.85b	3.3±0.88b	28.0±0.52b	2.0±0.57a	2.0±0.57a
D	9.3 ±0.12c	62.0±0.15b	6.0±0.78a	28.6±0.88b	1.6±0.66a	1.6±0.33a
E	8.1±0.13d	53.0±0.15c	7.6±0.33a	35.0±0.15a	1.6±0.33a	2.6±0.66a

Values (Mean±SE) in the column shown by different letters are significantly different (P≤0.05)

Table 5. *Hepatotoxic and Nephrotoxic effect in Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group	ALT (IU/L)	AST (IU/L)	Glucose (mg/dL)	Total Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Creatinine (mg/dL)	Urea (mg/dL)
A	15.8±0.42e	26.6±0.23e	62.3±0.45d	6.05±0.19a	4.2±0.12a	1.8±0.07a	0.19±0.013d	10.1±0.15c
B	21.08±0.77d	33.2±0.17d	68.6±0.45c	5.8±0.05ab	3.9±0.13b	1.9±0.07a	0.24±0.012c	12.3±0.63b
C	25.6±0.73c	40.5±0.07c	75.0±0.73b	5.4±0.24b	3.7±0.10b	1.6±0.33a	0.28±0.011b	12.6±0.84b
D	31.7±0.78b	47.7±0.84b	80.6±0.20a	4.2±0.08c	2.7±0.11c	1.5±0.17a	0.30±0.011ab	13.1±0.48ab
E	38.3±0.60a	55.2±0.34a	83.6±0.45a	3.8±0.04d	2.06±0.03d	1.7±0.07a	0.32±0.012a	14.6±0.21a

Values (Mean±SE) in the column shown by different letters are significantly different (P≤0.05)

the level with increase in AFB_1 concentration. Group E shows significant raise (P<0.05) in level of ALT and AST as compared to the control group A and other treatment groups (B, C and D). The significant increase (P<0.05) was also demonstrated among the different treatment groups such as group B, C, D and E with rise in AFB_1 concentration. Glucose concentration shows significant rise (P<0.05) in the level with the increasing concentration of AFB_1. Among group E and D there was no significant variance (P>0.05) but there was significant increase (P<0.05) in glucose level with increase in AFB_1 concentration. Total protein and albumin shows the drastic decrease in concentration with the increasing level of AFB_1, which show significant (P<0.05) inverse relationship. Globulin concentrations showing no significant increase (P>0.05) in varying levels of AFB_1 treatment in grass carp. The urea and creatinine levels significantly increase (P<0.05) with increasing level of AFB_1 concentration in the feed *(Table 5)*.

The micronucleus frequency percentage of grass carp of group A, B and C were zero percent but there was significant increased (P<0.05) in group E followed by group D *(Fig. 1, Table 2)*.

The liver tissue of grass carps exposed to different levels of AFB_1 showed hydrophic degeneration, fatty change, necrosis and leukocytic infiltration and necrotic cells with pyknotic nuclei *(Fig. 2)*. The fatty change was more severe in group E followed by group D. Mild leukocytic infiltration and pyknotic nuclei were also observed. Microscopically the kidney of grass carp show nephrosis, leukocytic infiltration, vacoulation of epithelial cell, necrotic cells with pyknotic nuclei and glomerular shrinkage in dose dependent manner *(Fig. 3)*. The increase in the level of AFB_1 in the diet causes severe changes. The histomorphology of intestine of grass carp show hypoplasia of goblet cells, villi sloughing, leukocytic infiltration with the increase in concentrations of AFB_1 in diet *(Fig. 4)*. The gills of grass carp were also affected. In group D and E there was sloughing of respiratory epithelium, degeneration of the lamellae, congestion, leukocytic infiltration and necrosis *(Fig. 5)*. The lesion scoring of liver, kidney, intestine and gills are presented in *Tables 6, 7, 8* and *9* respectively.

DISCUSSION

The present study revealed that weight gain and specific growth rate of grass carp fed with different concentrations of AFB_1 in the diet was significantly lower than control group (P<0.05) with no mortality. The results of current study were agreed with the study reported previously [12,20,21]. They

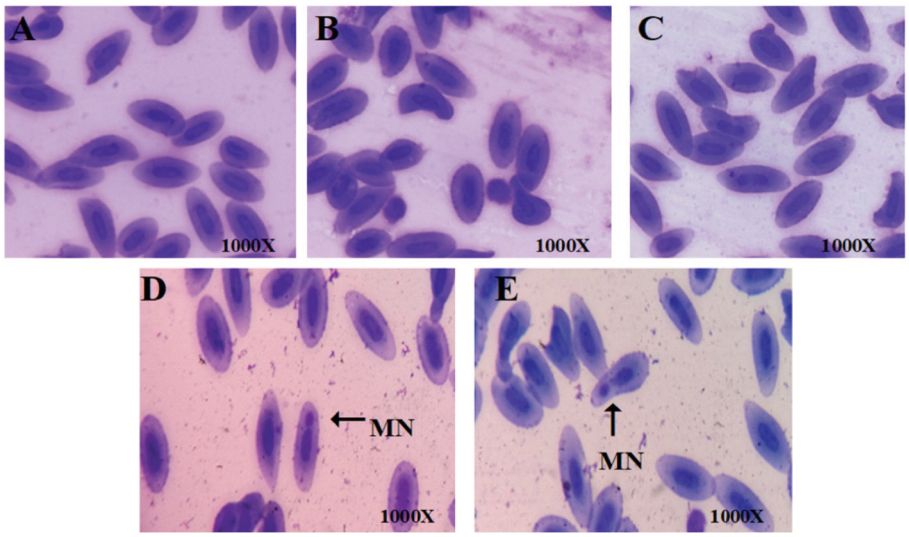

Fig 1. Erythrocytes of grass carp of Group A, and Group B are showing normal morphology. Group D and Group E showing micronuclei (MN), Geimsa stained (1000X)

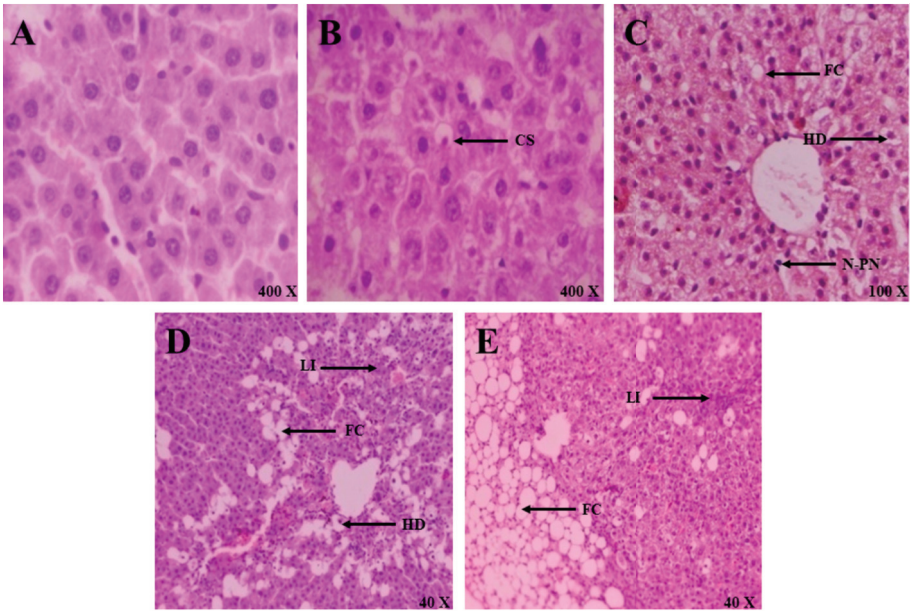

Fig 2. Photomicrograph of liver of grass carp exposed to different levels of AFB_1. **A)** Liver of control group; **B)** Liver of grass carp given 25 µg AFB_1 showing mild cloudy swelling of hepatocyte; **C)** Liver showing fatty change (FC), hydrophic degeneration (HD) and necrotic cell showing pyknotic nuclei (N-PN); **D)** Severe fatty change (FC), hydrophic degeneration (HD), leukocytic infiltration (LI); **E)** Showing severe fatty change (FC), leukocytic infiltration (LI). H&E stained, 40X, 100X, 400X

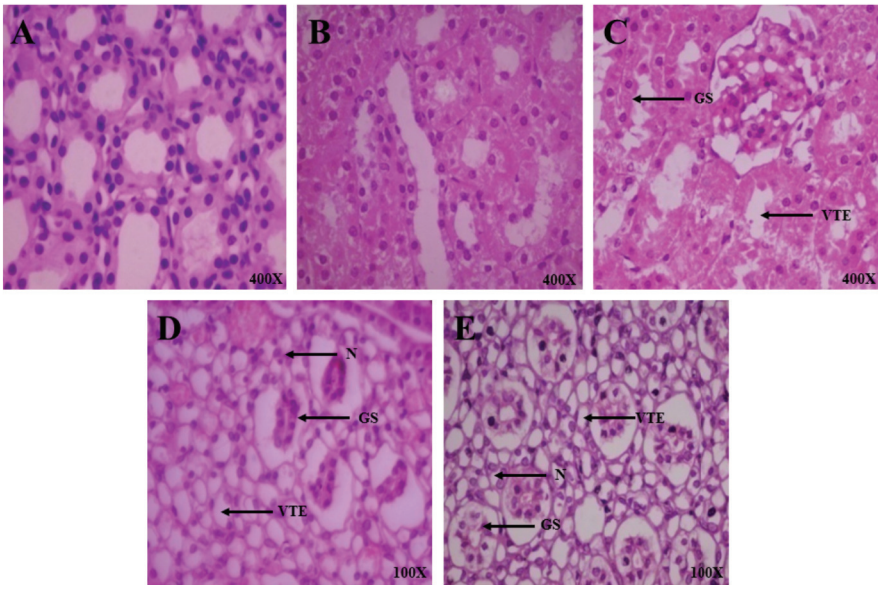

Fig 3. Photomicrograph of kidney of grass carp exposed to AFB_1. **A)** Kidney of normal group; **B)** Kidney of grass carp given 25 µg AFB_1 showing normal histomorphology; **C)** Kidney showing vacoulation of tubular epithelial cell (VTE) and glomeruli shrinkage (GS); **D)** Vacoulation of tubular epithelial cell (VTE), nephrosis (N) and glomeruli shrinkage (GS); **E)** Showing vacoulation of tubular epithelial cell (VTE), nephrosis (N) and Necrotic cells showing pyknotic nuclei (N-PN). H&E stained, 100X, 400X

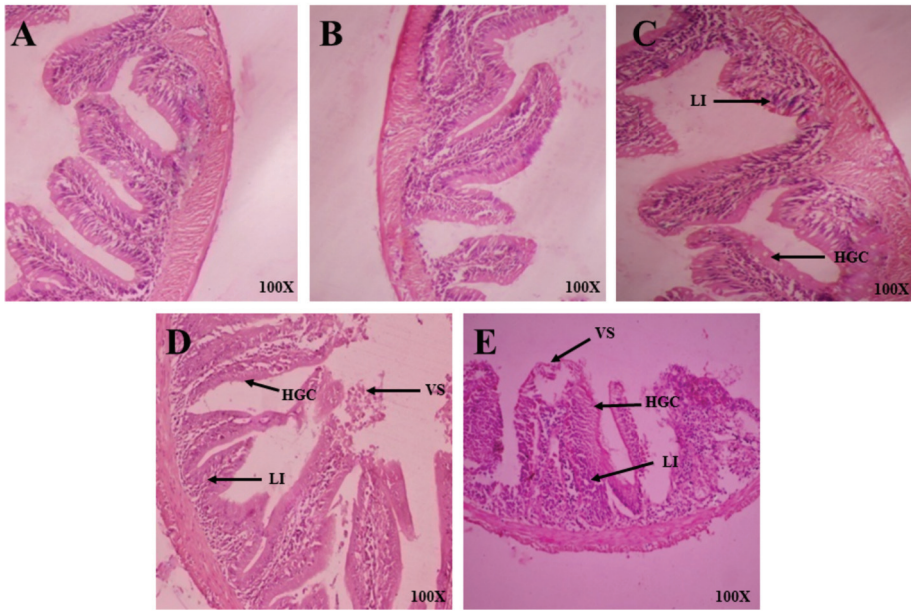

Fig 4. Photomicrograph of intestine of grass carp exposed to AFB_1. **A)** Normal intestine of control group; **B)** Normal intestine of grass carp exposed to 25 µg AFB_1; **C)** Showing hypoplasia of goblet cells (HGC), leukocytic infiltration (LI); **D)** Hypoplasia of goblet cells (HGC), villi sloughing (VS); **E)** Hypoplasia of goblet cells (HGC), villi sloughing (VS), leukocytic infiltration (LI). H&E stained (100X)

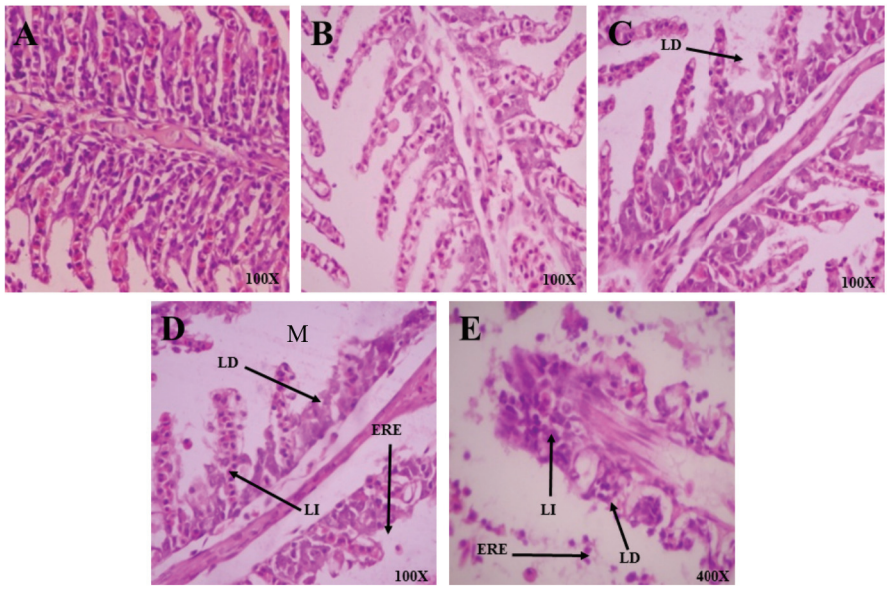

Fig 5. Photomicrograph of gills of grass carp exposed to AFB_1. **A)** Normal gills of control group; **B)** Normal histomicrograph of grass carp exposed to 25 µg AFB_1; **C)** Showing lamellae degeneration (LD); **(D-E)** Showing lamellae degeneration (LD), exfoliated respiratory epithelium (ERE), leukocytic infiltration (LI). H&E stained 100X and 400X

Table 6. *Histopathological scoring of liver of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group (AFB_1 µg/kg)	Lesion Score							Severity Percentage
	Hydrophic Degeneration	Fatty Change	Pyknotic Nuclei	Leukocytic Infiltration	Necrosis	Total Score	Max. Possible Score	
A	2	0	0	0	0	2	150	1
B	8	2	1	4	0	15	150	10
C	21	12	7	9	4	53	150	35.33
D	27	25	13	14	6	85	150	56.66
E	31	35	18	26	9	119	150	79.33

investigated that feeding of low to high concentrations of AFB_1 for a longer duration produces a decrease in feed intake and weight gain efficacy. Andleeb et al.[22] conducted study on fry Catlacatla and found the highest weight gain in control group as compared to aflatoxin treated groups. The survival rate was more than 90% in all AFB_1 treated group without any significant difference [23]. Sepahdari et al.[24] investigated that there was a decrease in weight gain and SGR in fish treated with 75 ppb and 100 ppb AFB_1 per Kg of diet as compared to control group after two months. The similar results were observed by Wang et al.[25] in Yellow catfish *(Pelteobagrus fulvidraco)* fed with

Table 7. *Histopathological scoring of kidney of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group (AFB_1 µg/kg)	Lesion Score								Severity Percentage
	Congestion	Vacoulation in Tubular Epithelial Cells	Pyknotic Nuclei	Glomeruli Shrinkage	Leukocytic Infiltration	Necrosis	Total Score	Max. Possible Score	
A	0	0	0	0	0	0	0	180	0
B	3	4	2	3	3	0	15	180	8.3
C	4	11	12	10	5	3	45	180	25
D	9	14	13	18	15	8	77	180	42.7
E	14	17	20	27	19	13	110	180	61

Table 8. *Histopathological scoring of intestine of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group (AFB_1 µg/kg)	Lesion Score						Severity Percentage
	Reduction in Goblet Cells	Leukocytic Infiltration	Sloughing of Epithelial Cells	Necrosis and Degeneration	Total Score	Max. Possible Score	
A	1	0	0	0	1	120	0.83
B	2	2	1	3	8	120	6.66
C	8	4	7	6	25	120	20.83
D	12	9	12	11	44	120	36.66
E	19	16	22	12	69	120	57.5

Table 9. *Histopathological scoring of gills of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group (AFB_1 µg/kg)	Lesion Score							Severity Percentage
	Lifting of Respiratory Epithelium	Degeneration of Lamellae	Congestion	Leukocytic Infiltration	Necrosis	Total Score	Max. Possible Score	
A	0	0	0	0	0	0	150	0
B	4	2	3	2	0	11	150	7.33
C	4	9	6	3	5	27	150	18
D	8	12	7	6	8	41	150	27.33
E	15	18	8	11	12	64	150	42.66

200, 500 and 1.000 µg kg^{-1} of AFB_1 in feed which showed decrease weight gain and growth rate and altered feed conversion ratio.

The hematological and blood biochemical profile in the current study was significantly affected by different concentrations of AFB_1. The hematocrit and hemoglobin concentration was reduced because of the decreased in total erythrocyte count. Due to damage to the lymphoid follicle the lymphocyte percentage declined. The effect of AFB_1 in low concentrations for long duration in Nile tilapia which revealed marked anemia and leucopenia [26]. Similar results were observed in channel catfish and common carp [22,24]. The results showed that AFB_1 causes increase in the serum AST, ALT, glucose, urea and creatinine level. The increase in AST, ALT and glucose is due to the damage to liver. AFB_1 causes injury to the hepatocytes which contain preformed AST and ALT released into the blood. The higher level of glucose in the blood indicates liver damage because the damage liver cannot respond to insulin. The findings of current study were agreed with the results of [12,27] which showed elevated levels of urea and creatinine in the blood of Nile tilapia exposed to different concentrations of AFB_1. The serum total protein and albumin levels decreased significantly in a dose dependent manner. This is due to the binding of AFB_1 metabolites with the macromolecules of cell and damage to the hepatocytes. Liver is mainly involved in the synthesis of different proteins. AFB_1 hepatotoxicity interfere the synthesis of proteins by bond formation of AFB_1 adducts to the macromolecules of cell [28].

The histopathological examination showed that higher concentrations of the AFB_1 can cause changes in the liver, including hydrophic degeneration, leukocytic infiltration, necrosis and progressive fat deposition particularly at 75 ppb and 100 ppb AFB_1/kg. The results are similar with the study of Evalvn et al.[29] who reported AFB_1 contamination in trout fish which showed microscopically basophilic hepatic cells with hyperchromatic nuclei in irregular cords. The gills showed degeneration of lamellae and lifting of respiratory epithelium. In kidney tissue nephrosis, glomeruli shrinkage and cell vacoulation were observed. The intestine showed hypoplasia of goblet cells, sloughing of epithelial cells and leukocytic infiltration. Histopathological studies revealed liver cell degeneration and necrosis and progressive fat deposition at a level of 75 ppb and 100 ppb AFB_1/kg of diets after 2 months [25]. The results are also in agreement with the study of [22], who reported that AFB_1 in *Catla catla*

fish causing histopathological changes in liver, kidneys and intestine. The higher concentrations of AFB_1 caused degenerative changes, pyknotic nuclei and increased vacoulation of liver hepatocytes. In kidneys necrotic changes in nucleus were quite prominent, the Bowman's space increased which was indicative of glomerular atrophy and there were eosinophilic proteinaceous material in the lumen of tubules. The intestine showed higher leukocytic infiltration, sloughing of epithelium and vacoulation in enterocytes.

The genotoxic effect of AFB_1 in grass carp was studied by micronucleus assay. The genotoxicity was only recorded at 75 ppb and 100 ppb AFB_1/kg, having micronucleus frequency percentage of 0.85 and 2.15% respectively. Abdullah et al.[30] investigated the DNA damage of AFB_1 in rainbow trout (sensitive) and channel catfish (resistant) using comet assay. Through intra peritoneal route 0.5 mg AFB_1/mL DMSO/kg body weight was administered to fish. The Comet assay was performed on total blood, kidney cells and hepatocytes of both channel catfish and rainbow trout after 4 and 24 h. Significant ($P<0.05$) and high genotoxicity was exhibited by trout kidney tissue and blood tested after 4 h which then reduced by 24 h. DNA damage gradually increased with time in liver cells.

The present study concluded that the production performance of fish is reduced in AFB_1 treated groups lead to economic loss. AFB_1 in higher concentrations affect the hematological and blood biochemical profile. The microscopic examination of tissues showed that AFB_1 causes pathological changes. AFB_1 is genotoxic and induced DNA damage in fish at higher concentrations (75 and 100 µg/kg of diet).

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Research Article

The Effect of the Supplemental Feeding of Queen Rearing Colonies on the Reproductive Characteristics of Queen Bees (*Apis mellifera* L.) Reared from Egg and Different old of Larvae

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Abstract

This study was carried out to determine the effect of supplemental feeding on the reproductive characteristics of queen bees reared from different stages of brood. Queen were reared from one and two-day-old larvae grafted by Doolittle method and were reared from the eggs were transferred by Karl Jenter set and given to the starter colonies prepared as queenless. The starter colonies are arranged as follows: B1, one-day-old larva were grafted; B2, two-day-old larvae were grafted; B3, two-day-old eggs were transfered; and F1, four grams of vitamin a, protein, and minaral mixture were added to the sugar syrup at a ratio of one to one (w/w) per day; F2, no supplemental feeding and the bees benefited only from natural resources. In general, supplemental feeding of starter colonies increased the acceptance rate of larvae and eggs. Colonies fed with a supplemental diet had a higher acceptance rate (82.35%) compared to unfed colonies (62.74%). The highest queen emergence weight (205.75±1.46 mg) was obtained from the two-day-old egg transfer. In the fed group, the average emergence weight of the queen bee was found to be 195.01±2.03 mg, while this value was determined as 186.30±2.09 mg in the group that was not fed. Supplemental feeding of the colonies increased the spermathecae diameter of the queens from 0.98±0.025 mm to 1.09±0.025 mm, while the number of spermatozoa in the spermathecae increased from 4.26±0.679 million to 4.54±0.648 million.

Keywords: *Egg transfering, Honeybee, Larva grafting, Queen rearing, Reproductive features, Supplemental feding*

Yetiştirme Kolonilerinde Ek Besleme Yapmanın Yumurta ve Farklı Yaştaki Larvalardan Yetiştirilen Ana Arıların (*Apis mellifera* L.) Üreme Özellikleri Üzerine Etkisi

Öz

Bu çalışma ek beslenmenin yumurta ve farklı yaşlardaki larvalardan yetiştirilen ana arıların üreme özellikleri üzerine etkisini belirlemek amacıyla yapılmıştır. Bir ve iki günlük yaştaki larvalar Doolitle yöntemiyle ve yumurtadan ana arı üretimi ise jenter seti yardımıyla transfer edilerek ana arısız olarak hazırlanan başlatıcı kolonilere verilmiştir. Başlatma kolonileri aşağıdaki şekilde düzenlenmiştir: B1, bir günlük larva transferi; B2, iki günlük larva transferi; B3, iki günlük yumurta transferi; F1, günlük bir litre bire bir oranında (bir suya bir şeker) şeker şurubuna dört gr vitamin, protein ve minarel karışımı ilave edilmiştir. F2, ek besleme yapılmayarak arıların sadece doğal kaynaklardan yararlanması sağlanmıştır. Genel olarak, başlatma kolonilerine yapılan ek beslemeler larva ve yumurta kabul oranını arttırmıştır. Tamamlayıcı diyetle beslenen koloniler (%82.35), beslenmemiş kolonilere (%62.74) kıyasla daha yüksek kabul oranına sahip olmuşlardır. Yetiştirme grupları içinde en yüksek ana arı çıkış ağırlığı (205.75±1.46 mg) iki günlük yumurta transferinden elde edilmiştir. Besleme yapılan grupta ortalama ana arı çıkış ağırlığı 195.01±2.03 mg olarak bulunurken, bu değer ilave beslenme yapılmayan grupta 186.30±2.09 mg olarak belirlenmiştir. Kolonilere ek besleme yapmak ana arıların spermatheca çapında 0.98±0.025 mm'den 1.09±0.025 mm'ye bir artış sağlarken, sparmatheka içindeki sperm sayısında ise 4.26±0.679 milyondan 4.54±0.648 milyona bir artış sağlamıştır.

Anahtar sözcükler: *Yumurta transferi, Larva aşılaması, Balarısı, Ana arı yetiştirme, Ek besleme, Üreme özellikleri*

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INTRODUCTION

Usually a honey bee colony has one queen, a few hundred drones and thousands of worker bees. However, due to its anatomical, physiological and behavioral characteristics and its functions within the hive, queen is the most important individual in a colony [1,2]. It is possible to identify colony performance with the performance of the queen bee [3].

Having quality queen bees can help to inctease the performance of honey bee colonies. However, the quality of a queen varies depending on many physical properties, such as emergence weight, diameter of spermatheca and sperm number stored in the spermatheca [4-6]. Physical characteristics that affect the quality of the queen are influenced by various factors such as rearing season, genotype, feeding, age and number of transferred larvae [6-8]. Many investigators have reported a negative relationship between larval age and quality of the queen [9-11].

Recently, supplemental feeding and dietary formulations of queen breeding colonies have become a common approach in beekeeping. Pollen, nectar, syrup and vitamins are important nutritional components for queen rearing colonies [12,13]. In addition, it has been reported that providing additional nutrients to the rearing colonies significantly increases the quality of the transferred larvae and hence the quality of the queen bees [14,15].

The need for large quantity and high-quality queen bee breeders in commercial queen breeding has led to different searches for methods to increase queen quality [7]. Doolittle [16] was the first person to instill worker larvae into artificial queen cells, and, since then, many studies have been conducted on the factors affecting the larval acceptance rate. Some researchers reported that the supplemental feeding of queen rearing colonies positively affected the larval acceptance rate [17,18]. Another researcher reported that the acceptance rate of egg transfer was lower than the transfer of larvae, but it was reported that the queen bees from the egg transfer had higher weights compared to the queen bees that reared from the larvae transfer [19,20].

Queen bee emergence weight can be used as a quality factor in the evaluation of queen bees because high emergence queen bees have a larger spermatheca diameter, a higher ovariol number, and a higher number of spermatozoa [4,21]. On the other hand, it is stated that queen body size or emergence weight can be used as a reliable index for determining the quality of the queen bee [22].

This study was carried out to determine effect of supplemental feeding on reproductive characteristics of queen bees reared from different stages of brood such as heights of sealed queen cell, emergence weight, spermatheca diameter and number of spermatozoa in spermathecae.

MATERIAL and METHODS

The research was conducted between June and August of 2017 in the beekeeping and research center of Ardahan University in Turkey. In the study, the Caucasian honey bee *(Apis mellifera caucasica)* colonies were used. The six sisters queen bees, which were reared in the previous year was used as starter colonies. Six colonies, each with ten frames, were reduced to five frames, and then the starter colonies were formed equalized according to adult bees, brood area, honey and pollen frame.

The starter colonies were arranged as follows: B1, one-day-old larva transfer; B2, two-day-old larvae transfer; B3, two-day-old egg transfer; F1, four grams/kg of a vitamin, protein, and mineral mixture were added to the sugar syrup at a ratio of one to one (w/w) per day (vitamin A, 6.000.000 I.U; vitamin D3, 1.200.000 I.U; vitamin E, 1000 mg; vitamin B12, 24 mg; vitamin C, 5000 mg; biotin, 10 mg; folic acid, 100 mg; choline, 3000 mg; inositol, 3000 mg; carotene, 100 mg; methionine, 3000 mg; lysine, 6000 mg; threonine, 3000 mg; tryptophan, 3000 mg; manganese, 300 mg; iron, 300 mg; zinc, 300 mg; copper, 100 mg; iodine, 50 mg; magnesium 10.000 mg; potassium, 20.000 mg; and phosphorus 3000 mg); F2, no supplemental feeding and the bees provided benefited only from natural resources. Eggs and larvae transfers to each group were given to six starter colonies. 34 larvae/eggs were transferred to each of these colonies. One and two-day-old larvae were transferred by the Doolittle method and queens reared from the egg were transferred by Karl Jenter set and given to the starter colonies prepared as queenless [19,20,22]. The starter colonies were fed two days before the transfers and feeding was continued until the queen cells were sealed [14]. In this case, eight days (eight liters of syrup) to the B1 starter colony, seven days (seven liters of syrup) to the B2 starter colony, and 10 days (10 liters of syrup) were given to the B3 starter colony.

The acceptance rate was determined by counting the larvae/eggs which were accepted two days after the larvae/egg transfer. Larvae/egg acceptance rates were calculated as a percentage with the following formula:

$$\text{Acceptance Rate} = \frac{\text{(Accepted Larvae/Egg)}}{\text{(Total Grafted Larvae/Egg)}} \times 100$$

On the tenth day, queen cells were measured with using calipers and then caged, and emergence time was provided. The queens were weighed in a sensitive scale (mg), and the emergence weights were determined. The selected queens were introduced to the combs that covered with worker in mating nucs and mated. Twenty days after mating, the spermatheca of ten laying queens were removed in each group, the trachea on the spermatheca was cleaned, the sperm sac was taken on a lamella slide, and the diameters of spermatheca were measured with

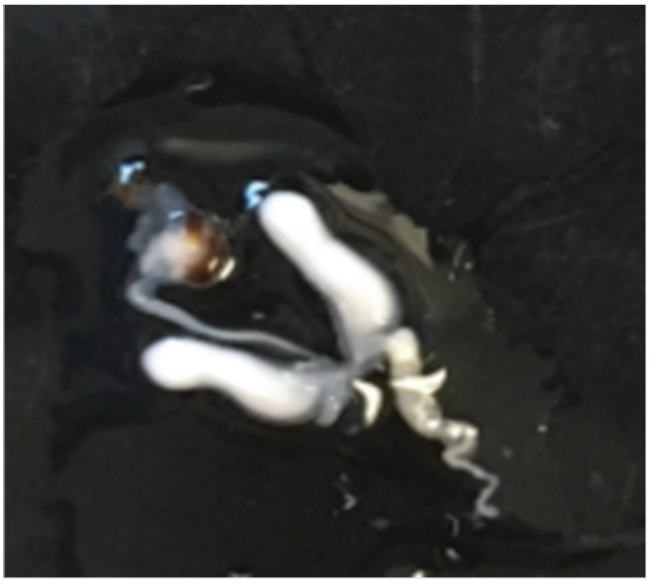

Fig 1. Queen bee reproductive organs

Fig 2. Queen bee and spermathecae

an ocular micrometer at a 4.5x10 magnification microscope [21,23-25]. The spermathecae were then discharged with a fine insect needle and fine forceps in 1 mL of saline solution (0.9%). Tap water was added to make 10 mL total volume. The sample that was taken from this mixture was dropped between the lamella and the lamella slide; then the number of spermatozoa in the square part of the Thoma slide were counted, and the total amount of spermatozoa (million pieces/queen) found in the 10 mL mixture and also in the sperm sac of the queen bee was calculated [23-27].

In the statistical analysis of the data heights of the sealed queen cell, the emergence weight, the diameter of the spermathecae and number of spermatozoa were compared according to one-way analysis of variance[28]. The chi-square test was used for the statistical analysis of cell acceptance rates. The DUNCAN multiple comparison test was used to determine the differences between the groups.

RESULTS

The acceptance rates in one, two-days-old larvae and two-day-old eggs groups were determined 72.06%, 83.82%, and 61.76%, respectively *(Table 1)*. The highest acceptance rate was obtained from two-day old larvae while the lowest acceptance rate was obtained from two-day old egg. The difference between larval acceptance rates was statistically insignificant while the difference between larval and egg transfer rates was statistically significant (P<0.01). The acceptance rate of egg transfer was lower than larva transfers.

In general, giving a feed containing proteins, vitamins, and minerals to the initiating starter colonies increased the larval and egg acceptance rate *(Table 1)*. The rate of acceptance in the fed colonies (82.35%) was found higher than the acceptance rate of non-fed colonies (62.74%). The difference was observed in the acceptance rate of larvae and eggs between the fed and non- the fed groups was found significant (P<0.01).

The results show that the longest sealed queen cell was obtained from two-days-old egg transfer group. The difference between the groups in terms of sealed queen cell was found to be statistically significant (P<0.01). Although the acceptance of egg transfer is low, it is thought to be an important application in terms of increasing the sealed queen cell length. While 29.05±0.24 mm long sealed queen cell was obtained from fed colonies, this value was determined to be 27.03±0.39 mm in non-fed colonies. The difference observed in terms of sealed queen cell between groups was found to be significant (P<0.01).

Table 2 shows emergence weights of queens raised from the one-day-old larvae, two-day-old larvae, and two-day-old eggs. The highest emergence weight (205.75±1.46 mg) was obtained from the two day old egg transfer. The difference observed between the emergence weights of the queens raised from different stages of the brood was significant (P<0.01). In other words, egg transfer was significantly effective for the emergence weight of the queen bee.

In the feeding group, the average (±S.E.) emergence weight of the queens was found to be 195.01±2.03 mg, and this value was determined to be 186.30±2.09 mg in the group which was non-fed. The effect of feeding on the emergence weight of the queen was found to be significant (P<0.05). Feeding the starter colonies influenced the emergence weight of the queen bee.

Table 3 presents the diameter of the spermathecae and

Table 1. *Average larvae/egg acceptance rates of the groups (%)*

Groups	Number of Grafted Larva and EGG	Number of Accepted Larvae and Egg	Grafting Success Rate (%)
Breeding Method (B)			
1 day old larvae (B1)	68	49	72.06[ab]
2 day old larvae (B2)	68	57	83.82[a]
2 day old egg (B3)	68	42	61.76[b]
Feeding Medhod (F)			
Fed colonies (F1)	102	84	82.35[a]
Unfed colonies (F2)	102	64	62.74[b]
B x F Interaction			
B1XF1	34	28	82.35[ab]
B1XF2	34	21	61.76[c]
B2XF1	34	32	94.11[a]
B2XF2	34	25	73.53[b]
B3XF1	34	24	70.58[b]
B3XF2	34	18	52.94[c]

[a,b,c] *Means with no common superscripts differ (P<0.01)*

Table 2. *The average (±S. E.) of sealed queen cell length and emergence weight of queen bees in research groups*

Groups	N	Sealed Queen Cell Length (mm)	N	Emergence Weight (mg)
		X±Sx		X±Sx
Breeding Method (B)				
1 day old larvae (B1)	49	27.52±0.30[b]	48	191.90±1.46[b]
2 day old larvae (B2)	57	24.20±0.37[c]	56	174.32±1.94[c]
2 day old egg (B3)	42	31.40±0.30[a]	42	205.75±1.46[a]
Feeding Medhod (F)				
Fed colonies (F1)	84	29.05±0.24	84	195.01±2.03[a]
Unfed colonies (F2)	64	27.03±0.39	62	186.30±2.09[b]
B x F Interaction				
B1XF1	28	28.25±0.34[c]	28	195.85±1.68[c]
B1XF2	21	26.80±0.41[d]	20	187.95±2.07[d]
B2XF1	32	25.30±0.48[e]	32	179.85±2.67[e]
B2XF2	25	23.10±0.44[f]	24	168.80±2.28[f]
B3XF1	24	32.60±0.34[a]	24	209.35±2.33[a]
B3XF2	18	30.20±0.32[b]	18	202.15±1.40[b]

[a,b,c,d,e,f] *Means with no common superscripts differ (P<0.01); (P<0.05)*

number of spermatozoa of queens raised from the one day, two-day-old larvae and two-day-old eggs. The spermatheca diameters for the queens raised from the one-day, two-day-old larvae and two-day-old eggs were determined to be 1.04±0.018 mm, 0.83±0.017 mm, and 1.23±0.021 mm, respectively *(Table 3)*. The largest spermatheca diameter was obtained from egg transfer (two-days old), while the lowest spermatheca diameter was obtained from the queens raised from two-days-old larvae. The difference between the breeding groups was statistically significant (P<0.01). The results indicated that the egg transfer method has been increased the spermatheca diameters in honey bee queens. In addition, the ages of grafted larvae have a significant effect on diameter of the spermathecae and number of spermatozoa stored in sperm sac.

When the feeding colonies (1.09±0.025 mm) were compared with non-fed (0.98±0.025 mm), the diameter of spermatheca increased significantly (P<0.05). Supplemental feeding to the colonies increased the spermatheca diameter of the queen bees *(Table 3)*.

The breeding and feeding method influenced the number

Table 3. *The average (±S.E.) spermathecae diameters and numbers of spermatozoa stored in sperm sac in groups*

Groups	Diameter of the Spermathecae (mm)		Number of Spermatozoa (×10⁶)	
	N	X±Sx	N	X±Sx
Breeding Method (B)				
1 day old larvae (B1)	20	1.04±0.018[b]	20	4.44±0.429[b]
2 day old larvae (B2)	20	0.83±0.017[c]	20	3.81±0.451[c]
2 day old egg (B3)	20	1.23±0.021[a]	20	4.95±0.271[a]
Feeding Medhod (F)				
Fed colonies (F1)	30	1.09±0.025[a]	30	4.54±0.648[a]
Unfed colonies (F2)	30	0.98±0.025[b]	30	4.26±0.679[b]
B x F Interaction				
B1XF1	10	1.10±0.019[c]	10	4.56±0.693[c]
B1XF2	10	0.98±0.025[d]	10	4.33±0.371[d]
B2XF1	10	0.88±0.22[e]	10	3.99±0.509[e]
B2XF2	10	0.78±0.23[f]	10	3.62±0.462[f]
B3XF1	10	1.29±0.032[a]	10	5.08±0.204[a]
B3XF2	10	1.18±0.030[b]	10	4.82±0.288[b]

[a,b,c d,e,f] *Means with no common superscripts differ (P<0.01); (P<0.05)*

of sperms in the spermatheca (P<0.01). Queens raised from two-day-old larvae have a significantly smaller number of spermatozoa than the queens raised from one-day-old larva or two-day-old eggs *(Table 3)*. The highest number of spermatozoa were obtained from the queens raised from the two-day-old egg *(Table 3)*. The difference in number of spermatozoa among the breeding groups was significant (P<0.01). When fed colonies were compared with non-fed colonies, it was observed that the queens had more spermatozoa in the spermatheca. Feeding of starter colonies had an important effect to increase the number of sperms in the spermatheca, and this increase was found to be significant (P<0.01).

DISCUSSION

In this study, average acceptance rates in one, two-days-old larvae and two-day-old eggs groups were determined 72.06%, 83.82%, and 61.76%, respectively. The larva acceptance rate lower than Okuyan and Akyol [11] findings which indicate that the average acceptance rates from one and two-day-old larvae 81% and 85% respectively. However, the finding that the larval acceptance rate was found to be compatible with Gençer et al.[14] which indicate that the average acceptance rates from one and two-day-old larvae 73.4% and 82.3% respectively. Although the acceptance rate of the two-day-old larvae was high, no statistically significant difference was found between larval transfers. The lowest acceptance rate (61.76%) was obtained from egg transfer. This result is similar to that of Şahinler [19] (64%). The acceptance rates could be affected by rearing season, rearing methods and transfer material. In general, feeding increased larval and egg acceptance rates in all groups. Feeding of the queen rearing colonies with a feed containing protein, vitamins, and minerals can be said to increase the acceptance rate. According to Gençer et al.[14] and Sagili et al.[29], adding pollen or a vitamin mixture to syrup increases the larval acceptance rate.

The average height of queen cell cups obtained from one, two-day-old larvae and two-day-old eggs was 27.52±0.30 mm, 24.20±0.37 mm and 31.40±0.30 mm respectively. The average height of queen cell cups higher than Genç et al.[30] findings which indicate that the average height of queen cell cups obtained from one and two-day-old larvae 25.70±1.4 mm and 23.90±0.3 mm respectively. However, the height of queen cell cups was lower than findings of Cengiz et al.[22] which indicate that average height of queen cell cups from one-day-old larvae was 30.71±0.14 mm. The average heights of queen cell cups obtained in this study are similar to results obtained by Emsen et al.[9] which indicate that the average height of queen cell cups from one and two-day-old larvae 29.98±0.08 and 24.27±0.78 respectively. The findings of these researchers illustrate that height of queen cell cups can have a high variability.

According to Vaziritabar and Esmaeilzade [31], there is a positive correlation between the sealed queen cell and the emergence weights of queen bees. When evaluated in this context, the sealed queen cell obtained from egg scales can be said to be an important application for increasing the emergence of queen bees. A sealed queen cell obtained from the fed groups are longer than from the non-fed groups. In other words, feeding increased the height of the sealed queen cell. According to Njeru et al.[8] and Mahbobi et al.[15] the supplemental feeding has a positive effect on all morphological characteristics of

queen bees. When these results are evaluated, it can be said that adding supplemental feeding to queen rearing colonies increases the sealed queen cell length.

The average reared queen from one, two-day-old larvae and two-day-old eggs weight was 191.90±1.46, 174.32±1.94 and 205.75±1.46 respectively. The queen weights are higher than Gençer et al.[14] findings which indicate that the average of queen weights reared from one and two-day-old larvae 166.6±1.74 mg and 160.8±1.22 mg respectively. However, the weight of queen was lower than findings of Akyol et al.[4] which indicate that classified reared queens into three different group as heavy, medium and light and the average weight of these were 207.63, 193.47, and 175 mg respectively. it was found consistent with the emergence weight reported by Cengiz et al.[22] and Dodoloğlu et al.[32] which indicate that the average of queen weights reared from one-day-old larvae 199.07±7.55 mg and 206.13±3.20 mg respectively.

In this study, the average emergence weight of the queen from the egg transfer was found to be 205.75±1.46 mg; while the average emergence weight reported by Şahinler [19] was lower than the average emergence weight in our study (informed as 187.6 mg), it was similar to the average emergence weight reported by Dhaliwal et al.[20] (informed as 201.88 mg). The weight of queen bees might be affected by supplemental feeding of starter hives, bee density in starter hives, genetic factors and season. It can be suggested that the transfer of the egg, which is seen to be effective in the emergence weight of queen, should considered for queen breeding. It can be said that it would be beneficial to apply feeding along with egg transfer, a very important queen quality criterion that positively affects the live weight.

In this study, average spermatheca diameters of 1.10±0.019 mm and 0.98±0.025 mm from one-day-old larvae were obtained from the groups with and without supplemental feeding, respectively. The average spermatheca diameter obtained in a study by Dodoloğlu et al.[32] was found to be similar to that of the non-fed group but lower than the fed group (informed as 0.98±0.01 mm). However, average spermatheca diameters from one-day-old larvae was lower than findings of Akyol et al.[4] for heavy grups (informed as 1.258±0.2 mm). The spermatheca diameter obtained from egg transfer higher than Şahinler [19] findings which indicate that the average spermatheca diameter obtained from egg transfer 1.132±0.040 mm. The average spermatheca diameter of 1.23±0.021 mm the queen bees obtained from egg transfer was found compatible with the spermatheca diameter reported by Akyol et al.[4] for heavy grup (informed as 1.258±0.2 mm). The spermatheca diameters queen bees might be affected by genetic factors and season. In terms of influencing the spermatheca diameter of queens raised with supplemental feeding, our findings were similar to the results of Njeru et al.[8] and Mahbobi et al.[15]. In this study, it was observed that egg transfer promoted a more effective diameter of spermathecae in the queen than other transfers. These results highlight the transfer of eggs as grafting material.

The average number of spermatozoa in the spermathecae 4.44±0.429 million/queen obtained from one-day-old larvae was found to be lower than the finding of Güler and Alpay [33] (informed as 5.08±0.18). However, our findings were consistent with the reports of Koç and Karacaoğlu [6] for Caucasian bees (informed as 4.24±0.599). The average number of spermatozoa in the spermathecae of the queens obtained from the two-day-old egg transfer was similar to the values reported by Akyol et al.[4] for medium grup and Kahya et al.[34] (informed as 4.75±0.2 and 4.87±78). It is estimated that the number of different spermatozoa reported by the researchers is due to the season and the number of adult drones. In our study, it was seen that more spermatozoa were obtained from the fed groups than the other groups. This result is consistent with the results reported by Njeru et al.[8] and Mahbobi et al.[15].

It has been shown that supplemental feeding increases the acceptance rate in all groups; therefore, supplemental feeding to queen rearing colonies is important in increasing the success rate. Although the rate of acceptance of egg transfer is low, it is thought to be an important application in terms of increasing the emerge weight of queen bees. In commercial queen rearing, we recommend that bee-keepers rearing queen bees by supplemental feeding from one day old larvae. As a result, the feeding and transfer age of larvae were found to impact the live weight of queens, the diameter of spermathecae, and the number of spermatozoa in the spermatheca. This finding is supported by studies conducted by many researchers. It can be said that it is possible to produce better quality queens by means of supplemental feeding of starter colonies and use of eggs as transfer material.

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Research Article

Effects of Deep-Frying Sunflower Oil on Sperm Parameters in A Mouse Model: Do Probiotics Have A Protective Effect? [1]

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Abstract

The aim of this study was to investigate the effects of dietary sunflower oil and fried sunflower oil on reproductive sperm parameters, blood lipid profiles, lipid peroxidation and to additionally investigate the protective effects of probiotics. Five experimental groups were established using BALB/c type mice (n=8). A standard pellet mouse feed for the Control group was used. The pellet feed was fortified with sunflower oil (Oil Group), oil and probiotic (Oilpro), fried oil (Fryoil), fried oil and probiotic (Fryoilpro). At the end of the sixty-three-day feeding period, blood samples were collected via cardiac venipuncture, mice were euthanized and testis tissue samples were collected for analyses. Consumption of sunflower oil, natural or fried, decreased seminiferous tubule score ($P<0.01$) and increased plasma MDA levels ($P<0.05$) when compared with the control group. Nevertheless, the probiotic use appeared to prevent side effects. Probiotics also increased sperm motility ($P<0.01$). Prolonged exposure to high fat and fried oil diets would increase oxidative stress levels and have negative effects on fertility levels. Probiotic use may ameliorate such adverse effects. Additionally, this study model may lead to a better understanding of the effects of fast-food dietary habits on global fertility rates.

Keywords: *Infertility, Probiotic, Frying oil, Sperm quality, Oxidative stress, Malondialdehyde*

Derin Kızartılmış Ayçiçeği Yağının Bir Fare Modelinde Sperm Parametreleri Üzerindeki Etkileri: Probiyotiklerin Koruyucu Etkisi Var mı?

Öz

Çalışmamız, ayçiçek yağı ve kızarmış ayçiçek yağı eklenmiş diyetin reprodüktif açıdan sperm parametreleri, kan lipid profili ve lipid peroksidasyonuna etkisi ve buna karşın probiyotik kullanımının koruyucu etkisini araştırmak amacıyla yapılmıştır. BALB/c tipi fareler kullanılarak 5 deney grubu oluşturuldu (n=8). Kontrol gubuna standart pellet yem verilirken diğer gruplara sırayla ayçiçek yağı (Oil), ayçiçek yağı ve probiyotik (Oilpro), kızartılmış yağ (Fryoil) ve kızartılmış yağ ve probiyotik (Fryoilpro) verildi. Altmış üç gün süren deneyin sonunda farelerden kardiyak venipünktür ile kan örnekleri alında ve daha sonra ötenazi işlemi yapılarak testisler alınarak analizlerde kullanıldı. Kontrol grubu ile kıyaslandığında hem kızartılmış hem de kızartılmamış ayçiçek yağı seminifer tubul skorunu düşürdü ($P<0.01$) ve plazma MDA seviyesini artırdı ($P<0.05$). Ancak, probiyotik kullanımı bu olumsuz etkileri düzeltmede etkili bulundu. Probiotikler sperm motilitesi üzerinde de olumlu etki gösterdi ($P<0.01$). Uzun süreli aşırı yağ ve kızartma yağı tüketimi oksidatif stresi arttırarak fertilite üzerinde olumsuz etki yapabilir. Probiyotik kullanımı ise bu gibi olumsuz etkileri düzeltebilir. Yine, bu çalışma modeli fast-food diyet gibi kötü beslenme alışkanlıklarının olumsuz etkilerini anlamada önemli olabilir.

Anahtar sözcükler: *İnfertilite, Probiyotik, Kızartma yağı, Sperm kalitesi, Oksidatif stres, Malondialdehit*

INTRODUCTION

Infertility is a global problem, with approximately 50% of infertility cases due to decreased sperm quality [1]. Various dietary factors and diet related obesity, increases the risk of male hypogonadism [2]. In practice, sunflower oil

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is principally used in the preparation of most popular fried foods [3]. It has been reported that intermittent and continuous frying using oils affect the physiological, histological and biochemical events, resulting in chemical changes in the oils [3–5]. In these studies, frying oils have been shown to cause increased reactive oxygen species (ROS) that can contribute to many pathological conditions [6,7]. Spermatozoa are very sensitive to oxidative stress and frying oils is likely to negatively affecting sperm production capacity, quality and number [8,9]. Insufficient antioxidant response to ROS causes lipid peroxidation (LPO) that results malondialdehyde (MDA) formation, a highly reactive molecule [10] and a predictable marker of plasma and tissue LPO [6].

Repeatedly heated edible oil can cause increase in ROS formation that leads to decreased radical scavenging and thereby oxidative stress [11,12]. To counteract this, it is essential to increase the level of antioxidant capacity. One of the latest concepts is the "gut-brain axis" [13]. Probiotics have been shown to enhance the body's antioxidant capacity and strengthen the intestinal health and immune system, making it possible for the organism to cope with many negative metabolic activities [12,14]. They have positive effects on antioxidant properties and free radical metabolism [15]. Studies on mouse models have reported that probiotics have an positive effect on fertility [16,17]. Some poultry science studies have shown that the antioxidative capacities of probiotics can enhance the number and quality of spermatozoa [18,19]. However, the use of probiotics in livestock rearing is uncommon. Additionally, people nowadays prefer to cook their fries using vegetable oils and this has been shown to have detrimental effects, one of which is sperm quality.

Negative health effects of fried oil and positive health promotions of probiotics have been widely investigated, especially in high fat diet-induced obesity models [3,8]. However, so far, very little attention has been paid to determine whether edible sunflower oil, either raw or cooked, may reduce spermatozoon function and the possible protective effects of probiotics in human or animal models. In view of such findings, the aims of the present study were as follows: to investigate the effect of fried oil on sperm parameters, testicular tissue damage, to evaluate alteration in blood lipid profile and to investigate possible protective effect of use probiotics.

MATERIAL and METHODS

Animals

The experimental protocol of this study was approved by the Animal Experiments Ethics Committee of Near East University (Approval No: 2016/2-1). Animals were kept at a constant temperature (22±1°C) with 12 h light/dark cycle and housed in plastic cages.

Experimental Design

A total of 40 BALB/c mice at their period of sexual maturation (90 days old) were divided into 5 groups, 8 in each group (n=8). Group 1, as control group, received only a standard pellet diet. Group 2 (oil) received a standard diet supplemented with sunflower oil. Group 3 (Oilpro) was fed the standard diet supplemented with sunflower oil in conjunction with probiotic. Group 4 (Fryoil) received a standard pellet diet supplemented with deep-frying sunflower oil. Group 5 (FryOilpro) received a standard pellet diet supplemented with deep-frying sunflower oil in conjunction with probiotics. To prepare deep-frying sunflower oil, a commercial sunflower oil was heated three consecutive times to +232°C (smoke point). Feed parameters and other applications for each of the groups of animals used in the experiments are given in *Table 1*. Feed parameters were analysed by a commercially licenced laboratory (Safyem Ar-Ge Lab., Eskisehir, Turkey). The mice were allowed free access to their respective diets and water *ad libitum* for 63 days.

Preparation of Probiotic Drinking Water

A commercial probiotic in the form of 1 g water soluble powder in aluminium sachets was purchased from a local pharmacy. Each sachet contained $2x10^9$ each *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Bifidobacterium lactis*. One sachet per adult person is the recommended daily dose. To confirm the number of live bacteria in each sachet, total content of the sachet was added to 100 mL sterile saline solution (0.9% NaCl, w/v) and gently mixed in a sterile Erlenmeyer flask. Total aerobic bacteria were counted from this water. The 10-fold increment serial dilution technique was conducted according to Miller and Wolin [20]. One mL of the homogenized suspension was then transferred into 9 mL of 0.9% saline solution (NaCl) and serially diluted

Table 1. *Experimental groups (n=8) and feeding regimes used in the study*

Groups	Feeds	Crude Protein (%)	Crude Fat (%)	Crude Cellulose (%)	Starch (%)	Ash (%)	Dry Matter (%)	Energy (Kcal/kg)
Control	Standard pellet diet	19.43	2.48	4.35	38.97	6.19	92.03	2789
Oil	Standard pellet diet + sunflower oil	17.51	13.77	4.65	34.37	5.63	93.11	3306
Oilpro	Standard pellet diet + sunflower oil + probiotic	17.51	13.77	4.65	34.37	5.63	93.11	3306
Fryoil	Standard pellet diet + deep - frying sunflower oil	17.21	14.1	4.99	33.94	5.64	93.22	3317
Fryoilpro	Standard pellet diet + deep - frying sunflower oil + probiotic	17.21	14.1	4.99	33.94	5.64	93.22	3317

from 10^{-1} to 10^{-8} by using the same saline solution tubes. From the last three diluted samples, 0.1 mL each was plated on the Trypticase soy agar (TSA, Merck, Germany) plates and the plates incubated at 37°C for 48 h. All the colonies grown on the plates were counted and results were expressed as $\log_{10}$ colony forming units (CFU) per gram probiotic product. The total of $1.7x10^8$ CFU/g live bacteria were detected in the probiotic source. After the count of CFU/g of probiotic product, the drinking water of mice was fortified by addition of powdered probiotic at a concentration of $1x10^7$ CFU/mL live probiotic bacteria. The probiotic drinking water was refreshed at 3 d intervals during the experimental period, using one new sachet in each time. The probiotic used in the study was stored at room temperature during use as per manufacturers recommendations. All mice had access *ad libitum* to their water during the experimental period.

Body Weight Gain (WG), Feed Consumption, Energy Consumption and Consumed Energy Per g of WG

The mice were weighted at day 0 and at day 63. Body weight gain was obtained by subtracting the animal weight at day 63 from the initial weight (day 0). Feed consumption of each group of mice were followed by weekly measurements. Energy consumption and consumed energy per g of WG were calculated using consumed feed and the separate energy values of the feed mentioned in *Table 1*.

Collection of Blood, Semen and Tissue Samples

Biochemical tests were performed at the Diagnostic Laboratory of Animal Hospital, Near East University. Blood samples were collected by cardiac venipuncture into vacuum tubes containing K_2EDTA and clot activator tubes. Serum and plasma samples were obtained by centrifugation at 1500 g for 10 min at +4°C. The mice were euthanized via cervical dislocation technique. Testicles and epididymis were collected by performing laparotomy for better visualization of cauda epididymis and ductus deferens. The aforementioned anatomical structures were excised and placed in 5 mL warmed to 37°C Dulbecco's PBS. The excised organs were cut out in small pieces inside in the medium, and coated with mineral oil (FertiCult®). These were incubated for 20 min to allow the spermatozoa to diffuse through the Dulbecco's PBS medium. Immediately following this, the solutions were taken from the prepared suspension to prepare a microscopic slide for evaluation of the motility of the spermatozoa [21]. Epididymal fat was removed from each animal and weighed using a balance (ATX-224 [d=0.0001 g], Shimadzu, Kyoto, Japan). Testicles were also removed to measure lipid peroxidation levels. Samples were kept at -80°C until analysis.

Sperm Parameters

Sperm motility (%), concentration ($x10^6$ count/mL) and morphology (% abnormal) were evaluated using previously described methods [22,23]. To evaluate morphology the fixed and dried smears were embedded with Giemsa stain for 5 min. All parameters were assessed under light microscope.

Histological Evaluation

Testicle tissue samples were fixed in 10% neutral formalin and then routinely processed for embedding in paraffin. 5 µm sections of the paraffin tissue blocks were stained with Haematoxylin and Eosin. Histological sections were examined using a Leica DM500 light microscope coupled with a Leica Microsystem Framework integrated digital imaging analysis system (Leica Application Suit version 3.0 Serial 38132019 Leica ICC50 HD, Heerbrugg, Switzerland). Seminiferous tubules were scaled (50 tubules per animals) according to Johnsen's Tubular Biopsy Scores (JS) [24].

Blood Parameters

Total cholesterol (in mg/dL) and triglycerides (in mg/dL) concentrations were measured in serum samples (respectively TC Lot. 141617003, TG Lot. 141717003, Mindray, Shenzhen, China) using an automated chemistry analyser (BS120, Mindray, Shenzhen, China). Competitive ELISA test (Testosterone, DE1559, Lot. 29K126, Demeditec, Kiel, Germany) was performed to measure serum testosterone concentrations (in ng/mL). The tests were carried out in accordance with the manufacturer's directions. The washing steps of ELISA test were performed using an automated microtiter washer (MW-12A Microplate washer, Mindray, Shenzhen, China) and results were obtained using a microtiter plate reader at 450 nm (MR-96A Microplate reader, Mindray, Shenzhen, China).

Assessment of Lipid Peroxidation

MDA levels were measured in plasma and testicle samples in order to assess lipid peroxidation levels using commercially available assay kits (TBARS Assay Kit, Item No. 10009055, Batch No. 0510196 and 0502129, Cayman Chemicals, Michigan, USA). Plasma samples were directly treated. However, testicle samples were firstly homogenized. This was performed according to manufacturer's protocol using RIPA buffer (Item No. 10010263, Batch No. 0490889-1, Cayman Chemicals, Michigan, US) and a Dounce tissue grinder set (D8938, Lot. 3110, Sigma-Aldrich, Missouri, US) on ice. Following homogenization, samples were centrifuged at 1600 g for 10 min at +4°C and supernatants were used for analysis. The principle measurement was based on the reaction with thiobarbituric acid (TBA) in boiling water for 60 min in acidic medium and measurement of the absorbance of the reaction mixture at 532 nm [25]. Absorbance were measured with a UV/VIS Spectrophotometer (Model T70, S/N:17-1814-01-0059, PG Instruments Ltd, UK). Plasma and tissue MDA concentrations were expressed as µmol/L and µmol MDA/g, respectively.

Statistical Analyses

Statistical analyses were carried out using GraphPad Prism

software (version 7.04, GraphPad Software, San Diego, CA, USA). All data were expressed as mean±standard deviation (±SD). Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. A difference of P<0.05 was considered statistically significant.

RESULTS

During 63 days, feed consumption was similar among all groups, with exception of the control group (P<0.001, *Table 2*). There was no difference between energy gain and energy gain rate to bodyweight gain. When the epididymal fat mass gains were examined, it was determined that Oil and Oilpro groups were significantly higher compared to the control group (P<0.05, *Table 2*). In addition, the FryOilpro group had lower epididymal fat mass compared to Oil group (P<0.05).

No statistically significant difference existed between the groups with regards to either sperm count or morphology *(Table 2)*. However, sperm motility in Oilpro and Fryoilpro group were significantly higher than both Control and Oil groups (P<0.01). The results taken from Fryoilpro group demonstrated the highest motility rate in comparison to the other groups. Probiotic use had a positive effect on the sperm motility *(Table 2)*.

Seminiferous tubules of the testicles were evaluated as Johnson score values *(Table 2)*. The JS values of Control, Oilpro and Fryoilpro groups were higher than that of Oil and Fryoil groups. The values of Control were also better than that of Oil and Fryoil values (P<0.001). Also, the results of Oil were better than that of Fryoil (P<0.05). Both Oilpro and Fryoilpro were better than Fryoil on the JS values (P<0.001). The JS values of Oilpro and Fryoilpro were better than that of Oil JS values (P<0.001). The consumption of sunflower oil, as natural or fried, decreased tubule JS score when compared with normal diet used for feeding the Control group. Nevertheless, the probiotic use appeared to significantly prevent the effects of high fat and frying oil diets *(Table 2)*.

Sperm morphology and testes tissue were histologically examined *(Fig. 1)*. Abnormal sperm morphology was observed in both control *(Fig 1.A)* and experimental groups *(Fig. 1.B-C)*. In the histologic evaluation of seminiferous tubules, normal structure was detected in control group *(Fig. 1D)*. Nevertheless, degenerative changes and gaps have been observed in the seminiferous tubules of Fryoil group *(Fig.1E)*. Ameliorative effects of probiotics observed that in Fryoilpro group *(Fig.1F)*.

Triglycerides concentrations were only significantly lower in Oilpro group in comparison with the control group (P<0.05). In Oil group, plasma MDA levels, the end products of lipid peroxidation were significantly higher than control group (P<0.05). There was a significant difference in the MDA values of Oilpro and Fryoilpro versus Oil (P<0.05). However, the MDA values between Fryoil and Fryoilpro groups did not differ statistically (P>0.05). Serum concentrations of total cholesterol, triglycerides and testosterone are shown in *Table 2*. Plasma MDA and testes MDA levels are presented in *Fig. 2*.

DISCUSSION

Infertility is a worldwide problem and no definable cause can be found in 25% of infertile men [26]. However, a relationship between increased ROS formation and decreased

Table 2. *Feed consumption, weight and energy gain, epidydimal fat increment and changes in sperm and biochemical parameters of the groups. Results are represented as ± SD (n=8)*

Parameters	Groups				
	Control	Oil	Oilpro	Fryoil	Fryoilpro
Feed consumption (g), 63 d	271±8.01	216±7.95 [c]	225±7.79 [c]	221±7.23 [c]	227±7.46 [c]
Energy consumption (KCal) 63 d	757	714	744	732	753
Consumed energy per g of WG	178	190	157	183	121
Weight gain (WG, g) 63 d	4.25±2.49	3.75±1.66	4.75±1.48	4.00±2.39	6.25±2.25
Epididymal fat (g)	0.36±0.05	0.55±0.08 [c]	0.49±0.18 [a]	0.45±0.21	0.43±0.13 [a]
Sperm count (x10^6 count/mL)	19.49±6.96	19.75±6.65	19.62±11.17	13.12±5.16	16.81±9.65
Sperm morphology (% abnormal)	28.71±3.81	23.57±7.42	21.75±5.40	28.29±6.58	24.29±9.79
Sperm motility (%)	58.57±7.89	61.14±11.86	70.38±8.76 [b,e]	61.43±13.29 [g]	73.14±4.73 [j]
Seminiferous tubules scale (JS score)	8.40±0.35	7.78±0.43 [c]	8.39±0.34 [f]	7.61±0.27 [c,d,h]	8.22±0.14 [f,j]
Total cholesterol (mg/dL)	99.19±24.77	121.09±12.02	129.51±16.10 [a]	109.23±14.26	118.60 ±30.97
Triglycerides (mg/dL)	106.40±.69	77.88±25.18	59.39±15.77 [a]	91.69±34.68	93.13±40.26
Testosterone (ng/mL)	2.70±3.69	2.96±4.14	3.38±3.55	2.30±1.74	3.74±3.10

[a] P<0.05 versus Control; [b] P<0.01 versus Control; [c] P<0.001 versus Control; [d] P<0.05 versus Oil; [e] P<0.01 versus Oil; [f] P<0.001 versus Oil; [g] P<0.01 versus Oilpro; [h] P<0.001 versus Oilpro; [j] P<0.001 versus Fryoil

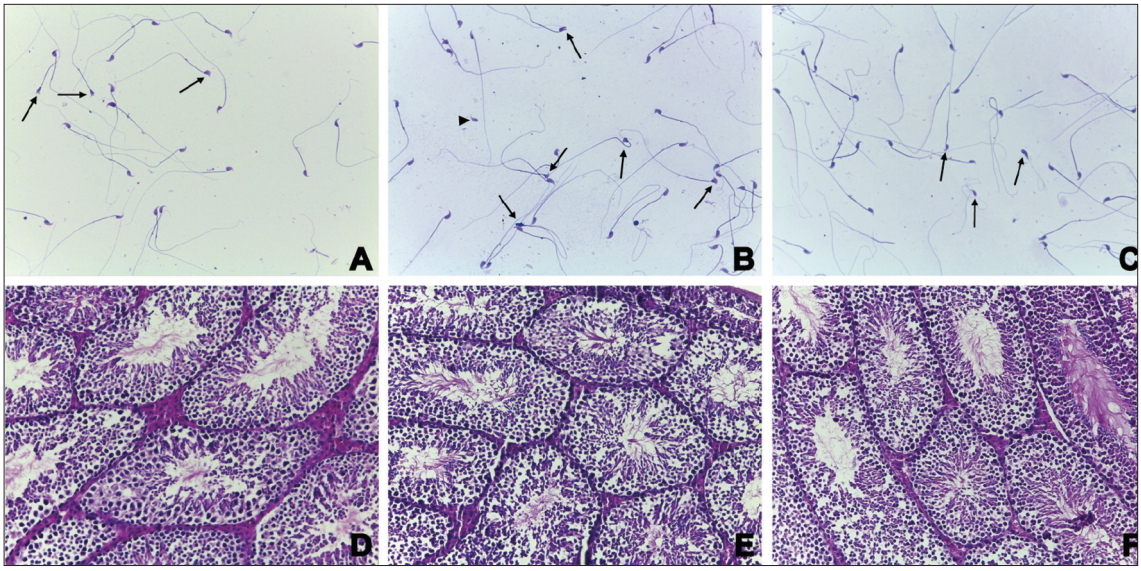

Fig 1. Representative images of sperm and testis tissue showing the effects of frying oil and probiotics. Sperm smear in control (A), Fryoil (B) and Fryoilpro (C) groups. Arrows indicate abnormal sperm, x400, Giemsa stain; Testis seminiferous tubules showed normal structure in control group (D), degenerative changes and gaps in the seminiferous tubules in Fryoil group (E) and ameliorative effects of probiotics observed that in Fryoilpro group (F). x200, H&E

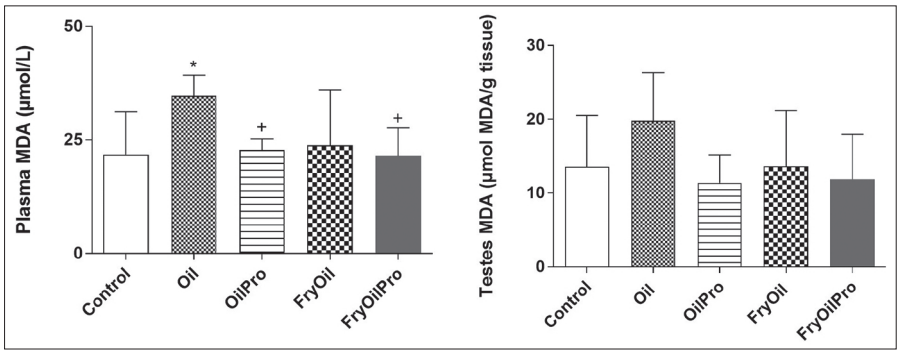

Fig 2. Plasma and testes MDA levels of the mice (± SD; n = 8). *P<0.05 compared to the "control", +P<0.05 compared to "oil" (n = 8)

sperm motility has been determined [27]. High-fat diets are known to negatively affect the antioxidant capacity and increase fat-related oxidative stress and ROS levels. It is possible to decrease the oxidative damage by increasing antioxidant levels [15]. Recently, antioxidant properties of probiotics have been highlighted. Studies on the protective effects of probiotics on fertility in a high-fat diet are limited for both humans and animals [2,16,17]. In this study, the detrimental effects of deep-frying oil, even non-heat-treated sunflower oil, on the reproductive organs of male mice and the protective effect of probiotic use were investigated and evaluated. Free radicals play an important role in the pathophysiology of reproductive dysfunction in male animals and humans. It is known that sperm cell membranes are rich in polyunsaturated fatty acids and are very sensitive to damage from free radicals [28]. High fat-diet model studies have shown that sperm motility decreased, normal morphology has been impaired and antioxidant supplementation increased spermatogonium and Sertoli cell count [29]. Another study reported that high fat diet decreased testosterone level, impaired semen quality, caused atrophy and degeneration in seminiferous tubules, and these effects were reversed with probiotic use [30]. In this study, it was determined that sperm motility increased significantly in probiotic fed groups. Sperm motility was better in the Fryoilpro group than that of Fryoil group ($P<0.01$). It has also been reported that high energy diets can cause mitochondrial dysfunction associated with overproduction of reactive oxygen species in testicular metabolism and that probiotics are a potential agent that can be used to eliminate these harmful effects [2,31]. Although the effects are not reflected in the statistics, abnormal sperm rates decreased in the Oil and Oilpro groups compared to the control group, also in the FryOilpro group compared to Fryoil and control groups ($P<0.01$). It has been observed that frying oil reduces the sperm concentration and the negative effect of the frying oil is reversed in the probiotic fed groups. Nevertheless, these results were not significantly reflected in the statistics.

Probiotics are recommended as an alternative to pharmacological products in many medical conditions, including modulation of obesity, which is often associated with poor semen quality. Dardmeh et al.[2] showed that *Lactobacillus*

rhamnosus PB01 has a positive effect on both weight loss and reproductive hormones, significantly improving sperm motility and kinematic parameters. Numerous studies have been published using various rodent species to investigate the effects of probiotics on health [16,32-35]. One study reported that probiotics improved seminiferous tubules with impaired morphology [36]. In this study, the negative effects of sunflower oil and fried sunflower oil on seminiferous tubules was demonstrated. These negative effects were ameliorated through the use of probiotics to the level of the control group. Additionally, sperm motility and seminiferous tubule scores increased in the probiotic high fat diet group.

An *in vivo* study conducted with rats, demonstrated that high fat diet did not alter cholesterol and triglyceride levels in rats. However, it significantly led to decreases in the testosterone levels and an increased fat mass of the epididymis [37]. Fernandez et al.[38] reported that sperm quality decreased and sperm counts were similar among groups of rats fed with high fat diet as sperm motility was impaired without any effect on other sperm parameters. Our study results showed similarities with those results. Our study showed that epidydimal fat mass was significantly higher particularly in the Oil and Oilpro groups when compared with the control group ($P<0.001$; $P<0.05$). Epidydimal fat mass was significantly higher in the FryOilpro than in the Oil group ($P<0.05$), and the same trend was seen between Oil group and Oilpro group. The use of probiotics reduced the epidydimal fat mass.

High fat diet decreases the levels of testosterone whilst increasing oxidative stress in rats [39]. Here we showed that the levels of testosterone did not change among groups. However, there were slight difference in MDA levels, an oxidative stress indicator between some groups. The levels of cholesterol in Oilpro group were significantly higher compared with control group. In the other groups, there were slight but not significant increases in cholesterol levels. It is thought that this situation may be related to the upregulation of cholesterol biosynthesis which is induced by increases in both lipid levels and the corresponding insulin expression [40]. In contrast to this, it is thought that the decrease in triglyceride levels in the experimental groups might be related to their metabolism. It is known that mice can affect lipid use and transport due to their higher metabolic rates compared with rats, hence this might be related to factors affecting insulin secretion stimulation [41]. Furthermore, excessive triglycerides do not accumulate in the liver of BALB/c mice fed with high fat diet and this may be associated with low fatty acid uptake [42]. Probiotics had no effect on both parameters among the experimental groups.

Diet and probiotics also had an effect on testosterone plasma levels. Although statistical differences were not significant, mean values were higher in probiotic fed group. This suggests that use of probiotics has positive effects on fertility. These positive effects have been demonstrated in different *in vivo* studies conducted on different animal groups [8,14]. Additionally, MDA levels, an LPO indicator, in testes were slightly increased in Oil and Fryoil groups but not in probiotic groups. This is possibly be related to the duration of the experiment, and results may be significant over a longer period of time. Another finding suggests this may be associated with plasma MDA levels. These results were also similar with in an *in vivo* study conducted on rats [8,15]. It is thought that this type of diet would increase oxidative stress in the testis tissue in the long term and alter MDA values. However, the use of probiotics would be beneficial against these negative effects.

In conclusion, high fat diet, or high-energy diet in male mice, could result in negative effects caused by oxidants on reproduction even though mice were not subject to obesity. This was concluded from the study findings on sperm motility, seminiferous tubule score, MDA levels in plasma and testes. Results indicate the use of probiotics had potential to reverse the negative effects of deep-fried sunflower oil in mice. When the duration and conditions of the study are taken into consideration, it may be suggested that prolonged feeding time and direct gavage of the fat would increase oxidative stress level and negative effects on fertility. This study model could be important for understanding the effects of fast-food dietary habits on human reproductive health globally.

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Research Article

Serum Thiol Disulphide Levels Among Sheep with Sarcoptic Mange

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Abstract

Sarcoptic mange, a notable parasitic disease, causes dermatological alterations among ruminants. Thiol-disulphite hemostasis is a novel oxidative stress parameter. The aim of this study was to evaluate dynamic thiol/disulfide homeostasis in sheep with sarcoptic mange. Total of thirty-six sheep (n=15 female, n=21 male) with sarcoptic mange (Group I), and twelve healthy sheep (Group II) were used in the study. A novel method was used to determine the thiol disulphide parameters. Native thiol, total thiol and Disulphide values were statistically lower in Group I. Disulphide/native thiol, Disulphide/total thiol, and Native thiol/total thiol proportions had no statistical differences in groups. Sarcoptic mange was probably affected by the thiol Disulphide hemostasis in infected sheep. Thus, the data obtained in this study might form base for further studies to include antioxidant molecules in the treatment protocols.

Keywords: *Oxidative stress, Sarcoptic mange, Sheep and Thiol disulphide*

Sarkoptik Uyuzlu Koyunlarda Serum Tiyol Disülfit Seviyeleri

Öz

Önemli paraziter bir hastalık olan sarkoptik uyuz ruminantlarda dermatolojik lezyonlara neden olmaktadır. Tiyol-disülfit dengesi yeni bir stress oksidatif parametresidir. Bu çalışmanın amacı sarkoptik uyuzlu koyunlarda dinamik tiyol/disülfit dengesinin değerlendirilmesidir. Bu amaçla sarkoptik uyuzla (grup I) enfekte 36 koyun (n=15 dişi, n=21 erkek) ve 12 sağlıklı koyun (grup II) çalışmaya dahil edilmiştir. Tiyol disülfitin belirlenmesinde yeni bir metot kullanılmıştır. Natif tiyol, total tiyol ve disülfit parametreleri grup I'de istatistiksel olarak düşük seyrederken disülfit/natif tiyol, disülfit/total tiyol ve natif tiyol/total tiyol oranları arasında gruplarda farklılık elde edilmemiştir. Sarkoptik uyuzun enfekte koyunlarda disülfit dengesini etkilediği düşünülmekte olup bu dengeyi sağlayacak antioksidan moleküllerin terapotik seçeneklere eklenmesi gerektiği öngörülmektedir.

Anahtar sözcükler: *Oksidatif stres, Sarkoptik uyuz, Koyun ve Tiyol disülfit*

INTRODUCTION

Sarcoptic mange, a well-known/significant parasitic disease, causes animal discomfort and dermatological alterations among ruminants. Due to parasite and host interactions [epidermal layer, *stratum corneum* and responsible agent as, *Sarcoptes scabiei* var *canis*] itching, alopecia and primary/secondary lesions exist [1]. Small ruminant animals are well known to adapt to unpleasant harsh conditions. Indeed, overcrowding, nutritional deficiencies and effects of various diseases can result in economic losses [2]. Among sheep diseases, mites are one of the most beneficial reasons of economic loss related to dermal system due to morphlogical changes [3]. Mite invasions are contagious skin diseases resulted with reduce in meat quality due to skin damage by hyperkeratosis, pruritus [4].

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Oxidative stress term describes the balance relation in oxidants and antioxidants, when the balance shift in oxidants. Thus, increased formation of free radicals and lipid peroxidation develops the oxidative stress. Oxidative stress might be estimated with malondialdehyde, sialic acid, total oxidant capacity and total antioxidant capacity [5].

Thiol, a well-known antioxidant, participate within the eradication of reactive oxygen molecules by enzymatic/non-enzymatic pathways [6,7]. Low molecular weight thiols, (i.e. homocysteine, cysteine, glutathione, and albumin), all involve within the plasma thiol pool. Thiols employ in oxidative response within oxidant molecules, establishing disulfide bonds. Regarding arrangement of enzymatic reactions, detoxification, apoptosis, regulation of signaling pathways, dynamic thiol/disulfide homeostasis is essential. Taking into account altered thiol/disulfide concentrations are associated with many inflammatoric conditions [8-14], determination of thiol/disulfide homeostasis were composed of classic Ellman method using 5,5¢-dithiobis-(2-nitro-benzoic) (DTNB) acid [15], high-performance liquid chromatography, fluorescence capillary electrophoresis, bioluminescent systems [16-19] and relatively novel method as described by Erel and Neselioglu [20]. In the present study the aim was to analyze dynamic thiol/disulfide homeostasis in sheep with sarcoptic mange.

MATERIAL and METHODS

Thirty-six sheep from both sexes (n=15 female, n=21 male) were admitted to Adnan Menderes University, Faculty of Veterinary, Department of Internal Medicine with a alopecia, crusting and scaling history. The diseased population enrolled in the present study at the age of 1 to 6 years of age, of both sexes (n=15 female, n=21 male). The vast majority of the sheep breed were composed of Sakiz. At clinical examination lesions typically showing sarcoptic mange appearance were severely excoriated [i.e. scratching, itching and biting/self-damage]. The lesions were located on the nose, ear and mouth edge. Other twelve sheep were involved as healthy control. Deep skin scrapings were collected from lesions (ear and face) for diagnosis of sarcoptic mange. For determining the mite examination 10% NaOH were used on slide to microscopy.

Afterwards, sheep were allocated in to two groups. Group I involved 26 sheep with sarcoptic mange and Group II healthy control animals (n=12) without obvious clinical signs. Blood samples were collected from jugular vein in to the tubes (Vacutte, USA) containing with clot activator. All samples were centrifugated at 3000 rpm for 10 min and sera were kept on -80°C until analyses. Thiol Disulphide parameters were analyzed with a commercial ELISA kit (Real Assay Diagnostics, Turkey) as described before [20].

Statistical Analyses

Native thiol, total thiol, disulphide, Disulphide/native thiol %, Disulphide/total thiol % and Native thiol/total thiol % levels in groups were tabulated as mean and standard deviation. Groups were compared with non-parametric Mann-Whitney U test since data did not showed normal distribution. All analyses were performed with SPSS 21.0 (IBM, Chicago) program and $P<0.05$ were considered significant.

RESULTS

Oxidative stress parameters native thiol, total thiol, and disulphide values were statistically lower in Group I. The calculated parameters Disulphide/native thiol, Disulphide/total thiol, and Native thiol/total thiol ratios had no statistical differences in Group I and II shown in *Table 1, Fig 1*.

DISCUSSION

During consultation sheep infested with sarcoptic mite showed significant exfoliative dermatitis, scaling and crusting along with intense pruritus, self-trauma and wool loss. Complete lesions observed in nonwoolly skin of the body determined on to the face, as described previously [21-23]. Primary/secondary skin lesions [24] comprising alopecia, mild crusting [lips, nostrils to those of extending to other parts of the head, face and ears] and significant erythema. The vast majority of the sheep presented self-trauma due to pruritus, alopecia namely wool loss, brown scabs on to the skin [22].

It has been well recognized that the oxidation of reactive oxygen radicals relatively causes disulfide bonds existing. Disulfide bonds might return to thiol groups, through a pathway involving thiol/disulfide homeostasis

Table 1. *Thiol/disulphide hemostasis parameters of healthy and sheep with sarcoptic mange*

Parameters	Group I	Group II	*P value*
Native thiol	188.64±99.21	276.03±46.81	0.002
Total thiol	198.93±97.92	324.89±32.79	0.000
Disulphide (SS)	18.95±7.44	33.67±5.86	0.000
Disulphide/native thiol %	25.59±33.97	12.78±4.43	0.429
Disulphide/total thiol %	14.15±10.64	10.49±2.38	0.982
Native thiol/total thiol %	85.05±26.76	84.69±11.16	0.228

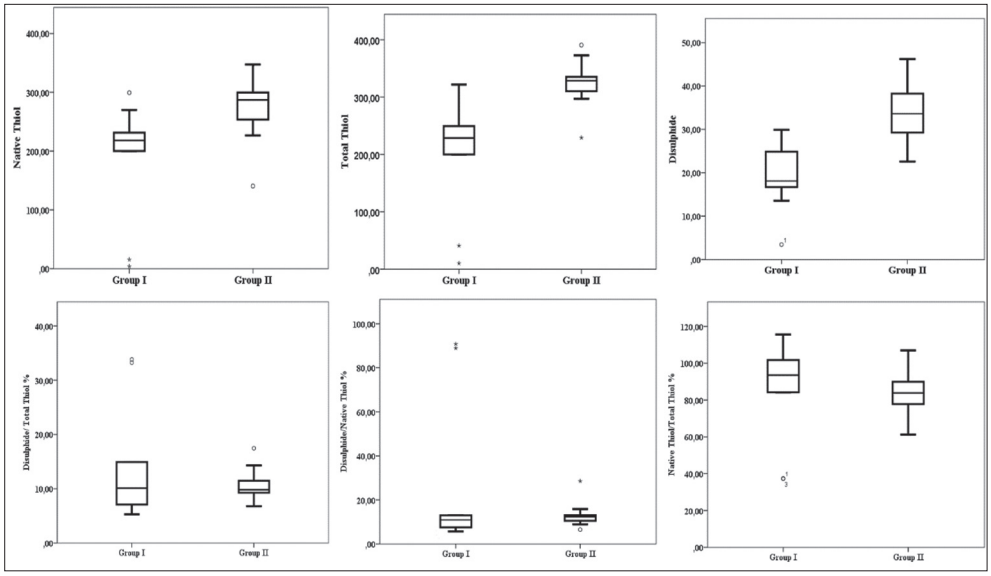

Fig 1. The box plot display of thiols/disulphide parameters of healthy and sheep with sarcoptic mange

maintenance [25,26]. Altered thiol/disulfide balance has been related to several diseases [8-14]. In the present study, thiol, as an important component of the plasma antioxidant system, was significantly lower in sheep with sarcoptic mange.

The results of the present study might be comparable to prior investigations [27]. In a previous research oxidant/antioxidant balance to those dogs with sarcoptic mange, composed of 30 cross-breed male dogs [n=15 with sarcoptic mange compared with n=15 healthy controls], lipid hydroperoxide level, total oxidant status and oxidative stress index in diseased dogs were statistically elevated (P<0.01, P<0.01 and P<0.05, respectively) when compared to healthy ones [27]. Taking into account sulphydril levels in mange mite infected cases statistically decreased levels (P<0.05) were striking. There was no statistical difference detected to those of total antioxidant capacity among groups. The researcher briefly concluded a probable relationship between oxidant/antioxidant imbalance and sarcoptic mange infestation in dogs [27].

A relatively novel research evaluating oxidative stress [by detecting malondialdehyde (MDA), total antioxidant capacity (TAC) and total oxidant status (TOS)] markers in 40 sheep naturally infected with *Psoroptes ovis indicated that* serum MDA and TOS increased significantly (P<0.01), whereas serum TAC decreased significantly (P<0.01) in diseased animals. Available evidence suggested a probable interaction between oxidant/antioxidant imbalance and *Psoroptes ovis* infection in sheep. The authors concluded that MDA, TAC and TOS might be interpreted for detecting the oxidative stress in naturally occurring *Psoroptes ovis* infection among sheep [28].

Another research designated for detecting the status of antioxidant alterations in 59 pigs naturally infected with sarcoptic mange, three groups were involved as follows; healthy control, subclinical sarcoptic mange and clinical sarcoptic mange. Lipid peroxides (LPO), reduced glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured. Regarding the latter study GSH, SOD, GPx concentrations in blood were significantly declined in the clinical and subclinical sarcoptic mange groups, in contrast to the healthy controls, whereas LPO content of diseased pigs was significantly higher. From the present study, it may be concluded that sarcoptic mange was related to remarkable alterations in the oxidative stress markers, which promptly necessitates correction of the antioxidant status of the infested pigs [29]. In another research thiol-disulphide hemostasis was examined on calves undergoing dehorning process with different analgesia protocols. This study stated the reduction on native thiol and total thiol levels in all analgesia groups without any significance and researches pointed out the pain management protocols might be influence the oxidative balance by thiols [30].

Thiol measurement is a growing era in basic and applied molecular life sciences. By measurement of thiol/disulfide homeostasis, it may be possible to understand and highlight the negative effects of oxidative stress in an attempt to make interpretation for disease activation. The results of this study might suggest that further researches directed to include antioxidant molecules in the treatment protocols of such cases may be of help.

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Short Communication

Brucella suis S2 Isolated from Aborted Sheep Fetuses in Northwestern China

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Abstract

This study aimed to investigate the cause of abortion in a traditional sheep farm. Here, 31 specimens were examined by PCR targeting the *Brucella* outer membrane protein gene 22 *(omp22),* and 25 (80.6%, 25/31) were found to be positive. Totally, 3 *Brucella suis* S2 and 10 *Brucella melitensis* were isolated from 31 aborted fetuses in which one *B. suis* S2 and *B. melitensis* were isolated from a same aborted fetus. All of these isolates were identified by PCR and conventional bacterial tests. These 3 *B. suis* isolates and the reference strain *B. suis* S2 were all identified as *B. suis* biovar 1 and the 10 *B. melitensis* isolates were all identified as *B. melitensis* biovar 3. This study suggests that *B. suis* S2 can partially induce abortion in pregnant ewes. Hence, for the sake of safety, it is need to develop a new *Brucella* vaccine to protect pregnant sheep from brucellosis.

Keywords: *Brucella suis S2, Sheep, Abortion, China*

Kuzeybatı Çin'de Atık Koyun Fötuslarından *Brucella suis* S2 İzolasyonu

Öz

Bu çalışma, geleneksel bir koyun çiftliğinde atıkların sebebini araştırmak amacıyla gerçekleştirilmiştir. Çalışmada, 31 hayvan PCR tekniği ile *Brucella* dış zar protein geni 22 *(omp22)* için taranmış ve 25'i (%80.6, 25/31) pozitif bulunmuştur. Otuz bir adet atık fötusun 3'ünden *Brucella suis* S2 ve 10'undan *Brucella melitensis* izole edilirken bir hayvanda *B. suis* S2 ve *B. melitensis* beraber tespit edilmiştir. Tüm bu izolasyonlar hem PCR hem de klasik bakteriyolojik testlerle yapılmıştır. Üç adet izole edilen *B. suis* ve referans suş *B. suis* S2 B. suis biovar 1 olarak, 10 adet izole edilen *B. melitensis* ise *B. melitensis* biovar 3 olarak identifiye edildi. Bu çalışma, *B. suis* S2'nin gebe koyunlarda atığa sebep olabileceğini göstermiştir. Bu nedenle, gebe koyunlarda koruyucu amaçlı brusellozise karşı yeni bir aşı geliştirmeye gereksinim olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Brucella suis S2, Koyun, Atık, Çin*

INTRODUCTION

The brucellosis causes great losses among domestic animals throughout the world, and it has been prevalent for decades in China [1]. Long-term serological studies have indicated that 5% of sheep and 0.8% of cattle are infected with brucellosis. *Brucella* melitensis *(B. melitensis)* infection is endemic, particularly in developing countries in the Mediterranean and Middle East and parts of Africa and Latin America [2]. It is also the main cause of sheep abortion in China. The seropositive rate was 0.66% for cows and 3.25% for sheep during the two-year period covering 2013-2014 in China [3]. In areas where the brucellosis morbidity of sheep and goats is high, vaccination is the best method of controlling the disease in animals [4].

In China, an attenuated strain of *Brucella* suis *(B. suis)* S2 was obtained by serial transfer of a virulent *B. suis* biovar 1 strain originating from swine [5]. It is widely used in sheep and goats and is administered orally in their drinking water.

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Although it has been assessed for safety under field conditions and found satisfactory by Chinese authorities, there remain a few controversial issues regarding specific risks. It has been reported that the infection and replication of *B. suis* S2 in goat trophoblast cells (GTCs) can induce apoptosis due to endoplasmic reticulum (ER) stress, which is involved in the mechanism underlying goat abortions [6]. Verger et al.[5]. have also reported that *B. suis* S2 induces a poor immunity against the *B. melitensis* infection of pregnant ewes.

This study was performed on a traditional farm in the Ili region, where located in the northwest of Xinjiang Uygur Autonomous Region (XUAR) in China. Previous works have observed that the rate of abortion is relatively high on a neighboring farm where brucellosis is epidemic. There were about 157 ewes on this farm and all of the animals were given adequate food and water. The purpose of the present work was to investigate the reason for these abortions on this farm.

MATERIAL and METHODS

Ethical Approval

All animals used in our experiment were treated humanely and in accordance with institutional animal care guidelines. Our study was approved by the Animal Care and Use Committee of Shihezi University.

Bacterial Strains

Reference strains of *B. melitensis* 16M and *B. suis* S2 were provided by the Anthropozoonosis laboratory in Shihezi University, *B. suis* 1330 was donated by the College of Veterinary Medicine, Northwest A&F University.

Sample Collection and DNA Extraction

The samples including sheep aborted fetuses (n=31) were collected from the Ili region northwest of Xuar in areas in which brucellosis is common between April and May in 2018. Samples of spleen, liver, and lung tissues were collected aseptically from aborted sheep fetuses. Then the tissue samples were cut into pieces weighing about 6 mg, suspended in 1 mL sterile saline, homogenized using a tissue grinder for 15 min, and centrifuged at 10.000 rpm for 1 min. The supernatants were discarded. DNA extraction procedures were performed using the TIANamp Genomic DNA Kit (Tiangen Biotech Co., Ltd., China) according to the manufacturer's instructions. DNA concentrations were determined by measuring the A_{260}, and the samples were stored at -20°C until further processing.

PCR Amplification

The forward (F) and reverse (R) primers of *omp22* gene were F 5'-TGATGGGAGGGACCGACTA-3' and R 5'- TGGTTC TTCAGGTTGTTACGC-3', which were used to screen *Brucella* spp. The Bruce-ladder multiplex PCR primers were used to identify the species of *Brucella* genus [7]. The duplex PCR primers were used to differentiate *B. suis* S2 from *B. suis* 1330 [8]. All samples were examined by PCR in a total volume of 30 µL, with 13 µL ddH_2O, 15 µL master mix, 0.5 µL of each primer and 1.5 µL DNA template. The reaction mixtures for *omp22* were first incubated for 5 min at 94°C. Then 37 cycles were performed as follows: 30 s at 94°C, 40 s at 55°C, and 5 min at 72°C. The reaction was performed in a DNA thermal cycler (Perkin-Elmer, USA) and 2 µL of the product was, fractionated in a 1.5% or 2% agarose gel, stained with 0.5 mg/mL ethidium bromide solution, and visualized under UV light [9]. All of these data were analyzed using SPSS version 17.0.

Bacterial Isolation

The tissue samples were homogenized before plating on the Brucella-selective agar (BD, USA). Then, 100 µL of the homogenized or lysed suspension was inoculated onto two Brucella-selective agar plates (BD, USA). The suspension was spread with a loop producing a depot followed by single colonies. All cultures were incubated at 37°C with 5% CO_2 for five days. *Brucella* identification and species differentiation were accomplished using PCR protocols [7]. *Brucella* was identified by conventional bacterial and typing methods [10]. This process was completed at the Center for Disease Prevention and Control (CDC) of China.

RESULTS

Of the 31 studies samples, PCR results targeting the *omp22* gene amplification demonstrated that 25 of the aborted sheep fetuses were found positive with *Brucella* spp. Among the positive results, 2 out of 25 aborted fetuses were identified as *B. suis* by Bruce-ladder multiplex PCR. PCR using DNA from *B. melitensis* amplified six fragments, of 1682, 1071, 794, 587, 450 and 152 bp in size; with *B. suis* by the presence of an additional 272-bp fragment *(Fig. 1)*. These isolates were further identified as *B. suis* S2 using duplex PCR. The *B. suis* 1330 was positive in the duplex PCR for the 285 bp amplicon. The vaccine strain *B. suis* S2 was positive in the duplex PCR for both the 285 bp and 497 bp amplicons *(Fig. 2)*. The remaining 23 samples were infected with *B. melitensis*, only partial results were presented *(Fig. 1)*.

Totally, *Brucella* was isolated from 13 out of 25 samples and the isolates were confirmed using the *omp22* gene-targeting PCR (data not shown). *B. suis* S2 was isolated from 2 out of 25 positive samples and identified using duplex PCR. *B. melitensis* was isolated from 9 out of 25 positive samples. Apart from these, *B. melitensis* and *B. suis* S2 were simultaneously isolated from one aborted fetus *(Table 1)*. Thus, totally 3 *B. suis* S2 and 10 *B. melitensis* were identified from aborted fetus. Three wild isolates and the reference strain of *B. suis* S2 were all identified as *B. suis* biovar 1 by conventional bacteriological methods. All of these three wild isolates and *B. suis* S2 were found positive for

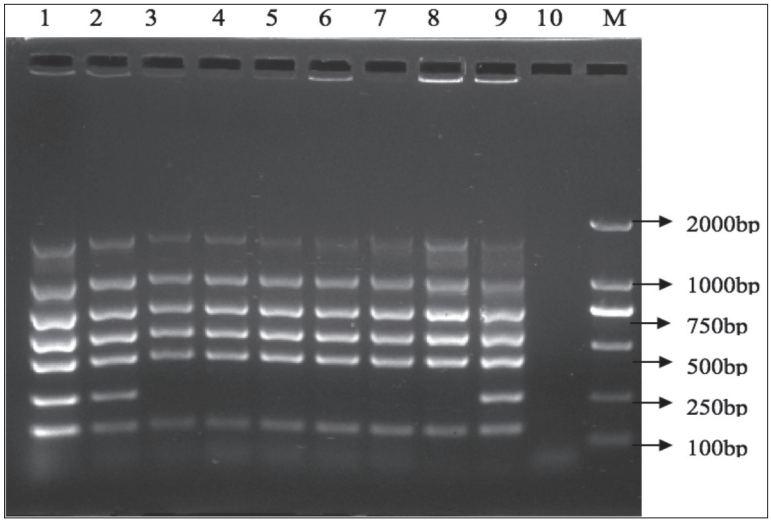

Fig 1. Bruce-ladder multiplex PCR: differentiation of *Brucella*, *B. melitensis* and *B. suis*

Lane 1-7: aborted sheep fetuses; Lane 8: *B. melitensis* 16M Lane 9: *B. suis* S2; Lane 10: Negative Control; Lane M: DL2000 (Takara)

Fig 2. PCR product for differentiation of vaccine strain *B. suis* S2 from *Brucella* wild strain 1330.

Lane 1-2: aborted sheep fetuses 1; Lane 3-4: aborted sheep fetuses 2; Lane 5: *B. suis* 1330; Lane 6: *B. suis* S2; Lane 7: Negative Control; Lane M: DM500 (Takara)

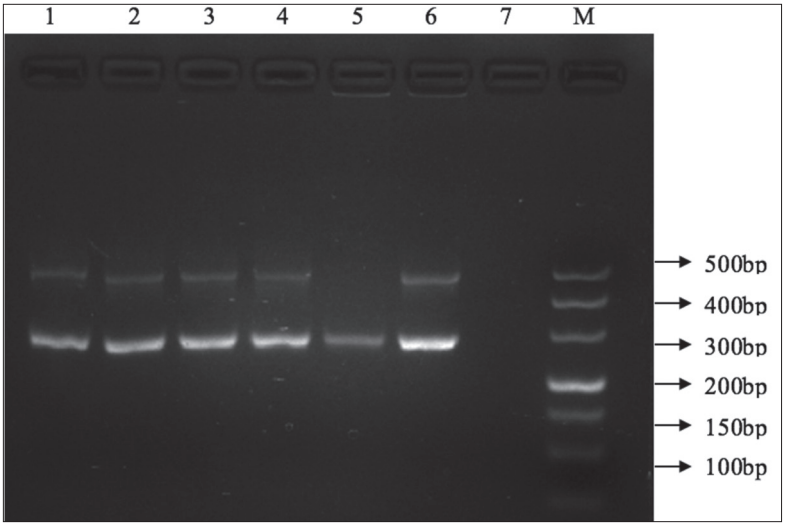

Table 1. *The isolation of B. suis S2 and B. melitensis in aborted fetuses*

Animal No	PCR Results	*B. melitensis*	*B. suis* S2
1	+	-	+
2	+	-	+
3-11	+	+	-
12	+	+	+
13-25	+	-	-
26-31	-	-	-

H_2S production, thionine sensitivity, tbilisi phage lysis RTD 10^4 and A monospecific sera agglutination. The growth of all the *B. melitensis* isolates on medium with thionin at only 40 µg/mL (1:25.000) concentration and basic fuchsin at all concentrations suggested these isolates as *B. melitensis* biovar 3.

DISCUSSION

Various pathogens, such as *Coxiella burnetii*, *Chlamydophila abortus*, *Salmonella enterica* serovar Abortusovis, *Toxoplasma gondii*, and *Neospora caninum*, have been found to induce abortions in pregnant sheep [11], but in this study, we could not find any of these pathogens in the samples after PCR identification and pathogen isolation (data not shown). Here, 25 (80.6%) out of 31 samples were identified as being infected with *Brucella* by the *omp22* target gene PCR assay. However, three isolates were further identified as *B. suis* S2, and the rest of 10 isolates were identified as *B. melitensis* according to the Bruce-ladder multiplex PCR assay [7]. These results suggest that *B. suis* S2 could partially induce abortion in pregnant ewes because *B. suis* S2 can infect and replicate in GTCs; the growth rates begin to accelerate at 12 h, with the bacterial load peaking after 24 h [6]. In addition, it is likely that the particularly high sensitivity of pregnant animals to brucellosis involves the local suppression of the immune response in the placenta [12], which leads to colonization and placenta damage, and ultimately abortion, in pregnant animals.

The bacteriological isolation is still the "gold standard" for diagnosis of Brucellosis [2]. The rate of isolation of *B. melitensis* from aborted cattle and sheep fetuses were found to be

28% in Ili region (northwest of XUAR) [13]. In this study, 10 *B. melitensis* (32.2%) and 3 *B. suis* S2 (9.6%) were isolated from 31 aborted sheep fetus samples in which one *B. suis* S2 and *B. melitensis* were isolated from a same aborted fetus *Table 1*. The co-existence of two different species of *Brucella* in the same animal is rare, and the mechanism by which they co-exist is not fully understood. However, there are still 13 samples positive for PCR but negative for culture because of the contamination decreased the rate of *Brucella* isolation. Thus, the techniques of *Brucella* isolation need to be improve in our laboratory. The Brucellosis is still enzootic in Ili region of XUAR and is the main cause of abortion in sheep and cattle. *B. melitensis* and *B. suis* strains are biotyped by 4 main tests such as H_2S production, CO_2 requirement, dye (thionin and basic fuchsin) sensitivity and agglutination with monospecific A and M antiserum [2]. *B. melitensis* biotype 3 is the predominant subtype of *B. melitensis* as documented before [13]. In parallel with this, current study revealed that 76.9% (10/13) isolates belonged to *B. melitensis* biotype 3 while *B. suis* biovar 1 made up 23.0% (3/13).{Alton, 1988 #168}

The live *B. suis* S2 vaccine has been used successfully in China to immunize sheep for decades. However, the occurrence of abortion in ewes after vaccination suggests that several factors may play a role in vaccine-induced abortions, including immune status, current health status, other diseases, and vaccines [14]. The present work shows that the vaccine strain could induce the abortion and be isolated from aborted sheep fetus. Hence, it is urgently necessary to develop a new *Brucella* vaccine to protect animals especially pregnant animals more safely.

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Short Communication

Angel Wings Syndrom in Swans *(Cygnus cygnus* and *Cygnus atratus)* [1]

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Abstract

In the present study 6 male swans two black *(Cygnus atratus)* and four white *(Cygnus cygnus),* weighing about 5.5-7.5 kg, with bilateral wing sagging and turning out of the wing tips belonging to Parks and Gardens Directorate of Metropolitan Municipality were used as materials. The swans were being fed on daily ration (pellet feed) as well as food residues such as bread, bagels, chips, etc. thrown by visitors. The clinical examination revealed that developing feathers and the tips of the wings of swans were stuck up from its normal position. The wings of the swans were bandaged adjacent to their bodies for one week and their rations were arranged. Angel wings have a high chance of being treated if the problem is diagnosed early.

Keywords: *Swan, Angel wings, X-ray*

Kuğularda *(Cygnus cygnus* ve *Cygnus atratus)* Melek Kanat Sendromu

Öz

Sunulan çalışmada, Büyükşehir Belediyesi Park ve Bahçeler Müdürlüğüne ait, yaklaşık 5.5-7.5 kg ağırlığında, kanatları bilateral olarak ventrale doğru sarkmış ve kanat uçları dorsale doğru dönmüş 6 erkek kuğunun [iki siyah *(Cygnus atratus)* ve dört beyaz *(Cygnus cygnus)*] tanı ve tadavi süreci ele alındı. Kuğuların günlük rasyonda (pelet yemi) ve ayrıca parkı gezenler tarafından (ekmek, simit, cips vb.) beslendiği öğrenildi. Klinik muayenede kuğuların kanatlarında yeni tüy gelişiminin olmadığı ve uç kısımlarında deformasyonlar bulunduğu görüldü. Kuğuların kanatlarını kullanamadıkları ve abdomenden uzak pozisyonda tuttukları dikkat çekti. Kuğuların rasyonları düzenlenerek, kanatları 7 gün süre ile abdominal duvara bandajlanarak hareketleri kısıtlandı. Melek kanatları sendromu erken teşhis edilirse, tedavi edilme şansının yüksek olduğu belirlendi.

Anahtar sözcükler: *Kuğu, Melek Kanatları, X-ışını*

INTRODUCTION

"Angel wing" (AW) is a deformity commonly found in ducks, geese, swans and other waterfowl [1]. There has been little scientific study done on the condition, yet most wildlife and waterfowl experts agree the overwhelming cause of angel wing is an unhealthily-high protein and/or carbohydrate-based diet. The disorder causes the last joint in one or both wings to unnaturally twist outward rather than lying flat against the bird's body. The resulting image is named in 20 different ways, depending on whether these primary flight flaps are twisted (angel wing, aircraft wing etc.) or hanging (low wing, sling wing etc.). But AW is the most common and accepted name for this deformity [1-4]. AW has widely been reported in domesticated birds raised in the hunting grounds as compared to wild birds. The vast majority of the cases have been reported in waterfowl however other species including parrots, rattles, toys, fishermen and tournaments are also predisposed [1,3]. The etiology of AW is unknown however some of the documented predisposing factors include nutritional deficiency or imbalance especially vitamins elevated concentration of dietary protein, increased levels of contaminants (polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans and perhaps a genetic predisposition due to relatives after a bottle neck event [1,5,6]. Kear [1] reported that inappropriate nutrition, high protein diet and lack of exercise were the

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main causes of AW in wild-water birds. If the AW is noticed early, the wing can be brought to its normal position for 3-5 days to improve. However, in cases of adulthood, the carpus can be traumatized leaving amputation as a is the best solution [7,8].

Swan with AW problem in same herd was not observed very often. In the present study, 6 swans with AW were considered with clinical, radiological and laboratory analysis to be shared with clinicians.

MATERIAL and METHODS

In the present study 6 male swans [(two black [*(Cygnus atratus)* and four white *(Cygnus cygnus)*], weighing about 5.5-7.5 kg, with bilateral wing sagging and turning out of the wing tips belonging to Parks and Gardens Directorate of Metropolitan Municipality were used as materials. As a result of the anamnesis, the swans (ages between 4-6 month) were being fed on daily ration (pellet feed) as well as food residues such as bread, bagels, chips, etc. thrown by visitors.

Clinic and Radiological Examination

The clinical examination revealed that developing feathers and the tips of the wings were stuck up from its normal position. The wings were mangled and directed away from the body. The wing joints *(A. carpo-phalangeal)* were twisted, deformed and no longer flat and smooth *(Fig. 1-a,b)*. The radiological examination revealed a deformation on the distal tip of carpo-metacarpus and bending on the dorsolateral side of the primary flight flaps in the wings *(Fig. 2)*. The wings were bilaterally shaped in 5 cases. In the black swans the wing twist, twist was advanced in dorsolateral direction. In white swans, primary flight flaps were found to be pendulous in the ventral direction.

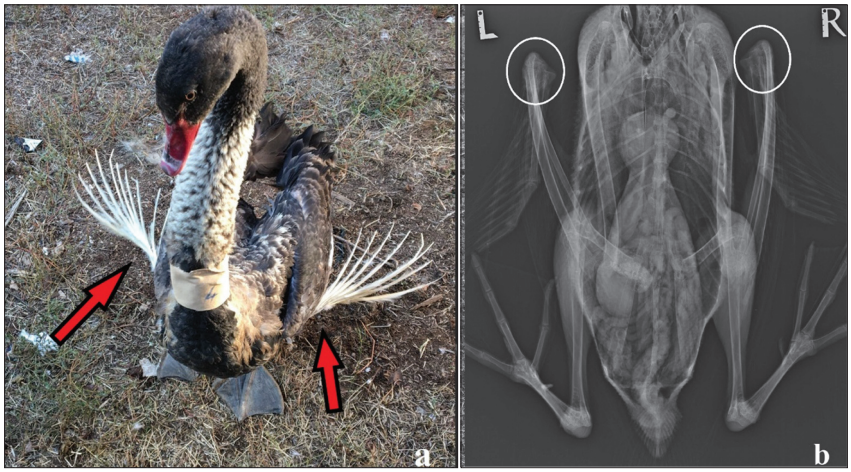
Laboratory Analysis

Blood (4 mL) was collected from brachial wing vena *(v. brachialis)* in all cases for hematological and biochemical analysis. In all the affected birds, significant findings of hematologic analysis were including leukocytosis consisting of lymphocytosis, granulocytosis and monocytosis. Additionally, a slight increased MCHC and marginally low MCH values were also recorded. However, red blood cell count remained normal in all birds *(Table 1)*.

As a result of biochemical analysis, sodium value was found above or above the upper limit at all of the cases, again calcium value was found to be well below the lower limit in all cases *(Table 2)*.

Daily feed analysis of swans has been analyzed in laboratory in animal Nutrition Department *(Table 3)*.

Fig 1. The clinical examination revealed that the wings were mangled and directed away from the body. (a) Case 4, clinical appearance of wings *(red arrows)* and (b) deformation on the carpo-metacarpus joints were evident in the radiological position (D/V) *(white circles)*



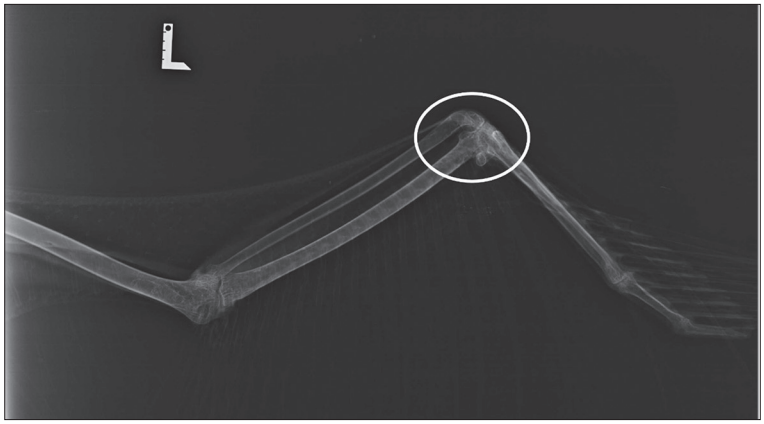


Fig 2. Radiological image of the left wing of case number 4 (M/L). Deformation on the carpo-metacarpus joints were evident in the radiological position *(white circle)*

Table 1. *Hematological outcomes of cases*

Hematological Values	Reference Values	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
WBC (x10^9/L)	6.3-22	36.71	38.39	41.25	37.03	33.6	31.85
Lym. (x10^9/L)	0.9-9.77	11.41	11.59	11.79	11.14	10.95	10.73
Mon. (x10^9/L)	0.05-1.39	3.63	3.76	3.87	4.77	3.12	4.20
Gra. (x10^9/L)	3.33-14.6	21.67	23.4	25.59	21.12	19.53	16.92
RBC (x10^{12}/L)	1.96-2.9	2.36	2.59	2.74	2.8	2.05	2.22
MCV (fl)	164-200	123.5	121.2	130.1	127	125.6	117.2
Hct	-	29.1	31.3	35.6	35.5	25.7	26.0
MCH (pg)	52.9-65.5	50.0	49.4	52.9	50.7	54.1	46.8
MCHC (g/dL)	29-36.5	40.5	40.8	40.7	40.0	43.1	40.0
RDW	-	6.7	7.4	8.1	8.5	6.6	7.2
Hb (g/dL)	11-16.5	11.8	12.8	14.5	14.2	11.1	10.4
THR (m/mm^3)	-	29	429	432	475	530	330
MPV (fl)	-	7.3	6.7	6.7	6.4	6.6	6.7

Table 2. *Results of biochemical analysis of swans*

Analyses	Range	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Total protein (g/dL)	3.55-5.45	4.2	4.3	4.4	4.5	4	4.2
Albumin (g/dL)	1.2-2.15	1.9	2	2.3	2.4	1.8	1.8
Creatinin (µmol/L)	18-89	8.84	17.68	17.68	8.84	8.84	17,68
Urea (mmol/L)	0.1-2.4	1.43	1.07	1.78	1.78	1.78	1.43
ALT (SGPP) (U/L)	10-59	33	47	113	61	41	35
AST (SGOT) (U/L)	17-122	25	24	40	27	45	27
GGT (U/L)	4-26	9	9	5	5	10	9
CK (U/L)	124-894	276	250	182	277	399	217
LDH (U/L)	165-724	337	355	394	493	559	374
Glucose (mmol/L)	6.2-12.6	10.49	12.21	10.43	11.99	9.04	10.54
Cholesterol (mmol/L)	3.7-8	6.68	6.68	5.44	5.21	5.44	5.96
Calsium (mmol/L)	2.19-2.89	0.98	0.86	0.88	0.92	0.95	0.84
Sodium (mmol/L)	132-150	154	157	160	161	148	146
Potassium (mmol/L)	3-5	4.5	4.0	3	3	3.3	3.4

Treatment

For the treatment, the wings of the swans were bandaged adjacent to their bodies for one week (7 days) and their rations were arranged *(Fig. 3)*. In addition to feeding balanced protein, carbohydrate, fat, vitamins, minerals, green vegetables (lettuce, etc.) and clean drinking water.

RESULTS

It was determined that the success rate of the applied treatment was high in early diagnosed cases *(Fig. 4)*. In 4 swans determined to be in the early stage of the AW, bandage application and regulation of the ration prevented the wing degeneration and corrected wing position. No improvement was observed in the wings of 2 swans which were determined to be in the advanced stage.

Table 3. *Feed analysis results*

Physical Analysis	Result	
Appearance	Normal	
Color	Normal	
Smell	Normal	
Foreign body	None	
Chemical Analysis	**Natural State**	**In Dry Matter**
Dry matter, %	92.19	-----
Raw ash, %	12.51	13.57
Raw fat, %	4.43	4.80
Raw cellulose, %	7.04	7.64
Raw protein, %	17.2	18.46
ME, kcal/kg	2546	2762

Fig 3. Bandage application of the swans were bandaged adjacent to their bodies for one week

Fig 4. Case No. 1 after the bandage is removed, on the 7th day of treatment

However, the progression of the problem was stopped by the regulation of the ration. Among all the affected birds, clinical improvement was seen in 4 white swans as compared to 2 black swans with no improvement. The partial and complete wing amputation is recommended for these two cases. But it was not accepted.

DISCUSSION

Angel Wings or Slipped Wing is more commonly observed in swans and geese and is to a lesser extent reported in ducks [1,4,5,7,8]. The deformed wing developed during growth, results in one or both wings sticking out from the body leaving the bird unable to fly. The left wing is more commonly affected than the right wing. In our cases, the frequency of bilateral appearance was very obvious. This condition becomes apparent while the flight feathers are growing, with the weight of the primary feathers appearing to be too great for the carpal joint muscles, leading to the dropping wing tip. The primary flight feathers may become damaged. In this study, deformities wings have been permanent since the long-standing of events for in the wrong nutritional feeding [8,9]. The recommendations for blood tests in waterfowl are similar to those commonly performed in other birds and include the following: complete blood count, advanced serum chemistry panel with bile acids, serum protein electrophoresis [10].

If the treated patients are very young, the condition can sometimes be minimized by splinting and repositioning the affected wing while feeding them a proper diet for optimal growth. Even then, a full recovery is not guaranteed. For rehabilitators, it can be emotionally taxing to see birds denied the chance for a full and productive life because people didn't know about the dangers of improperly feeding them. But, as the swans evaluated in this study were adults, there was no desired improvement. Although a mild improvement was seen in the swans that were in acute phase of the disease [9,11,12]. Male birds seem to be more prone to this condition than females. Although it looks odd, it is not painful to the bird. It can occur with one wing or both. Radiological examination showed deformation of the carpometacarpal joints in all cases [11]. The principles of positioning the avian patient for radiography are the same as for other species. At least two views, 90° to each other, are suggested. Positioning may be maintained by taping the patient directly to the cassette or to a radiolucent plexiglas board. Masking tape is usually sufficient in the chemically immobilized patient and has the advantage of not pulling out feathers when it is removed [13]. It was shown that in adult wild birds the disease is incurable and usually leads to an early death as affected birds are rendered effectively or totally flightless. However, adult swan has been adversely affecting the solution of the problem. Among all the affected birds, clinical improvement was seen in 4 white swans as compared to 2 black swans with no improvement. These results brought to mind the question that the propensity of the swan might be different between the swan species, even though it is in the same age range in the course of the problem. The partial and complete wing amputation is recommended for these two cases. But it was not accepted.

In young birds wrapping the wing and binding it against the bird›s flank for a few days and feeding more natural diet can reverse the damage. If diet is the primary issue, reducing the protein by adding wheat to the birds› feed may be recommended. A diet that provides sufficient amounts of vitamin D (the «sunshine» vitamin), vitamin E and manganese may also be indicated [6,14].

Another interesting fact of this study is that it appears in a swan colony living in the same place. AW respond well if treated early [12,13]. In the case of late-onset and long-onset

treatment, the treatment will often fail. These phenomena must be rehabilitated in certain regions. Because these birds cannot fly and move fast.

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Case Report

Diagnosis of Multiple Congenital Cardiac Defects in a Newborn Calf

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Abstract

This case report presents the clinical diagnostic work-up and surgical approach via cardiopulmonary by-pass technique in 15 day-old, male, Simmental calf with multiple cardiac defects. Calf suffered from respiratory stress and poor growth. Cardiac auscultation revealed a loud pansystolic murmur with cardiac trill of both transthoracic area of the heart. Cardiomegaly and pulmonary artery bulge (x-ray) and atrial fibrillation were observed. Color Doppler transthoracic echocardiography revealed musculomembranous ventricular septal defect (VSD), atrial septal defect (ASD) and patent ductus arteriosus (PDA) with left to right shunt. Routine hematological and serum biochemistry profiles were non-specific. Calf underwent to lateral thoracotomy for surgical correction of multiple cardiac defects. During the cardiopulmonary by-pass, calf was dead due to hemodynamic imbalances and ventricular fibrillation. Necropsy confirmed the presence of multiple cardiac defects in this case.

Keywords: *Calf, Multiple cardiac defect, Echocardiography, Cardiopulmonary by-pass*

Yenidoğan Bir Buzağıda Çoklu Kongenital Kardiyak Defektlerin Tanısı

Öz

Bu olguda çoklu kalp defekti olan 15 günlük, erkek Simmental buzağıya diyagnostik yaklaşım planı ve olguya kardiyopulmoner by-pass tekniği ile cerrahi yaklaşımın sonuçları sunulmuştur. Buzağı solunum stresi ve büyüme geriliği şikayeti getirildi. Kardiyak oskültasyonda kalbin her iki transtorasik alanında titreşimle beraber yüksek pansistolik üfürüm belirlendi. Ayrıca kardiyomegali, pulmoner arter dolgunluğu (röntgen) ve atriyal fibrilasyon gözlendi. Renkli Doppler transtorasik ekokardiografide muskulomembranöz ventriküler septal defekt (VSD), atriyal septal defekt (ASD) ve soldan sağa şant ile beraber patent duktus arteriozus (PDA) tespit edildi. Rutin hematolojik ve serum biyokimyasal profiller nonspresifikti. Buzağıda çoklu kardiyak defektlerin cerrahi yolla düzeltlmesi için lateral torakotomi yapıldı. Kardiyopulmoner by-pass sırasında, buzağı hemodinamik dengesizlikler ve ventriküler fibrilasyon nedeni ile hayatını kaybetti. Nekropside çoklu kardiyak defekt varlığın ortaya konuldu.

Anahtar sözcükler: *Buzağı, Çoklu kalp defekti, Ekokardiografi, Kardiyopulmoner by-pass*

INTRODUCTION

Congenital cardiac malformations have been reported rarely in calves. Ventricular septal defect (VSD), a more common congenital heart disease among calves can be seen either alone or in combination with more complex abnormalities such as atrial septal defect (ASD) and patent ductus areteriosum (PDA) [1,2].

In physical examination, cyanosis of the mucous membranes and a loud systolic heart murmur are important to be suspected for congenital cardiac defect in neonatal calves [3,4]. In these cases, thoracic x-ray may present cardiac chamber dilations (cardiomegaly) and pulmonary edema. Based on these clinical findings, it could be possible to suspect the presence of cardiac defect(s), however echocardiography is still the most reliable diagnostic tool to confirm or rule

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out the preliminary diagnosis. Congenital cardiac defects can lead to poor growth, un-responded to medical therapy and sudden death (economical losses). Early diagnosis of the cardiac defects can improve the disadvantages by removing from breeding and unnecessary usage of antibiotics or etc. Medical therapy can transiently improve the clinical signs such as respiratory distress and exercise intolerance, whereas surgical correction of cardiac defect has been considered for definitive solution. There are limited case reports on multiple cardiac defects and their surgical correction in calves. Thus, this paper is a report on diagnostic and surgical approaches in a Simmental calf with a combination of congenital cardiac defects (ASD, VSD and PDA).

CASE HISTORY

A 15 day-old Simmental calf was presented to Animal Teaching Hospital of Faculty of Veterinary, Balıkesir University with the complaint of respiratory stress, exercise intolerance, dyspnea and poor growth. After the diagnostic work-up, a PDAwas suspected, and to be able to confirm the diagnosis, patient was referred to cardiology clinic of Animal Teaching Hospital of Faculty of Veterinary, Bursa Uludağ University. Clinical examination revealed cyanosis of mucous membranes, a loud gallop cardiac rhythm and pulse deficit. Cardiac auscultation revealed a grade-5 pansystolic murmur with cardiac trill of both trans-thoracic area of the heart. Complete blood cell count was within reference ranges, except a mild neutrophilia ($7.38x10^9$/L, reference range: 0.6-$6.7x10^9$/L). Biochemical profile (VetScan® Large Animal Profile, 10 parameters, Abaxis) was unremarkable. Coagulation status was evaluated by use of thrombo-elastography (TEG® 5000 Hemostasis System, USA). Of TEG, reaction time (R time: 18.8 min) and kinetic time (K time: 5.3 min) prolonged, compared to healthy control *(Fig. 1)*.

Electrocardiographic evaluation (base apex leads) showed atrial fibrillation. Increased heart size and cardiac globalization, dorsal deviation of trachea, diffuse pulmonary edema in caudal lung lobes were detected on left lateral and ventro-dorsal thoracic radiographies *(Fig. 2)*. After restraining lateral recumbence, phased array medium frequency echocardiography probe (3-5 mHz) was used (Esaote, Caris Plus, Italy) for the final diagnosis of case as mentioned previously [5]. Standard echocardiographic techniques were used for measurements *(Table 1)*. Coupling gel applied on hair clipped area between the fourth and fifth intercostal spaces behind the olecranon. On right parasternal long axis and left apical 5 chamber views showed the presence of inlet VSD that was detected just below the aortic valve between two ventricles. A passage with strong left to right sided turbulence flow (Vmax: 1.54 m/s) was visualized in color flow Doppler of the left parasternal long axis view of the abnormal passage between ventricles *(Fig. 3)*. An agitated saline contrast (bubble) study was performed right before injection by moving it rapidly back and forth between the 2 syringes that were connected to the 3-way stopcock. Positive echo contrast was seen 5 to 10 s after intravenous injection of mixture. The contrast of the air-saline mixture was visible as hyperechoic content

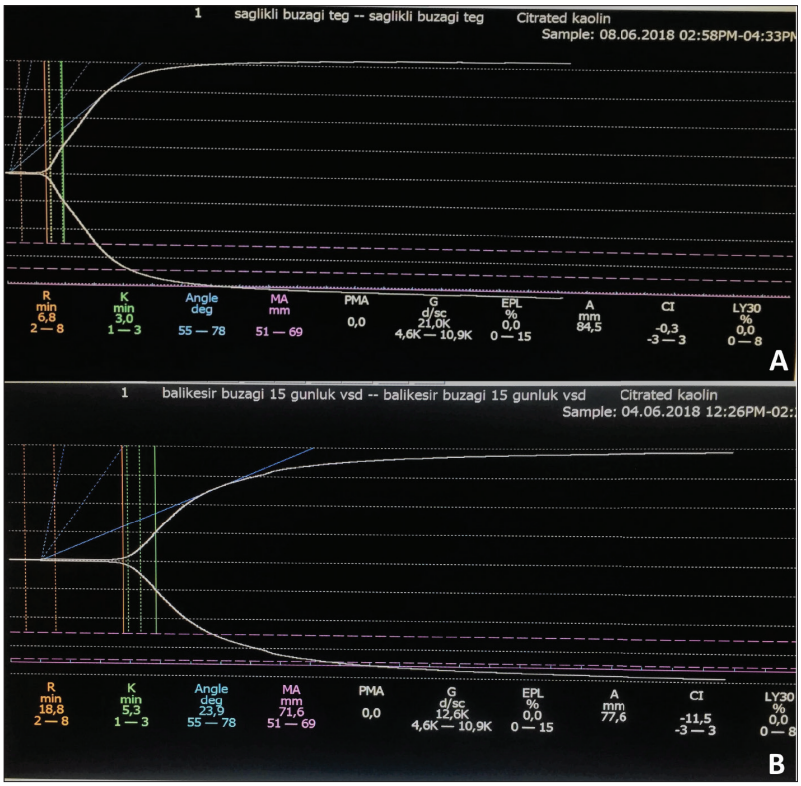

Fig 1. TEG evaluation of the calf **(B)** shows prolongation in Reaction time (R: 18.8 min) and Kinetic value (K: 5.3 min) compared to healthy control **(A)**

respectively in right atrium (RA), right ventricle (RV) and left ventricle (LV) that proved the abnormal passage between both atriums and ventricles respectively. Color flow Doppler examination showed a turbulent flow and low frequency regurgitant jet (Vmax: 1.11 m/s) and high pulmonary artery flow velocity (Vmax: 2.19 m/s) relating with PDA, a connection between ascending aortic root (Ao) and PA. VSD to Ao diameter ratio was 0.74 and PA to Ao ratio was 0.9 on right parasternal short and long axis views.

Surgery of cardiopulmonary by-pass was decided, and until the operation calf was treated with diuretic (furosemide, 2 mg/kg, im, 2x1, for 5 days) to reduce pulmonary edema and cardiac volume overload. After that, calf underwent right lateral thoracotomy to correct and repair multiple cardiac defects. Preoperatively, atropine sulphate (0.01 mg/kg sc) and xylasine HCl (0.01 mg/kg im) were injected respectively. Ketamine HCl (4 mg/kg, im) was administered for general anesthesia and to facilitate the orotracheal intubation. After intubation, isoflurane was inhaled during surgery using 2% concentration of oxygen. Respiration was assisted by mechanical ventilation (Anesthesia Ventilator 900 Series, AMS 200 Anesthesia Workstation, AMS Ltd. Şti., Ankara, Turkey). Patient was closely monitorizated (Datex-Ohmedia Cardioscap/5, GE Healthcare, Helsinki, Finland) and all vital parameters were controlled and peripheral arterial (left femoral artery) and central venous pressures (left jugular vein) were checked and recorded at 15 min intervals during surgery. Urinary output was also controlled following urethral catheterization.

Table 1. *Some echocardiographic parameters of the calf evaluated*

Echocardiographic Paremeters	Case	Reference Value	References
LVDd (cm)	7.50	4.15±0.12	[5]
RVDd (cm)	1.98	1.34±0.05	[5]
FS (%)	35	36.9±1.6	[5]
Ao (cm)	2.86	2.65±0.08	[5]
LA (cm)	6.35	2.12±0.05	[5]
LA/Ao	0.9	0.81±0.3	[5]
PA (cm)	3.11	F	-
PA/Ao	1.01	<1.0	[6]
PA V max (m/s)	2.19	F	-
Ao V max (m/s)	1.67	F	-
Kg	34.7	33.9±1.7	[5]
Age (days)	15	18.18±2.5	[5]

***LVDd:** left ventricular diastole diameter, **RVDd:** right ventricular diastole diameter, **EF:** ejection fraction, **FS:** fractional shortening, **Ao:** aorta, **LA:** left atrium, **RA:** right atrium, **PA:** pulmonary artery. **F** Reference value could not be found*

Following the routine right 4th intercostal thoracotomy, peripheral cannulations were completed from right jugular vein, right femoral artery and caudal vena cava. After clamping of aorta, cranial and caudal vena cava, anti-coagulant (Clexane®, 6000 IU/0.6 mL Anti-XA, Sanofi Aventis) was administered to circulation and cardiopulmonary by-pass was started. Cardioplegia solution (Cardioplegia Solution A, Baxter Healthcare Ltd) was applied through the aorta to stop the cardiac manners. And then atrial and ventricular septal defects were observed following to right atriotomy and incision of tricuspital septal annulus. Defects of atrial and ventricular septums were repaired by pericardial patches, and right atrial incision was sutured

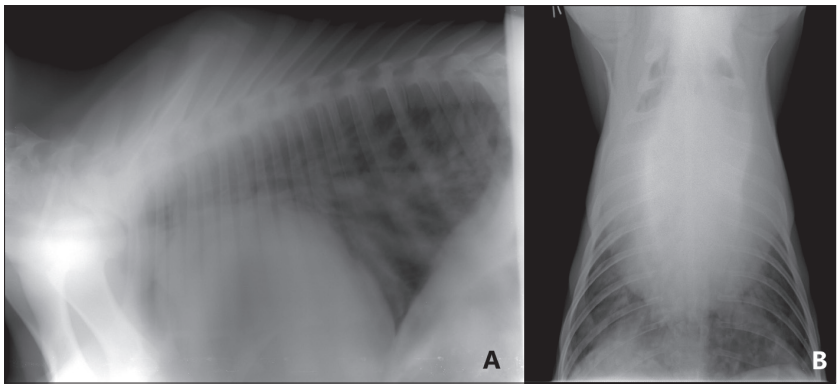

Fig 2. Increased heart size and cardiac globalization, dorsal deviation of trachea, diffuse pulmonary edema in caudal lung lobes on lateral **(A)** and ventrodorsal **(B)** x-ray of the thorax

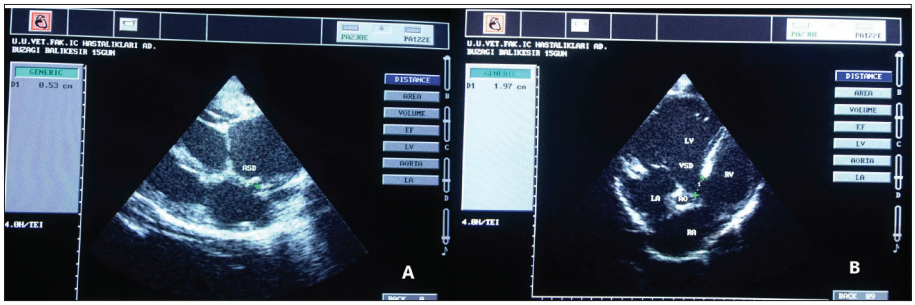

Fig 3. Passages between atriums **(A)** and ventricles **(B)** on echocardiographic view of the heart. **VSD:** ventricular septal defect, **ASD:** atrial septal defect, **LA:** left atrium, **RA:** right atrium, **LV:** left ventricle, **RV:** right ventricle, **Ao:** Aorta

routinely. At that time, before right thoracotomy incision, the blood in the cardiopulmonary by-pass pump was re-loaded the circulation system, and cardiac pulsations were attempted. Pace was applied the on the myocardium to constitute the normal cardiac rhythm; however, unresponsive atrial fibrillation formed, and the calf was dead despite having medical and intracardiac defibrilations which turned itself to ventricular fibrillation (VF). Necropsy showed the presence of PDA on the left side of the heart as well as ASD and VSD in this case.

DISCUSSION

Congenital cardiac defects such as ASD and membranous VSD and their relation with breed and sex predispositions were reported previously in calves [1-4,6-9], but the diagnostic steps and cardiopulmonary by-pass procedures of a combination of three congenital cardiac defects (ASD, VSD and PDA) in newborn calves has not been reported yet.

In this case, clinical (tachypnea, cyanosis and cardiac murmur), electrocardiographic (atrial fibrillation) and thoracic x-ray findings (cardiomegaly and pulmonary edema) were suggestive of congenital cardiac defect(s). Transthoracic echocardiography was performed as a gold standard for definitive diagnosis. VSD to Ao diameter ratio (0.74) was greater than 0.6 suggestive for large, unrestrictive VSD [8].

Several views of the main PA from both the right and left imaging windows have taken [5]. Decreased cardiac output that was characterized by low FS value *(Table 1)* due to overt cardiac size and diuretic usage to clear pulmonary edema before the evaluation of the calf might decrease the velocity of pulmonary artery; therefore there was not a serious turbulence (presence of low frequency regurgitant flow) in pulmonary trunk. It seems that volume and pressure change in RV resulted with right-sided heart failure and pulmonary hypertension in short life span of our patient. Due to low frequency pulmonary artery regurgitant flow, Eisenmenger's syndrome was not present in this case, in which a long-term left-to-right cardiac shunt caused by a congenital heart defect (VSD, ASD and/or PDA) changes to a cyanotic right-to-left shunt with pulmonary hypertension [3]. In this case, PA to Ao ratio (0.9) and peak pulmonary artery flow velocity (2.19 m/s) were not compatible with the presence of pulmonary hypertension [6].

The patient was treated with diuretic to improve clinical signs by decreasing pulmonary edema and cardiac volume overload for 5 days, before the cardiac surgery. In that period, coagulation status of calf was evaluated by thrombo-elastography (TEG) which could provide useful information of global platelet function and coagulation cascade [10]. In our case, mild hypocoagulable state was observed due to prolongation of R time (the time from the test start until the start of clot or fibrin formation) and K time (speed of the band formation between fibrin and platelets). This observation showed that coagulation status could be changed by multiple congenital cardiac defects in calves, as reported in neonatal calves with endotoxemia [11,12].

Calf was dead during the cardiopulmonary by-pass due to AF and then VF that was un-responsive to medical therapy. AF is also one of the intra and postoperative arrhythmia causing mortality in human medicine [13]. It seems that another possible reason of death was prolonged mechanical ventilation process in our case.

In conclusion, a calf with multiple congenital cardiac defects (ASD, VSD and PDA) underwent to surgical correction via cardiopulmonary by-pass has been reported. By this case report, diagnostic evaluation steps of the calf with multiple congenital cardiac defects, operation possibilities and disadvantages of the procedure were reported briefly.

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Case Report

Mandibular Hypertrophic Osteodystrophy Fibrosa in a German Shepherd Puppy

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Abstract

Hypertrophic osteodystrophy is a developmental disease of unknown etiology and affects primarily young rapidly growing large and giant breed dogs. The present study reports a case of mandibular hypertrophic osteodystrophy fibrosa in a 6-month-old male German shepherd puppy admitted with a history of one month decreased appetite, difficulty in chewing, and open mouth with continuous drooling. Clinical examination revealed thickened mandible and filling of the intermandibular space with hard tissue. Radiography revealed bone-like material filling the intermandibular space. Biopsy from the thickened mandibular mass demonstrated marked osteoclastic activity and resorption of bone trabeculae that were replaced by fibrous connective tissue. The marrow cavity was filled with massive fibrous tissue mixed with hemorrhage and osteoclasts. Hypertrophic osteodystrophy fibrosa should be taken into consideration in dogs with mandibular masses. Radiography and histologic evaluation are suitable tools to differentiate hypertrophic osteodystrophy. fibrosa from mandibular neoplasms and cranio-mandibular osteopathy.

Keywords: *Bone resorption, Dog, Hypertrophic osteodystrophy, Mandibular masses, Osteoclasts, Teeth*

Bir Alman Çoban Köpeği Yavrusunda Mandibular Hipertrofik Fibröz Osteodistrofi Olgusu

Öz

Hipertrofik osteodistrofi, etiyolojisi bilinmeyen bir gelişim dönemi hastalığıdır ve birincil olarak genç ve hızlı büyüyen büyük ırk köpekleri etkiler. Bu çalışma ile, bir aydır süren iştahta azalma, çiğnemede zorlanma, ağzın sürekli açık kalması ve salya birikimi semptomları olan 6 aylık bir erkek Alman çoban köpeğinde mandibular fibröz hipertrofik osteodistrofi vakası bildirildi. Klinik muayenede mandibulanın kalınlaştığı ve intermandibular boşluğun sert doku ile dolu olduğu belirlendi. Radyografide intermandibular boşluğun kemik benzeri materyal ile dolduğu görüldü. Kalınlaşmış mandibular kitleden alınan biyopside belirgin osteoklastik aktivite ile birlikte kemik trabeküllerinin rezorpsiyonu ve fibröz bağ dokusu ile yer değiştirdiği gözlendi. Kemik boşluğu, kanama ve osteoklastlarla karışık halde masif fibröz doku ile doluydu. Mandibular kitle belirlenen köpeklerde fibröz hipertrofik osteodistrofi dikkate alınmalıdır. Radyografi ve histolojik değerlendirme, fibröz hipertrofik osteodistrofiyi mandibular neoplazilerden ve kranio-mandibular osteopatiden ayırmak için uygun yöntemlerdir.

Anahtar sözcükler: *Kemik rezorpsiyonu, Köpek, Hipertrofik osteodistrofi, Mandibular kitle, Osteoklastlar, Diş*

INTRODUCTION

Canine hypertrophic osteodystrophy fibrosa is a developmental disease of unknown etiology affecting young growing large and giant breed dogs [1]. It has been reported in Great Dane, Irish Wolfhound, Saint Bernard, Boxer, Dalmatian, Irish setter, Weimaraner, Doberman pinscher, German shepherd, Labrador retriever, Collie, Greyhound and even the Bassett hound and some Terrier types [2-4]. It is characterized by extensive bone resorption, proliferation of fibrous connective tissues, and insufficient mineralization of the bones [3,4]. The disease affects long bones as well as those with high rates of renovation such as the mandible and the maxilla that may develop gradual swollenness and deformities [3,4].

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This report records the clinical, laboratory, radiographic and histologic findings of a rare case of mandibular hypertrophic osteodystrophy fibrosa in a six-month-old German shepherd puppy.

CASE HISTORY

A six-month-old male German shepherd puppy was admitted to the clinic of the Department of Surgery, Anesthesiology and Radiology, Faculty of Veterinary Medicine, Cairo University, Egypt, with a history of one month decreased appetite, difficulty in chewing, and open-mouth with continuous drooling of saliva. The owner had declared no history of oral and mandibular trauma. The puppy was fed on a commercial dry food of high protein content (Protein; 27% fat; 12%, calcium; 1.85%, phosphorus; 1.27% and crude fiber; 5.7%) and completed its routine vaccination against Canine Distemper virus, Canine Adenovirus Type 2, Canine Parainfluenza virus, Canine Parvovirus, Corona virus, bacterin of *Leptospira canicola* and *L. icterohaemorrhagiae* (VANGUARD 5/VC-L®, Zoetis Ltd, Sandton, South Africa) and rabies vaccine (Defensor® 3, Zoetis Ltd, Sandton, South Africa) at 4 months of age.

Oral examination revealed a fixed, firm, painless mandibular mass filling the intermandibular space extending from the mandibular symphysis at the level of tooth 301 to tooth 310 (the left incisors to the left second mandibular molar tooth, *Fig. 1)*.

The left mandibular teeth were loose when compared to the right ones. No detectable abnormalities were recorded when palpating the right mandibular body and ramus. Bilateral symmetric non-edematous firm swellings were also noticed over the distal extremity of radius and ulna of both fore limbs without obvious lameness. The rectal temperature was 37.8°C, the heart rate was 92 beat/min and the respiratory rate was 32 breath/min.

Open mouth ventro-dorsal mandibular radiograph revealed unilateral increased bone thickness and density of the mandible, the left half of the intermandibular space was filled with radiopaque bone-like material compared to the right half. This radiopaque material was extending from the level of left mandibular first incisor tooth (tooth 301) to the level of left mandibular second molar tooth (tooth 310, *Fig. 2)*.

Cranio-caudal and medio-lateral radiographic examinations of the swollen fore limbs revealed an increased bone thickness of the distal ulna of both fore limbs and irregular periosteal reactions with palisading extra-periosteal cuff of mineralization along the distal ulna. Sclerosis of the metaphysis adjacent to the growth plate parallel to the physis was also recorded *(Fig. 3)*. Survey radiographs of the thorax and abdomen were unremarkable.

Ultrasonographic examination of the thyroid gland did not

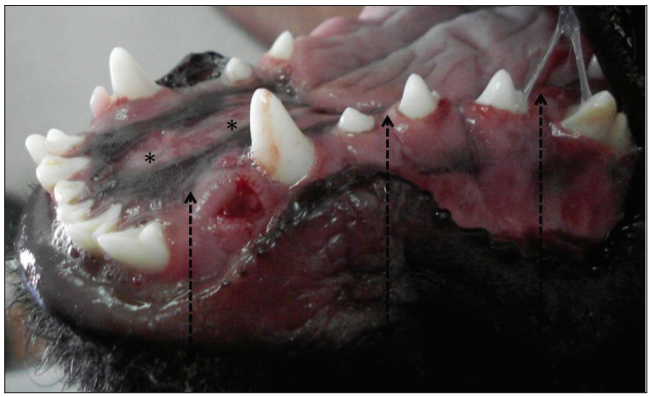

Fig 1. Photograph demonstrating the presence of profuse salivation, thickening of the mandible *(arrows)* and filling of the intermandibular space with firm tissue *(asterisks)* with loosening of left mandibular teeth

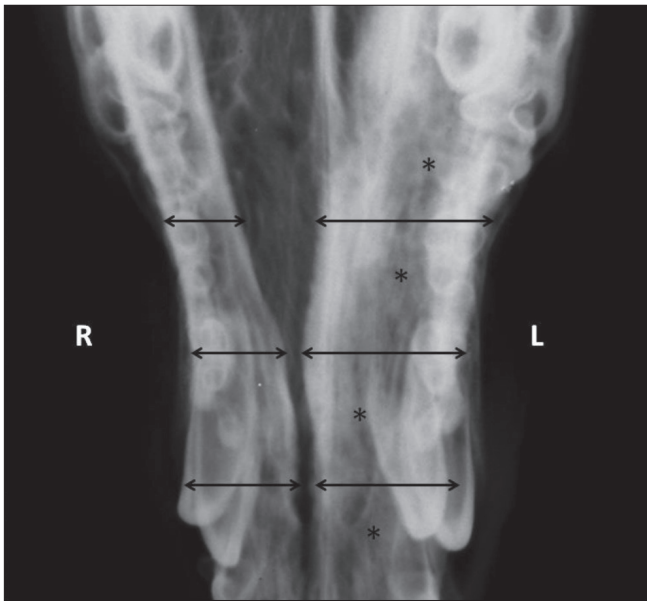

Fig 2. Intra oral ventro-dorsal radiograph of the mandible demonstrating thickening of the left mandible *(arrows)* with increased bone opacity of the left side of the mandible *(asterisks)*

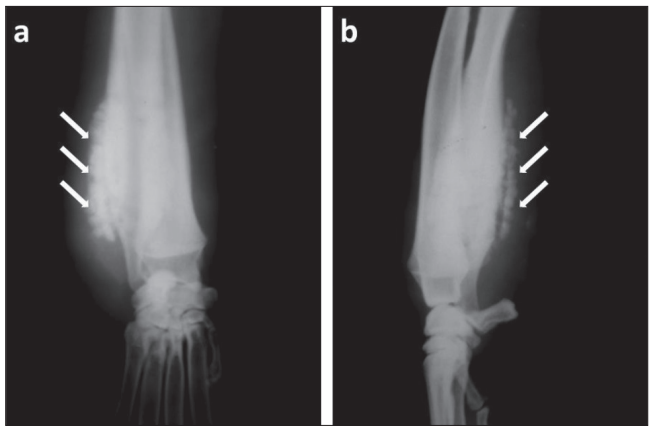

Fig 3. Cranio-caudal (a) and medio-lateral (b) radiographs of the distal radius and ulna demonstrating paraperiosteal mineralization *(arrows)* with sclerosis of the metaphysis adjacent to the growth plate

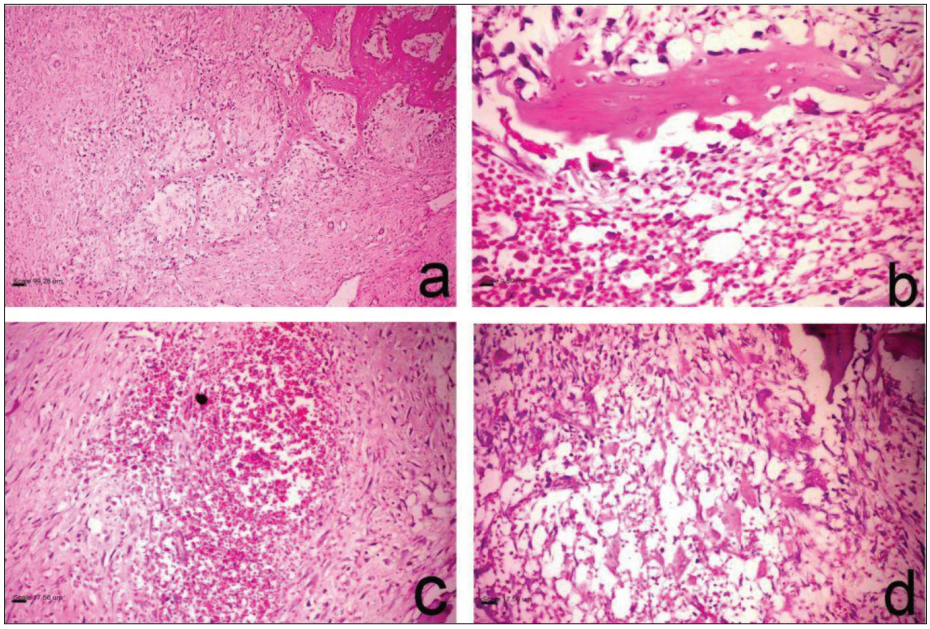

Fig 4. (a & b) Histopathologic photomicrographs of the bone biopsy demonstrating numerous osteoclastic activities and resorption of bone trabeculae that were replaced by fibrous connective tissue. The marrow cavities were filled with a massive fibrous tissue mixed with hemorrhage **(c)** and osteoclasts **(d);** (H&E)

reveal any abnormalities. The thyroid gland was visualized as a homogenous bi-lobed hypoechoic fusiform structure surrounded by echogenic capsule located between the common carotid artery and trachea. The parathyroid glands could not be identified.

Complete hematologic and biochemical examinations were done including CBC, liver (ALT 35 IU/L, AST 55 IU/L, total protein 6.2 g/dL) and kidney function tests (urea 19 mg/dL, creatinine 1.7 mg/dL), thyroid function (T3 130 ng/dL, T4 2.1 µg/dL), parathyroid function (PTH 15 pg/dL), serum alkaline phosphatase (45 IU/L), serum calcium (9 mg/dL) and phosphorus (4.3 mg/dL) values. All of the laboratory findings were within normal reference range.

Intra-oral biopsy was obtained through deep incisional needle bone biopsy performed under strict aseptic precaution and general anesthesia as follows: The puppy was premedicated with Atropine sulphate (Atropine sulphate® 1%, ADWIA Co., Egypt) at a dose of 0.05 mg/kg body weight given SC and Xylazine HCl (Xylaject® 2%, ADWIA Co., Egypt) at a dose 1.1 mg/kg body weight given IM. Then the puppy was given Thiopental sodium (Thiopental sodium®, EPICO, Egypt) 2.5% at a dose of 25 mg/kg body weight given IV. Histopathological examination of the biopsy sample revealed resorption of the bone trabeculae that were replaced by fibrous connective tissue and were associated with numerous osteoclastic activities *(Fig. 4-a,b)*. The marrow cavity was filled with massive fibrous tissue mixed with hemorrhage *(Fig. 4-c)* and osteoclasts *(Fig. 4-d)*. Based on histopathologic examination, the diagnosis was confirmed that the puppy had mandibular hypertrophic osteodystrophy fibrosa.

Conservative management of the puppy was carried out. Oral Meloxicam at a dose of 0.2 mg/kg once daily for 7 days (Mobic®, 7.5 mg tablet, Boehringer Ingelheim International GmbH, Germany), vitamin C 1 g daily (Vita C®, Misr Pharmaceutical Co., Egypt) and vitamin D 5 mg daily (Devarol S®, Memphis Co. for Pharm. Chem. Ind., Egypt) were given to the puppy. One week later, the dog was completely off food, hypothermic (36°C), lethargic and reluctant to move with a tendency to lay down. The owner was advised to feed the dog soft diet through a feeding tube along with the fluid therapy. Maintenance fluid therapy was performed by intravenous administration of dextrose 5% solution at a dose of 5 mL/kg/h. The puppy was unresponsive to treatment and died as a result of severe cachexia and debility.

DISCUSSION

The present report describes the clinical, laboratory, radiographic and histopathologic findings of a case of mandibular hypertrophic osteodystrophy fibrosa in a six-month-old German shepherd puppy.

Similar to a previous report [5], the exact cause and pathogenesis of such a condition remain unknown. Proposed causes may include nutritional imbalance, vaccination, canine distemper virus and heritability in some dog breeds [6]. Puppies between 5-8 months of age are at the highest risk of disease progression [5]. Puppies are usually presented with multisystemic clinical signs including anorexia, depression with swollen painful metaphyses [7].

The recommended treatment for hypertrophic osteodystrophy is usually nonspecific and directed to alleviate the non-specific clinical signs. The use of nonsteroidal anti-inflammatories with supportive care is considered appropriate [2]. However, some dogs may fail to respond to nonsteroidal anti-inflammatories and necessitates switching to corticosteroids [8]. The exact cause of hyper-

trophic osteodystrophy remains unknown [2]. The disease was first believed to be due to vitamin C deficiency due to its similarity with infantile scurvy [2], and the decreased plasma levels of vitamin C in dogs with hypertrophic osteodystrophy [4]. So it was advised to include vitamin C in treatment plan. Previous studies recorded that dogs may recover from hypertrophic osteodystrophy whether supplemented with vitamin C or not [4]. On the contrary, other studies reported that vitamin C, D and mineral supplementation may accelerate the rate of dystrophic calcification and diminish the rate of bone remodeling [4]. Evidence suggests that all treatment plans are somewhat successful with correction of dietary imbalance with no proof of a uniform cure [4].

The decreased appetite and excessive salivary secretion reported in the present case was mainly attributed to severe pain, teeth loosening as well as swelling of mandibular symphysis which prevented effective prehension and chewing of food. The teeth of the affected left mandible were loose as a result of bone resorption and fibrous tissue replacement.

The radiographic findings of hypertrophic osteodystrophy should be differentiated from mandibular neoplasm, cranio-mandibular osteopathy and fibrous osteopathy [6]. Neoplastic bone growths could be differentiated by displaying lytic appearance and the locally aggressive behavior of oral and maxillofacial canine osteosarcomas [9]. Moreover, maxillofacial osteosarcoma is a disease of older ages; the mean age for diagnosing dogs with maxillofacial osteosarcoma is 9-10 years [9]. Cranio-mandibular osteopathy is a bilaterally non-neoplastic proliferation of bone on the mandibular body, ramus of the mandible and/or tympanic bulla without osteophyte growths in the mandibular symphysis [2-4]. The new bone is similar in appearance to the bone found in hypertrophic osteodystrophy and may result in lameness. Fibrous osteopathy is a metabolic disease associated with hyperparathyroidism (primary, secondary, or nutritional) with persistent hypercalcemia and hypophosphatemia Disturbance in thyroid and parathyroid function was excluded in the presented puppy through ultrasonographic and biochemical examinations.

Radiographic changes of the distal ulna in both forelimbs are similar to previous reports describing hypertrophic osteodystrophy fibrosa [10]. These changes provided guidance that the mandibular swelling may be a mandibular form of hypertrophic osteodystrophy fibrosa. The presence of periosteal new bone formation separated from the underlying cortex has been reported in early stages of the disease. The newly formed bone begins at the metaphysic and may extend to the diaphysis in severe cases [11]. Radiographic changes of hypertrophic osteodystrophy should be differentiated from hematogenous osteomylitis and panostitis.

In the current case presentation, the diagnosis was confirmed by histopathological examination, which revealed osteoclastic resorption of bone trabeculae that were replaced by fibrous connective tissue.

In conclusion, hypertrophic osteodystrophy fibrosa should be taken into consideration in dogs with mandibular swellings and should be differentiated by radiography and histology from other mandibular masses including mandibular neoplasms and cranio-mandibular osteopathy. Histopathological examination provided a definite diagnosis of mandibular hypertrophic osteodystrophy fibrosa. The main limitation of the present study is the absences of long term follow up due to death of the animal.

Conflict of Interest Statement

All authors declare no conflict of interest for the presented case report.

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Letter to the Editor

The First Record *Pseudolynchia canariensis* (Diptera: Hippoboscidae) in An Eurasian Eagle Owl *(Bubo bubo* Linnaeus, 1758) in Turkey

(Türkiye'de İlk Kez Bir Bayağı Puhu'da *(Bubo bubo* Linnaeus, 1758) *Pseudolynchia canariensis* (Diptera: Hippoboscidae) Olgusu)

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Dear Editor,

Considering host preferences and morphologies, the Hippoboscidae family, known as a louse-fly, bird fly, tick fly, forest fly is a blood-sucking ectoparasites of domestic and wild animals. Although they have certain host preferences, when they need it they can suck blood from other hosts or even people. The Hippoboscidae family includes 3 subfamilies: Lipopteninae, Ornithomyinae and Hippoboscinae, *Pseudolynchia canariensis* (Mcquart, 1840), which one of the species belong to Ornithomyinae sub-family parasitic in poultry, plays a role in transmission of *Haemoproteus columbae* to domestic pigeons [1]. *Bubo bubo* known as Eurasian Eagle-Owl take part in Bubo genus which belong to Strigidae family, which has a variety of habitats showing distinct morphological and anatomical features and activating at night are birds. They often live alone and feed in various arthropods such as fish, reptiles, birds and mice [2].

A wounded puhu *(Bubo bubo)* brought by Directorate of Nature Conservation and National Parks Hatay Branch to University of Hatay Mustafa Kemal, Faculty of Veterinary Medicine, Department of Surgery on 29.11.2017, at the during the inspection were detected one hippoboscid fly. The fly was caughted by hand and for the diagnosis was taken into a glass bottle containing 70% alcohol. It was determined that the captured hippoboscid fly was *Pseudolynchia canariensis* by observing the distinctive morphological features [3] in the regions of the meta-basisternum *(Fig. 1-a)*, the head *(Fig. 1-b,c)*, the scutellum *(Fig. 1-d)* and the wing *(Fig. 1-e)*. For this study, the legal permission numbered 42742938-045.01-E.1990278 was obtained from the Directorate of Nature Conservation and National Parks Hatay Branch.

According to various studies in the world, *O. unicolor, O. stipituri, O. exilis, O. podargi, O. curvata, Ornithophila metallica, Ornithoctona plicata O. erythrocephala, Ornithomya avicularia, O. anchineuria, O. aobatonis, O. nigricornis, O. fuscipennis, O. parva, O. chloropus, O. fringillina, O. biloba, Olfersia fossulata, Icosta albipennis, I. angustifrons, I. chalcolampra, I. fenestella, I. longipalpis, I. nigra, I. zumpti, I. americana, I. rufiventris, Pseudolynchia canariensis, P. garzettae* the species of hippoboscid flies are reported from owls. Within these species *Ornithoica exilis, Ornithomya avicularia, O. chloropus, Pseudolynchia garzettae* [4] was announced from genus of *bubo* owl. In Turkey such as type of parasites *Haemoproteus* sp.[5], *Dispharnx nasuta* [6], *Strigiphilus cursitans* [7], *Strigiphilus barbatus, Columbicola columbae* [8], from owls and such as type of lice *Strigiphilus strigis*, *Kurodaia longipes* [9] has been reported from type of *Bubo bubo* owls. *Pseudolynchia canariensis* from Hippoboscid flies have been reported from mice and pigeons [10].

As a result, in this lettering, *Pseudolynchia canariensis* has been reported for the first time in species of owl the *Bubo bubo* in Turkey.

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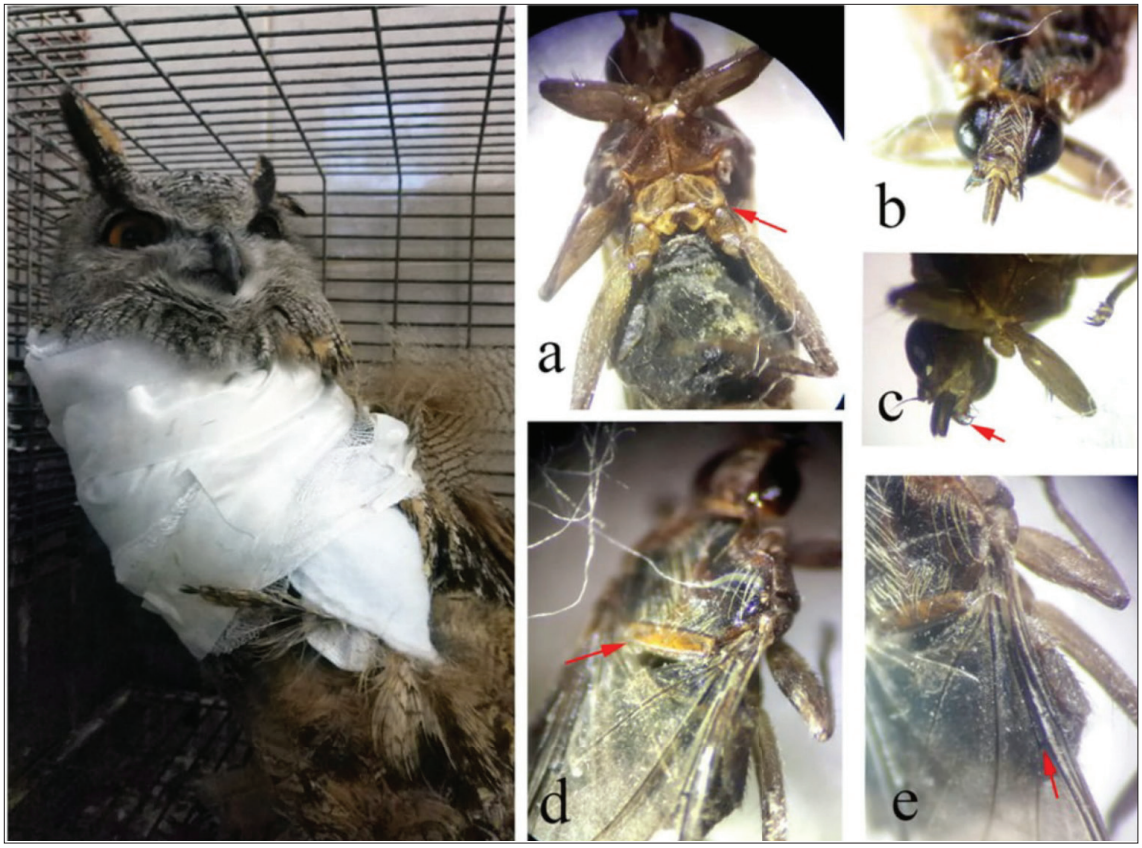

Fig 1. a) *Pseudolynchia canariensis*, posterolateral spur-like protrusion extending from the metabasisternum towards the posterior part of the coxa, ventrally, **b)** head, frontal vitta longer than vertex region, **c)** the vibrissal protrusion of the head has a strong and sharp angle, **d)** the structure at the scutellum is rectangular, **e)** there is one cross vein (r-m) in the wing

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Review

Present and Future Implications of Crimean Congo Haemorrhagic Fever Disease Emergence in Turkey

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Abstract

Crimean-Congo haemorrhagic fever virus (CCHFV) is an emerging tick-borne zoonosis and a public health concern in Turkey since its first confirmation in 2002. The virus displays great genetic diversity and tends to expand its release to new areas. The presence of the biological tick vectors harbouring the virus and a suitable habitat are the predisposing factors for disease emergence in Turkey and elsewhere. As Turkey is one of the most endemic countries for CCHF disease, deliberate studies should be conducted to monitor all aspects of virus circulation and diversity in all endemic and non-endemic areas of the country. This will help to gain valuable information to predict the fate of the disease, and to develop effective vaccines and treatment facilities. Owing to the zoonotic nature of the virus, it offers a good prospect for collaboration of human and veterinary medicine with the view to fight the disease based on the one health initiative. This review was focussed on CCHFV diversity, perspectives of disease occurrence in Turkey, and the present and future implications of the disease.

Keywords: *Crimean Congo Haemorrhagic Fever Virus, Recombination, Reassortment, Zoonosis, Turkey*

Türkiye'de Kırım Kongo Kanamalı Ateşi Hastalığının Güncel ve Gelecekteki Etkileri

Öz

Kırım-Kongo kanamalı ateş virüsü (KKKAV), Türkiye'de 2002 yılında ilk kez tespit edilmesini takiben, halk sağlığı açısından oldukça önemli, kene kaynaklı bir zoonoz olmuştur. Etken oldukça geniş bir genetik çeşitliliğe sahip olup, gittikçe daha geniş alanlara yayılım eğilimi göstermektedir. Virüs için biyolojik kenenin varlığı ve uygun ortam şartları Türkiye'de hastalığın ortaya çıkmasında temel faktörleri oluşturmuştur. Türkiye, Kırım Kongo Kanamalı Ateş hastalığının görüldüğü en endemik ülkelerden biri olduğu için, virüs dolaşımının ve çeşitliliğinin endemik ve endemik olmayan bölgelerde ciddi araştırmalar ile sürekli izlenmesi büyük önem taşımaktadır. Bu çalışmalar hastalığın ve virüs dolaşımının gelecekteki durumunun değerlendirilmesi açısından gerekli olduğu kadar, hastalığa karşı etkili aşı ve tedavi seçeneklerinin geliştirilmesi yönlerinden de önemlidir. KKKAV, zoonotik bir etken olduğundan, insan ve veteriner tıp alanlarında Tek Sağlık yaklaşımına dayalı çalışmalar, hastalıkla savaş açısından mükemmel bir örnek oluşturmaktadır. Bu derlemede, Türkiye'de KKKAV çeşitliliği ve hastalığının ortaya çıkışı, güncel ve gelecek ile ilgili durumlar tartışılacaktır.

Anahtar sözcükler: *Kırım Kongo Hemorajik Ateş Virüsü, Rekombinasyon, Reassortment, Zoonoz, Türkiye*

INTRODUCTION

The enhancement of global trade and travel, increase in population density, environmental and climate changes are the predisposing factors for appearance of emerging diseases in the world. Many of these diseases have viral origin and display zoonotic potential and some of them have biological arthropod vectors and intermediate hosts. One of the greatest concerns about these diseases is their expansion potential to spread to different parts of the world from their place of origin [1].

Crimean-Congo haemorrhagic fever virus (CCHFV) has been classified as an emerging tick born zoonosis affecting many parts of the world. The causative agent of the disease is the RNA virus of genus Orthonairovirus, in the *Nairoviridae* family [2]. In humans, CCHFV is known to be extremely infectious and is associated with an acute haemorrhagic disease called Crimean Congo haemorrhagic fever (CCHF), with mortality rates as high as 30% [3,4]. Historically, the disease associated outbreak characterised acute febrile disease with a high incidence of bleeding and shock syndrome, which was first observed in Soviet soldiers

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during the summer of 1944, in Crimea [5]. A similar disease presentation occurred in 1956, in the Belgian Congo (Democratic Republic of Congo). In 1969, it was recognised that viruses associated with haemorrhagic syndromes in Crimea and Congo were identical and thus, the name Crimean-Congo haemorrhagic fever virus was nominated [6].

Ticks primarily belonging to the genus *Hyalomma*, play an imperative role in CCHFV survival and maintenance by acting as biological vectors. Human infection may occur either by tick bites or by contact with tissues or blood of the viremic individuals or animals in disease endemic areas. Additionally, it is thought that migratory birds and livestock trade between countries could play parts in disseminating the virus to new areas [3,4,7,8].

In comparison to other tick-borne viruses, CCHFV has been most frequently disseminated and disease cases have been documented in many countries in three continents (Africa, Asia, the Middle East and Eastern Europe) of the world [6,9]. CCHFV displays an important feature of expansion to new geographical areas, as evident by its recent emergence in Spain [10].

Disease occurrence coincides well with the presence of the tick vector *Hyalomma marginatum marginatum*. The presence of a vector tick is considered essential to establish endemic foci [3,4,11]. To date, majority of the cases have been reported in Turkey [12]. It is important to note that imported CCHF cases have also been reported in countries including France [13], Germany [14], United Kingdom [15] and Greece [16].

Owing to the lack of a prophylactic vaccine and some beneficial effect of antiviral ribavirin [17,18], an affective outbreak response depends on early confirmation of disease cases and suitable control response. In particular, the provincial health care institutions and reference laboratories play a very critical role [19]. Human infections are an extremely critical public health concern and possess the potential risk of causing nosocomial outbreaks; hence, all disease cases must be brought to the attention of public health authorities [20].

Since CCHFV infection is presented by a complex cycle that includes both human and several vertebrate host and tick vector, a collaborative action involving multiple disciplines, particularly human and veterinary medicine, based on the one health initiative is extremely important to combat the disease [21]. One health initiative to deal with this pathogen is extremely important, not only in Turkey, but also for all disease endemic and potentially endemic regions in the world.

GENETIC DIVERSITY OF CRIMEAN CONGO HAEMORRHAGIC FEVER VIRUS CIRCULATING IN THE WORLD

Crimean-Congo haemorrhagic fever virus is enveloped, spherical shaped and almost 90 nm in diameter. It harbours a single stranded and tree segmented RNA genome consisting of small (S), medium (M) and large (L) gene segments. The S segment encodes nucleocapsid protein (NP) with endonuclease activity [22-24]. The M segment encodes a glycoprotein precursor that undergoes post-translational cleavage to give rise to two structural glycoproteins (Gn- 37 kDa and Gc- 75 kDa) and three non-structural proteins (NS_M, mucin-like domain and GP38). It is important to note that M segment is the most variable, as compared to S and L segments [23-26]. Glycoproteins Gn and Gc are responsible for virus attachment to host cells and contain epitopes for eliciting neutralising antibody response [27-30]. The L segment encodes L protein displaying viral RNA-dependent RNA polymerase activity [4].

In comparison to other arboviruses, CCHFVs display a wide genetic diversity as evident in the phylogenetic analyses. The diversity of CCHFV is related to the recombination and reassortment events that inevitably occur in the segmented RNA genome [6,31-33]. Recombination events are suggested to occur between the S segments of local topotype viruses circulating in Turkey [34]. In addition to recombination event(s), reassortment events have been observed, primarily in the M segments. Phylogenetic studies based on M segment sequences differ from those based on S and L segment sequences, as reassortment often occurs by chances in the M segments of viruses [6,7]. Reassortment events associated with M segment may result in the generation of novel isolates with enhanced virulence. Thus, studies of M segment variations are of critical importance to evaluate viral virulence mechanisms attributed to respective isolates [28,30,33].

The high genetic diversity observed in the CCHFVs circulating in the world has led to the classification of viruses in genetic groups or genetic lineages. Phylo-genetic analysis of CCHFVs based on mostly partial and more limited number of whole gene segments of viruses have shown that the viruses are classified into seven genetic lineages or groups in association with geographical regions. These include Africa 3 (South Africa, Iran, Mauritania, Senegal) Africa 2 (South Africa, Democratic Republic of Congo, Uganda, Namibia), Africa 1 (South Africa, Namibia, United Arab Emirates, Senegal, Mauritania, Nigeria, Burkina, Faso), Asia 1 and Asia 2 (Iran, Pakistan, United Arab Emirates, Madagascar, Oman, Iraq, China, Uzbekistan, Tajikistan, Kazakhstan), Europe 1 (Turkey, Russia, Greece, Kosovo, Bulgaria, Albania, Iran), and Europe 2 (Greece, Turkey) [6,31,33,35,36]. In addition, it was reported that two isolates characterised by whole genome analysis in China were formed as an independent group with reference viruses in phylogenetic analysis [37].

Phylogenetic studies involving CCHFVs across the world suggest that the ancestor of all genetic lineages emerged approximately a few thousand years ago, probably in Africa [35,38]. It is thought that the virus first reached south and central Asia during the middle ages and then, spread

to China, India, and Russia. Viruses belonging to European I lineage reached south Russia from Astrakhan 280-400 years ago and in less than 150 years extended from Russia to Turkey and Balkans [38].

EMERGENCE OF CRIMEAN CONGO HAEMORRHAGIC FEVER DISEASE IN TURKEY

The emergence of CCHF disease was first recognised in the Tokat province in the Kelkit Valley in northern part of Turkey in 2002 [39,40]. Since 2002, CCHF disease reports have increased and expanded to occur in several provinces in Kelkit Valley, which has been the endemic region for the disease. Between 2002 and 2017, a total of 10.562 confirmed cases and 501 deaths were recorded by the Turkish Ministry of Health (http://www.thsk.gov.tr). In general, the mean mortality rate is around 5% in Turkey [18,41].

Since the first confirmation of the disease in 2002, the majority of cases have been reported from the north of middle Anatolia and Black Sea region, having similar climate and environment conditions, especially from the provinces of Tokat, Yozgat, Sivas, Gumushane, Artvin, Bayburt, Erzurum, Erzincan, Amasya, Cankiri, Corum, Kastamonu [12,40,42]. It is assumed that the emergence and increase in disease incidence are facilitated by multiple predisposing factors associated with disease endemic regions including climate change as well as anthropogenic factors such as changes in agricultural habits and increase in wildlife population. In particular, the climate change along with anthropogenic factors may have caused significant effect on reproduction rate of *Hyalomma* tick. This inevitably resulted in extensive amplification of virus through tick vertebra tick cycle (along with increase of wildlife population) [20,43-45]. Owing to security reasons, several activities including, agriculture, farming, and hunting were limited in the rural areas in Tokat and neighbouring provinces between 1995 and 2001. In particular, the restriction of hunting probably resulted in increased wild animal population such as wild boars, hares, and other animals that served as amplifying hosts for virus. After the re-opening of these areas for use, people and livestock such as cattle and sheep might have been exposed to a large number of *Hyalomma marginatum marginatum* ticks, resulting in disease emergence [12]. Importantly, a high landscape fragmentation and warm climate conditions are optimal habitats for the vector, *Hyalomma* ticks, and coincide well with the high disease risk areas in Turkey [43]. It is very likely that potential factors contribute to disease emergence in Turkey, and may also be attributed to emergence of disease in Crimea, Bulgaria, Albania, and Kosovo [9,43,46,47]. In the previous years, CCHF cases were usually reported between April and September, with the highest number of cases in July, in Turkey. Recently, the case report period has been extended from March to November [44].

Tick bites are considered to be the most common route of CCHFV infection in Turkey [9,12,41]. Approximately, two thirds of CCHF disease cases have been reported among farmers and home makers in disease endemic areas and these people were probably exposed to vector ticks during their daily life [44].

Despite the lack of the CCHF disease report before 2002, it was very likely that the virus had been circulating in enzootic vertebrate tick vertebrate cycle in nature. In particular, some studies indicated the detection of virus specific antibodies among individuals in some parts of country suggesting virus circulation prior to disease emergence [44]. A survey carried out by Bodur et al.[48] in 2009 indicated that seroprevalence of CCHFV infection was 10% in disease endemic area. Their survey also reported the increase of the seropositivity with older age and presence of subclinical infections. In another survey, seropevalence was found to be 12.8% in disease endemic areas [42]. Koksal et al.[49] reported that seroprevalence was 13.6 in relatives and close neighbours of CCHFV infected patients, as a result of possible exposure of the virus. Epidemiological studies are undoubtedly indicative of virus circulation and subclinical infections prior to disease emergence in Turkey.

It is known that migratory birds harbouring infected ticks could play a role in introducing the virus to new areas [8]. Anatolian peninsula is located on the Black Sea and Mediterranean flyway of migratory birds. The presence of CCHFV infected nymph on migratory birds was detected by a study carried out by Leblebicioglu et al.[50]. Phylogenetic analysis revealed that viruses carried by migratory birds are closely related to European-Russian viruses belonging to European lineage I. It may be assumed that the migratory birds from which infected ticks were recovered, such as the great reed warbler, have the potential to move to Russia, Turkey, Europa, Africa and North Africa and the European robin can migrate to Russia, Turkey, Europa, and North Africa. Thus, it is not unlikely that migratory birds carrying infected ticks may contribute to further outbreaks [12].

Livestock trade and/or movements may also lead to CCHFV introduction in countries, through infected ticks. CCHF disease is also common in a neighbouring country, Iran, but studies indicated that viruses belonging to different genetic lineages were circulating in Iran [34,51]. A study conducted to characterise CCHFVs obtained from ticks on small ruminants near Turkish border in Iran revealed that the viruses belonged to European lineage I and displayed phylogenetic similarity with viruses characterised from human cases in Turkey. This is suggestive of transborder CCHFV transmission between these countries [52].

Owing to the extremely infectious nature of the virus, human infections possess the potential risk of causing nosocomial outbreaks and transmission of the disease to health care professionals [20]. A majority of nosocomial infections have been acquired while dealing with CCHFV

patients [9,53]. Transmission of CCHFV to health care workers has been occured in Turkey, and some cases, resulted in the fatal outcome [12].

PHYLOGENETIC ANALYSIS OF CRIMEAN CONGO HAEMORRHAGIC FEVER VIRUSES CIRCULATING IN TURKEY

In Turkey, phylogenetic studies mostly based on partial S, M and L segment sequences of CCHFV isolates derived from infected individuals and ticks revealed that a majority of isolates belonged to the European lineage I, including viruses characterised in Eastern Europe and Balkan Peninsula [28,29,34,54-56]. Kalaycioglu et al.[54] and Kalaycioglu et al.[55] reported two studies on molecular characterisation of CCHFVs harvested from infected individuals in disease endemic areas, between 2009-2012, in Turkey. Their study confirmed that the circulating viruses belonged to European lineage I, including viruses characterised previously in Turkey. Importantly, Kalaycioglu's studies agreed with the circulation of closely related viruses called local topotype as suggested by Ozkaya et al.[34].

The existence of AP92-like viruses that was first isolated from *Rhicephalus bursa* ticks in 1975 in Greece and classified within European lineage II were also detected in Thrace region (European part) of Turkey [57,58]. In particular, Gargılı et al.[58] reported the co-circulation of strains belonging to European I and European II lineages among ticks in the European part of Turkey. A recent study based on surveying the tick-borne viruses in Turkey showed that AP92-like viruses were found to be circulating in areas spanning the south and eastern Anatolia regions [59]. Although this group of viruses was initially thought to be non-virulent for humans, some mild clinical cases associated with AP92-like viruses were reported in rural Balkanian (Thrace) part of Istanbul [57,60] and Corum province located in central Anatolia region in Turkey [34]. Importantly, an AP92-like viral RNA was detected in a case, resulting in death in 2015, in Iran [61]. This suggests that there may be possible virulence differences between AP92-like strains, resulting in serious disease conditions. This possibility needs to be further investigated by case-based surveillance studies using whole genome sequence analysis of respective isolates.

FUTURE PATTERNS OF CRIMEAN CONGO HAEMORRHAGIC FEVER DISEASE IN TURKEY

Molecular Insights for Possible Virus Introduction Belonging to Different Lineages

The circulation of viruses from different genetic lineages in the same regions suggests the potential of the viruses to spread through migratory birds and/or trade of farm animals between countries [8]. This situation is critically important in terms of the ability of viruses from different genetic groups to circulate in a region and to infect the ticks and provide a suitable environment for ressortment [6,33].

The reassortment events were reported between and south and west African isolates and between Asian and southern African isolates of CCHFVs. It is interesting to note that the reassortments between west African and southern African viruses were associated with the L segment, while reassortment events between southern African and Asian isolates were associated with the M segment of the RNA genome [33].

A study carried out by Deyde et al.[31] suggested potential reassortment events between Turkish (200310849) and Russian (Kashmanov and Drosdov) strains. In their study, the phylogenetic analysis of M segment sequences of these viruses displayed a close relationship and clustered in European I genetic lineage while Drosdov and Kashmanov strains formed closely associated groups in phylogenetic analysis based on the S and L segments. Another recent study conducted by Lukashev et al.[38] also suggested possible reassortment events between European lineage I viruses.

The M-segment coding for glycoproteins is essential for binding to host cell receptors and also harbours neutralizing epitopes. These features make the M segment associated genetic variations and especially reassortment events more critical. In particular, reassortment events related to the M segment may lead to generation of viruses with an increased virulence [28,32,33]. In this respect, the viruses being circulated in any part of the world and importantly in Turkey need to be constantly monitored and followed up by molecular analyses.

Due to the expansion tendency of CCHFVs, Turkey may not only be considered as a "donor" country for Europe [8] but also as a "recipient" of new virus isolates from neighbouring countries such as Eastern Europe and particularly from Iran [54,55]. It was determined that viruses belonging to Asia I lineage were common in Iran and its neighbouring country Iraq, and the circulation of Asia II lineage viruses was also detected [62,63]. In addition, the presence of viruses belonging to European lineage I, and European lineage II viruses as a new genetic group have also been detected in Iran [62,64,65]. Interestingly, phylogenetic analysis of CCHFVs obtained in ticks collected from small ruminants in Southwestern region of Iran showed that viruses belonged to European lineage I, which was similar to viruses characterised in Turkey. This highlights the possibility of virus introduction between neighbouring countries by livestock trade and/or movement [52].

Turkey is located on the migratory routes of birds and has borders with Balkan and Middle Eastern countries.

Therefore, it is not unlikely that viruses in different genetic lineages may participate in circulation. This could facilitate reassortment event(s) between CCHFVs and the generation of reassortant viruses. In order to investigate the presence of viruses with different genetic lineage and possible genetic variations between viruses circulating in Turkey, it is essential to carry out molecular analyses based on whole genome sequences of CCHFVs. Whole genome-based characterisation studies involving Turkish isolates will provide invaluable insights to define constant and variable segments of the genome. This would also contribute significantly to antivirals and vaccine development studies.

Crimean Congo Haemorrhagic Fever Outbreak Risks in Non-Endemic and Potentially Endemic Parts in Turkey

The expansion potential of the CCHFVs and their tendency to establish new niches is not only critical for the world, but also important for Turkey. A study carried out by Tuncer et al.[66] reported 33.1% CCHFV antibody prevalence in livestock, in parts of South Marmara region of Turkey. A tick survey carried out by Yesilbag et al.[67] also confirmed the existence of CCHFV circulation in the same region. Two CCHF disease cases were confirmed in Bursa and Canakkale provinces of South Marmara region in Turkey [68]. Importantly, an outbreak and human infections were reported in Aydın province located in Aegean region of Western Anatolia [69]. Seroprevalence studies using human sera obtained from volunteers indicated that seropositivity were 19.6 and 19.7 in potentially endemic and non-endemic parts of Aydın province, respectively [70,71]. The presence of CCHFV antibodies in livestock, wild animals, and occurrence of disease cases in non-endemic parts of the country highlight the widespread distribution potential of CCHFV. Importantly, this was indicative of the presence of potential endemic regions in addition to Kelkit Valley in Turkey. Thus, further research regarding detection of CCHFV circulation in ticks, wild animals and livestock is essential to define all disease potential areas in Turkey. Potential predisposing factors could initiate new outbreaks in non-endemic areas. Therefore, precautions and public awareness has to be taken to minimise future outbreaks in Turkey.

Public Health Concern of CCHF Disease During Eid-Al-Adha

One of the most important public health aspects of CCHFV is its potential to cause disease cases and outbreaks during the time of Eid-Al-Adha. Thousands of livestock are transferred, and many people are involved in sacrificing activity nearly in every province of Turkey. In fact, CCHF disease outbreaks have been reported during this religious time in Pakistan [72]. According to the early drift of 10-11 days every year, this period will lie during summer and spring months for the next 10-15 years, when the vector ticks are active and prevalent [12]. This will not only be an important health concern for people in endemic areas but also for people residing in non-endemic parts of the country. In particular, the transport of livestock will definitely lead to transfer of infected vector ticks and viremic animals from endemic areas [73]. This will enhance the risk of transmission of CCHFV to humans. Hence, veterinary control for animal movements and training of staff involving animal sacrificing procedures is essential and all necessary precautions are imperative to minimise the risk of virus transmission.

ONE HEALTH INITIATIVE TO COMBAT CCHF DISEASE

Since CCHFV possesses a zoonotic behaviour, it is an excellent subject to establish a one health initiative-based campaign, which requires multidisciplinary collaborative studies including human and veterinary medicine and other related disciplines. Because of the lack of the effective prophylactic vaccine and limited treatment facilities, it is essential to implement all possible protective measures to prevent and control future outbreaks [21]. Implementation of one health disease surveillance and interdisciplinary actions investigating circulation of CCHFVs in ticks, wild animals and livestock by well-designed molecular epidemiologic studies in both disease endemic and potentially endemic areas in Turkey will lead to quicker disease recognition, efficient outbreak response and disease control.

The detection of virus specific antibodies is an important mediator to detect the presence and circulation of virus, which should be combined with tick based studies to evaluate the risk of future outbreaks in any potential area. This is particularly important to map areas where outbreaks could occur in future and to alert public health systems. If the prevalence of CCHFV increases in ticks, in conjunction with virus specific antibody circulation in wild and domestic animals in any given area, cases of disease outbreak may occur [9,44,45]. These examples highlight the importance of veterinary medicine in the one health initiative, particularly in case of CCHFV disease.

CONCLUSION

Since CCHF disease has been an important public health priority in Turkey, the disease surveillance is a fundamental issue for public health actions to detect, prevent and respond to health threats effectively in time. Early diagnosis of disease and all preventive measures are essential to minimise disease related disorders. One health initiative is the most ideal way to deal with CCHF disease and its public health consequences.

The existence of high genetic diversity in CCHFV strains has resulted in the generation of different genetic lineages, distributed in various disease reported regions. The investigation of possible co-circulation of virus strains that belong to different lineages is a critical issue for

CCHFV research in Turkey. This issue can be addressed by whole genome sequence analysis of isolates derived from ticks and infected individuals. The whole viral genome characterisation studies including viruses detected in Turkey are also imperative to gain valuable results and essential for vaccine and antiviral development.

Turkey's considerable experiences and efforts to deal with this tick-borne zoonosis have been a beacon for other disease endemic countries and also to countries that are at risk of being endemic in the future.

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AUTHOR INDEX - 2019

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