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Detection of *Clostridium perfringens* and determination of enterotoxin genes (*cpa* and *cpe*) in traditional turkish chicken doner kebab

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Abstract: The demand for the fast-food industry in the world is increasing day by day. In this sense, chicken doner kebab becomes frequently preferred food source in daily life. At the same time, chicken doner kebab is both a good animal origin protein source and a cheaper option. Therefore, it is prepared and consumed in high amounts in Turkey. In this study, it was aimed to determine the presence of *Clostridium perfringens* and its toxin genes in traditional Turkish chicken doner kebabs purchased from restaurants and modified atmosphere packaged (MAP) samples collected from markets. For this purpose, 100 ready-to-cook and 100 ready-to-eat, totally 200 doner samples have been used as material. As a result, the prevalence of *C. perfringens* has been found 29%, 6% in ready-to-cook and ready-to-eat samples, respectively. The *cpa* gene was detected in all isolates. However, both *cpa* and *cpe* gene was found only in 4% of isolates.
Keywords: *C. perfringens*, *cpa*, *cpe*, doner kebab, ready-to-eat.

Geleneksel türk tavuk döner kebaplarında *Clostridium perfringens*'in tespiti ve enterotoksin genlerinin (*cpa* ve *cpe*) belirlenmesi

Özet: Dünyada fast-food beslenmeye olan talep her geçen gün artmaktadır. Bu anlamda tavuk döner kebap günlük hayatta sıklıkla tercih edilen bir besin kaynağı haline gelmektedir. Aynı zamanda tavuk döner kebap hem iyi bir hayvansal protein kaynağı hem de daha ekonomik bir seçenek olması nedeniyle Türkiye'de yüksek miktarlarda üretilmekte ve tüketilmektedir. Bu çalışmada, restoranlardan satın alınan geleneksel Türk tipi tavuk döner kebapları ve marketten toplanan modifiye atmosfer paketli (MAP) örneklerde *Clostridium* perfringens ve toksin genlerinin varlığının belirlenmesi amaçlanmıştır. Bu amaçla 100 adet pişirilmiş ve paketlenmiş ve 100 adet restoranlardan alınmış ve yenmeye hazır olmak üzere toplam 200 döner örneği materyal olarak kullanılmıştır. Sonuç olarak, pişirilmiş ve paketlenmiş örneklerde *C. perfringens* prevalansı %29 ve restoranlardan alınmış örneklerde ise %6 olarak bulunmuştur. Tüm izolatlarda *cpa* geni tespit edilmiş olmasına rağmen hem *cpa* hem de *cpe* geni izolatların sadece %4'ünde belirlenmiştir.
Anahtar sözcükler: *C. perfringens*, *cpa*, *cpe*, döner kebap, yemeye hazır.

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Introduction

Turkish chicken doner kebab (also called gyro, donair, yeeros, and shawarma) is the traditional and quite common meat product both in Turkey and in countries populated densely by Turks (20, 38). Because of a well-known gastronomic value of our country it is produced and consumed in high amounts in Anatolia (36). Doner kebab's history goes back to the 19th century in Bursa/Turkey. Because of industrialization, urbanization, and globalization, its consumption spreads out to world (2). This product is usually made from breast meat and fat of chicken. Before the chicken meat is impaled on a doner stick, it is marinated with salt, pepper, cumin, onions, tomato paste, and several spices (21). A cone or cylinder shape is given to chicken meat on doner stick before cooking on the fire (34).

In addition, besides its high digestibility, it is also an important protein source in terms of being animal-originated and it contains many nutrients which are necessary for human body such as essential amino acids, fatty acids, and high amounts of minerals (17).

Due to the vertical and surface cooking process, Turkish chicken doner kebab always contains raw materials inside. Because of that, the applied heat treatment penetrates only to a certain depth. Cutting doner meat slices too thick or insufficient cooking time also affects the microbiological quality of the product negatively. This characteristic cooking process may cause the risk of consuming undercooked products. Therefore, it has been reported that it seems probable to encounter food poisoning cases caused by foodborne pathogens such as *Clostridium perfringens*. This is an essential risk that needs attention in terms of public health (19).

In recent years, it has been reported that the food demand has increased rapidly due to the increasing population. In last five years, Organisation for Economic Co-operation and Development (OECD) has announced that chicken consumption has increased 6.88% in worldwide (29). For this reason, it has been determined that there is an increase in the incidence of foodborne outbreaks (10). It has been reported that chicken meat contains many foodborne pathogens depending on the slaughter process (14, 32). According to the Centers for Disease Control and Prevention (CDC), *C. perfringens* is the fifth pathogen that most frequently cause foodborne poisoning after norovirus, *Salmonella*, Shiga toxin-producing *E. coli* (STEC), and *Campylobacter* in the United States between 2009-2015 (3).

Clostridium perfringens is gram-positive, ubiquitous, anaerobic, not able to motile, rod shaped and subterminal heat resistant spore forming bacterium. Also *C. perfringens* metabolizes gelatin, reduces nitrates to nitrites, and generates black colonies by its ability of sulphite reduction (28). It has been reported that, the bacteria have seven toxigenic types from A to G and it can produce a lot of extracellular toxins such as alpha, beta, epsilon, iota, cpe, and netb according to type of it. The agent is responsible for intoxication-type foodborne poisoning due to toxin consumption and disease occurs when one or more toxins are taken with foods (22). *C. perfringens* type A, B, and F have been found responsible for foodborne intoxication in humans (35). And also *C. perfringens* type A has been determined to be the most frequently identified strain among the other seven types (9, 31). *C. perfringens* toxin-based typing scheme and responsible genes for toxin synthesis is shown in Table 1.

In consequence of consuming improperly cooked or stored foods *C. perfringens* related food poisonings may occur. Due to the consumption of clostridial bacterial toxins, intoxication symptoms such as watery diarrhea and abdominal pain can be observed in humans. It has been reported that symptoms usually disappear within 12-24 hours. Besides, vomiting and fever are not the typical symptoms that can be observed (28).

The objectives of the present study were to investigate the presence of the *C. perfringens* in both MAP and unpacked chicken doner kebab samples and to confirm the presence of cpa and cpe toxin genes by multiplex PCR.

Materials and Methods

Traditional Turkish Chicken Doner Kebab Samples: In this study, between October 2019 – March 2020, 100 ready-to-cook (modified atmosphere packaged) and 100 ready-to-eat, totally 200 traditional Turkish chicken doner kebabs which were collected from restaurants, supermarkets, and butcher shops in Samsun, Turkey were used as material. Both ready-to-cook and ready-to-eat samples were bought at least 350 – 500 grams and samples were transported into the laboratory under cold chain conditions (4°C) as soon as possible after purchased.

Table 1. *C. perfringens* toxin-based typing scheme and genes (shown in parenthesis) (31).

Toxin type	Toxins					
	Alpha (*plc* or *cpa*)	**Beta (*cpb*)**	**Epsilon (*etx*)**	**Iota (*iap* and *ibp*)**	**CPE (*cpe*)**	**NetB (*netB*)**
A	+	-	-	-	-	-
B	+	+	+	-	-	-
C	+	+	-	-	+/-	-
D	+	-	+	-	+/-	-
E	+	-	-	+	+/-	-
F	+	-	-	-	+	-
G	+	-	-	-	-	+

The Isolation and Identification of Clostridium perfringens: Culture based isolation technique was used for isolation and identification. For this purpose, all samples were weighed 10 g into sterile jars under aseptic conditions and diluted with 90 ml Perfingens Enrichment Medium (PEM: Fluid Thioglycolate Medium + Perfringens (TSC) Supplement Oxoid SR 88E). Then, for generation of anaerobic conditions samples were covered with sterile paraffin and incubated at 46°C 20 h (Sanyo, MCO. 18 AIC). After this enrichment, a loopful sample was taken from turbidity and gas production positive enrichment jars and streaked on to Tryptose Sulphite Cycloserine (TSC) agar (Oxoid CM 587). Later, plates were incubated at same anaerobic conditions above. Typical colonies like 2 – 4 mm in diameter and black colored in TSC agar were qualified as suspected. Three or 5 suspected colony were selected and streaked on to TSC agar for biochemical identification test. According to biochemical tests, gram, reverse CAMP, lactose, gelatin, nitrate positive, catalase and motility negative colonies were identified as *C. perfringens* (1). For confirmation, PCR test was performed.

Confirmation of Clostridium perfringens

DNA Extraction: For the aim of procuring template DNA from *C. perfringens* isolates, GENESpin DNA Isolation Kit (eurofins, genescan 5224400605) was used in this study. For this purpose, isolates which were stored in – 20°C were revived in PEM anaerobically for 24 h at 37°C. Fresh cultures were used for DNA extraction process as mentioned in the manual of kit.

Primers Used for Detection: For this purpose, the presence of *cpa* and *cpe* genes were investigated in biochemically identified isolates. As recommended by Meer and Songer (26) *cpa* (400 bp) (5'- GCTAAT GTTACTGCCGTTGA 3' / 5'-CCTCTGATACATCGT GTAAG 3') and Mahamat Abdelrahim, Radomski (20) *cpe* (178 bp) (5'- ATAGATAAAGGAGATGGTTGGA 3' / 5'- CCATATTCTACAGATGCTTGTA 3') primer pairs were used for confirmation and enterotoxin characterization.

Multiplex PCR Conditions: *Clostridium perfringens* NCTC 8239 was applied as positive control in our study because of harboring both *cpa* and *cpe* genes.

The amplification was performed in thermal cycler (Bio-Rad MJ Mini) and conditions were initial denaturation 95 °C for 10 min, followed by 45 cycles 95 °C for 10 sec, annealing at 55 °C for 10 sec, extension at 72 °C for 20 sec, and for cooling step 40 °C for 30 sec. Electrophoresis of amplicons were separated at 2% agarose gel at 80 volts (Bio-Rad Power Pac-Basic & Bio-Rad electrophoresis tank). The PCR products were visualized under UV light (Wise-UV-Wuv-L50, Korea).

Results

In this study, a total of 200 chicken doner kebab samples, 100 ready-to-cook (MAP) and 100 ready-to-eat were analyzed. As a result, the prevalence of *C. perfringens* was 29%, 6% in ready-to-cook and ready-to-eat samples, respectively. We totally obtained 50 *C. perfringens* isolates, 12 from ready-to-cook and 38 from ready-to-eat samples (Table 2). The *cpa* gene was detected by PCR in all 50 isolates (100%). However, *cpe* gene was detected only in 2/50 isolates (4%) which were only ready-to-cook sample originated (Figure 1).

Table 2. The Prevalence and Toxin Gene Presence of *C. perfringens* in Samples.

Chicken Doner Kebab	Sample (n)	*C. perfringens* positive samples (%)	*C. perfringens* isolates	*cpa* positive isolates	*cpe* positive isolates
Ready-to-cook	100	29 (29%)	12	12	2 (5.2%)
Ready-to-eat	100	6 (6%)	38	38	-
Total	**200**	**35 (17.5%)**	**50**	**50**	**2 (4%)**

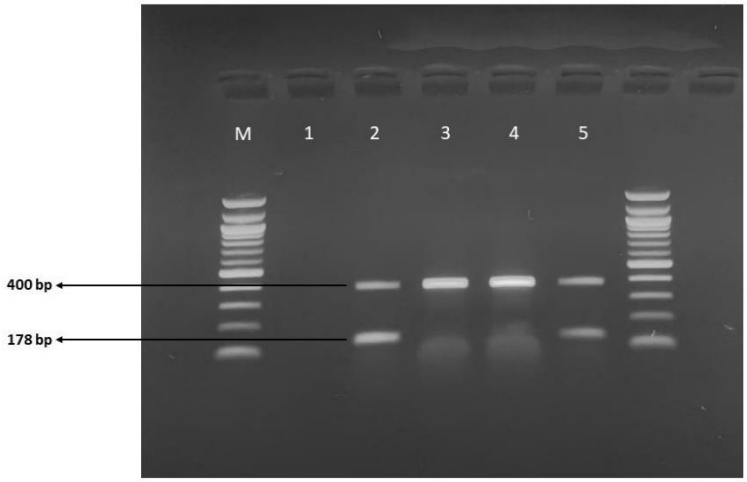

Figure 1. M: 100 bp DNA Marker, 1 distillated water, 2 *Clostridium perfringens* NCTC 8239, 3-4 *cpa* gene positive isolates, 5 *cpa* and *cpe* genes positive isolates.

Discussion and Conclusion

In our study, we investigated the traditional Turkish chicken doner kebab, which is immensely popular street food in our country. Our results indicate that 17.5% of our samples were contaminated with *C. perfringens*. This contamination level was 29%, and 6% in ready-to-cook (MAP) and ready-to-eat, respectively. The higher contamination level in modified atmosphere packed samples was attributed to the fact that the agent has anaerobic metabolism.

Similarly, in several studies, the presence of *C. perfringens* was investigated in chicken doner samples. Kayisoglu et al. (18), Elmali et al. (5), Katsurayama et al. (16), and Vazgecer et al. (37) isolated *C. perfringens* in traditional Turkish chicken doner kebab 60%, 32%, 12%, and 7%, respectively. The differences between the outcomes of the studies were thought to result from the regional discrepancy, different cooking procedures, microbiological quality, and the number of food samples taken. Moreover, the difference between results can be attributed to the formula, quality, and possible antibacterial effect of spices which were used during marination of doner. However, the number of studies in which MAP samples were studied is quite limited. This situation also contributes to the originality of our study.

Contrary to this, Haskaraca and Kolsarici (12), Lopašovský et al. (24), Öksüztepe and Beyazgül (30), Bostan et al. (2), Hampikyan et al. (11), Küpeli Gençer and Kaya (23) were used chicken doner kebab as material and the researchers reported that *C. perfringens* was found below the detection limit in their studies. These results can be linked to the cleaning and disinfection of equipment and hygienic conditions of food establishments as well.

In the present study, all our isolates were confirmed by mPCR due to housing the *cpa* gene. It has been reported that all toxigenic types of *C. perfringens* contain the *cpa* gene as well (31). In addition to this, *C. perfringens* type A is the predominant type in most of investigations and also it is the first toxigenic type in food intoxications related to *C. perfringens* in the US, Europe, and Japan (27). In parallel to our results, in Finland, Heikinheimo and Korkeala (13) detected *cpa* gene in all isolates as well. However, none of their isolates possessed *cpe* gene. Correlatively, Jang et al. (15) investigated *C. perfringens* in meat products and they got 33 isolates from chicken meat and *cpa* gene was also found in all isolates. In another study, Gholamiandehkordi et al. (8) analyzed *C. perfringens* in broilers in Belgium and they identified *C. perfringens* type A in all 71 isolates due to presence of *cpa* gene.

C. perfringens enterotoxin (CPE) is critical for human food intoxication and CPE is encoded by *cpe* gene as well (31). In our study, the *cpe* gene was detected only 2/50 (4%) of our isolates. In many studies, parallel to our results, *cpe* was rarely detected. Our results are also in agreement with Zhang et al. (40) as they detected *cpe* gene 3% as well. In India, Dar et al. (4) isolated 51 *C. perfringens* isolates from 184 chicken samples and all the isolates were identified as *C. perfringens* type A by a multiplex PCR. Accordingly, none of them carried *cpe* gene. Egyptian scientists have reported several data recently. In one of their investigation, the *cpe* gene was also carried by 1/10 (10%) of chicken isolates by genotyping analysis. However, all isolates contained *cpa* gene and were identified as *C. perfringens* type A (7). Also in another study, Shaltout et al. (33) reported that *C. perfringens* type A was detected 8/27 (29.6%) but *cpe* gene was not appointed by multiplex PCR assay. In our country, *cpa* gene was detected in all 22 isolates from turkey meat, according to Erol et al. (6). Nevertheless, *cpe* gene was not determined in their study.

Contrary to this, in Bursa-Turkey Yibar et al. (39) detected *cpe* gene 7/22 (31.8%) higher than our results in their investigation. This difference may be related to micro floral variation and microbiological quality of samples.

In conclusion, *C. perfringens* is an important food-borne pathogen which contains wide spectrum of toxins. In our study, it was isolated from ready-to-eat and ready-to-cooked (MAP) samples. The detection of *C. perfringens* in ready-to-eat foods has been the most important and critical outcome of our investigation in terms of public health.

Based on all results of our investigation, we would like to emphasize that precautionary warnings such as good hygiene and good manufacturing practice are dramatically essential for public health. Also, we highly recommend effective disinfection of equipment in the phase of preparation of foods. Further investigations are needed for the existence of *C. perfringens* in traditional Turkish chicken doner kebab samples. In addition, the detection of an important food pathogen such as *C. perfringens* in ready-to-eat foods is examined as a critical situation that should be remarkably underlined in terms of public health.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of interest

The authors declared that there is no conflict of interest.

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Determination of goat milk cost and assessment of factors affecting the profitability of Saanen goat enterprises in Çanakkale province, Turkey

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Abstract: The aim of this study is to determine the cost of goat milk in Saanen goat enterprises in Çanakkale province and to examine the factors affecting profitability in enterprises. The material of the study consists of 92 Saanen goat enterprises. The enterprises have been grouped according to their animal density; as 25-75 heads (small-scale), 76-150 heads (medium-scale) and 151 heads and above (large-scale). The findings of the enterprises for 2017 were determined by descriptive statistics, and multiple regression analysis was used to determine the factors affecting the total profitability of the enterprises. Since feed costs represented 46.22% of total production cost, it was followed by labour costs (27.19%), fuel costs (5.44%) and veterinarian-health service costs (5.19%). The cost of 1 L milk and absolute profit in enterprises was determined as 0.42/0.01 $/kg in small-scale, 0.41/0.02 $/kg in medium-scale and 0.38/0.05 $/kg in large-scale enterprises, respectively. It was observed that the share of inputs in enterprises generally decreases as the scale of the enterprise increases and large-scale enterprises were found to be more successful in solving technical, health and economic problems.

Keywords: Cost of milk, dairy goat, economic analysis, profitability, Saanen goat.

Çanakkale ili Saanen keçi işletmelerinde keçi sütü maliyetinin saptanması ve işletmelerde kârlılığa etki eden faktörlerin değerlendirilmesi

Özet: Bu çalışmanın amacı; Çanakkale il ve ilçelerindeki Saanen keçisi işletmelerindeki keçi sütü maliyetinin hesaplanması ve işletmelerde kârlılığa etki eden faktörlerin incelenmesidir. Çalışmanın materyalini 92 adet Saanen keçisi işletmesi oluşturmaktadır. İşletmeler sahip oldukları hayvan yoğunluğuna göre; 25-75 baş (küçük), 76-150 baş (orta) ve 151 baş ve üzeri (büyük) işletmeler olarak gruplandırılmıştır. İşletmelerin 2017 yılına ait bulguları, ortalama, yüzde, dağılım gibi tanımlayıcı istatistikler ile belirlenmiş, işletme toplam kârlılığında etkili faktörlerin saptanması amacıyla ise çoklu regresyon analizi kullanılmıştır. İşletmelerde maliyeti oluşturan unsurların %46,22'sini yem masraflarının, bunu sırasıyla işçilik (%27,19), akaryakıt (%5,44) ve veteriner-sağlık hizmet masraflarının (%5,19) oluşturduğu belirlenmiştir. İşletmelerde 1 L sütün üretim maliyeti ve mutlak kâr sırasıyla küçük ölçekli işletmelerde 0,42/0,01 $/kg, orta ölçekli işletmelerde 0,41/0,02 $/kg ve büyük ölçekli işletmelerde 0,38/0,05$/kg olarak saptanmıştır. İşletmelerde girdilerin payının genelde işletme ölçeği büyüdükçe düştüğü, teknik, sağlık ve ekonomik sorunların çözümünde ve kârlılık oranlarında büyük ölçekli işletmelerin başarılı olduğu saptanmıştır.

Anahtar sözcükler: Ekonomik analiz, kârlılık, Saanen keçisi, süt keçisi, süt maliyeti.

Introduction

Goats are animals that can make good use of low quality pasture and scrubland and turn it into meat, milk and other products (17). It is usually known as a traditional livestock breeding activity that forms the livelihood and essential food source of low-income families in rural areas (22). Animal products produced from goat breeding also contribute significantly to the national economy in terms of adequate and balanced nutrition of the growing population, supply of raw materials for industry and the revenues provided through exports (27). Goat milk production in Turkey is derived from the vast majority of hair goats. While 98.30% of 5 471 086 head goats milked in 2019 were hair goats, 99.40% of 577 209 tons of milk produced was obtained from hair goats (30).

In recent years in the Western Anatolian region, the activities of intensive enterprises, which mainly produce cheese and provide milk to dairies, are becoming

widespread. The breed used in these enterprises is generally high Saanen hybrids in terms of milk yield (22). Saanen goat breeds compliance with certain regions of Turkey has been provided and a very good and high milk yield has been achieved (16). Çanakkale is the province where Saanen goat breeding is intensively carried out and the number of animals is higher. In fact, 25.51% of the total of 852 871 head of Saanen goats in Turkey is located in Çanakkale province (5).

Dairy goat breeding activities are mostly based on small-scale and scattered family enterprises in Turkey (23). The utilization rate of advanced technology and qualified workforce is usually higher in large-scale enterprises. It is supported with many studies that enterprises work more profitably and cost-effectively with the increase in the scale (1, 15, 28). The technical and economic analysis of dairy goat enterprises have been handled within the scope of different provinces, and it is seen that these studies mainly focus on the hair or mohair goat enterprises (4, 6, 7, 11, 13, 28). Although studies analyzing the cost of unit goat milk and profitability in enterprises are limited, studies in this area are important in terms of determining appropriate enterprise scales and ensuring sustainability in the sector.

In this context, the aim of this study is to calculate the unit cost of goat milk in Saanen goat enterprises in Çanakkale province and districts and to examine the factors affecting profitability based on the enterprise scales.

Materials and Methods

Study area, selected enterprises and questionnaire structure: The primary data was obtained from Saanen goat enterprises which were registered in the system of Ministry of Agriculture and Forestry and members of Sheep and Goat Breeders' Association of Çanakkale province by using a questionnaire.

Çanakkale province is located in the west and coast side of Turkey (Figure 1) and is generally characterized by "dry-summer subtropical" climate that referred to as "Mediterranean". The mean annual temperature of Çanakkale is between 3° and 10 °C. Summers are dry and hot with mean 18° and 24°C. The average yearly rainfall is 618 mm (26, 29).

The owners who want to benefit from the animal support system must have 25 or more animals. For this reason, enterprises with 25 heads or more goats were included in the sampling in terms of both a good evaluation of production records and reliable data supply. In determining the sample, the average of each stratum and variance weights were taken into account using the simple random sampling method, and a single sample volume was determined for all strata (25).

Because the size of the population is known;

$$n = \frac{N.\sigma^2.Z\alpha}{(N-1).d^2}$$

N = 801 enterprises were included in the formula, and n = 87 enterprises were determined as a sample in the range of 1.55 standard deviations.

The distribution of enterprises in the sample by districts and scales was given in Table 1. The sample size consisted of 92 enterprises including back-up enterprises obtained by simple random sampling method. The enterprises were grouped as 25-75 heads (small-scale), 76-150 heads (medium-scale) and 151 and overheads (large-scale) according to their animal density. To provide the assumptions of statistical analysis from each scale in enterprises, at least 20 and more enterprises were included in the sample. The fieldwork was carried out in 2017-2018, covering the data of the year 2017.

Table 1. Distribution of enterprises in the sample by districts and scales.

District	Small	Medium	Large	Total
Ayvacık	1	2	2	5
Bayramiç	8	17	9	34
Biga	2	2	1	5
Çan	1	-	-	1
Ezine	3	6	7	16
Lapseki	2	3	3	8
Merkez	3	12	8	23
TOTAL	20	42	30	92

Figure 1. Display of study area.

Saanen goat enterprise owners were interviewed face-to-face, and a data supply form was applied to all 92 Saanen goat enterprises. In order to determine the data that will constitute the economic analysis, the amount and price information of the cost factors that constitute the unit cost of the milk, as well as questions regarding income factors, amount of milk produced and the sales price were included in data supply form.

Calculation of unit milk cost and profit: Unit milk cost was calculated according to the combined cost calculation method. For this, firstly, the costs per milk were determined in total production. The share of milk in the gross production value (main product + by-product value) was taken into account. Milk incentive premium and supports (supports for breeding goats between 15-90 months of age and supports comes from the National Sheep & Goat Breeding Project) were deducted from the production cost per milk, the remaining value was divided by the total amount of milk obtained at the end of a production period, and the cost of unit milk (with considering supports) was calculated (15). Absolute profit was obtained by subtracting the unit cost from the milk sales price, and proportional profit was obtained by dividing the absolute profit by the milk sales price (15).

Statistical analysis: SAS / STAT (Inst.Inc., Cary, NC), XLSTAT (Addinsoft, New York, NY) and SPSS Inc PASW Statistics 18 package programs were used in the analysis of the data. Descriptive analysis consisting of frequency, percentage and average was applied, and the correlations between the variables were examined. Whether the difference between group averages is significant was determined by using Variance Analysis (ANOVA). In hypotheses, independent variables were formed by scale groups, while dependent variables consisted of 1 L milk cost, milk yield per animal, producer milk sales price, total milk production, absolute profit, proportional profit, the income of milk sales and duration of the lactation period.

Regression Analysis: Multiple regression analyzes were conducted in order to analyze the input-output relationships of the enterprises for the production period in 2017. The formula where x_i indicates independent variables and Y indicates dependent variable was set up as follows:

$$\mathbf{Y_i = a_0 + a_1x_{i2} + a_2x_{i2} + \ldots + a_kx_{ik} + e_{ij}}$$

i = 1,2, …n j= 1,2…k

In formula;

Y_i: The observed i-th value of the dependent variable

X_{ij}: The value of the j-th independent variable at level i

a_j: j-th regression coefficient

e_{ij}: Error term

k: It refers to the number of independent variables.

The backward method was applied in the regression model. In the first stage, all variables were included. The process has been continued discarding the independent variable with the lowest partial F value in the following steps (20).

One of the most important problems encountered in the multiple regression model is whether there is a strong relationship (multiple correlations) between the independent variables included in the model (3). A correlation of 0.80 and above between the independent variables was considered as an indication of multiple correlations between variables, and this is undesirable (19).

Durbin Watson value, which explains whether there is an autocorrelation between terms in the model, is expected to be between 1.5-2.5 coefficients (18). However, VIF values indicating whether there are multiple correlations in the model are expected to be below 10 (9). Therefore, these points have been taken into account while including variables.

The model where the scales were considered as a dummy variable is as follows:

$$\mathbf{Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + a_6x_6 + a_7x_7 + a_8x_8 + a_9x_9 + a_{10}x_{10} + a_{11}x_{11}}$$

The dependent variable (Y) expresses the total profit obtained from milk sales income in US dollars.

Independent variables in the model was determined as feed costs (x_1), labour costs (x_2), veterinarian and health services costs (x_3), electricity-water costs (x_4), fuel costs (x_5), building-equipment maintenance and amortization costs (x_6), other costs such as credit interests, insurance costs, general administrative costs (x_7), 1 Lt milk cost (x_8), producer milk sales price (x_9) and small & medium scale (x_{10}), medium & large scale (x_{11}) as a dummy variable.

Results

Table 2 shows that the feed costs were the main cost factor in the enterprises with a ratio of 46.22%. Labour costs, fuel costs and veterinarian and health services costs were followed by respectively, with the rate of 27.19%, 5.44% and 5.19%.

Table 3 shows that the main component of the sub-income factors across enterprises was the increase in inventory value with the ratio of 51.53%. Goat kids sales, other incomes and cheese sales were followed by respectively, with the rate of 20.29%, 9.91% and 9.60%.

Table 4 presents the distribution of the values related to producer milk sales prices, 1 Lt milk cost and profit/loss in enterprises by scales. Producer milk sales price was found to be 0.44 $/kg while 1 Lt milk cost as 0.41 $/ kg. While milk sales income was 15 495 $ on average absolute profit was 0.03 $ / kg, and proportional profit was 6.68%. It was observed that the cost of production of 1 L of milk decreases as the enterprise scales increase. In contrast, enterprise profitability was found to be directly proportional to the enterprise scales (Figure 2).

The results of some milk production and yield values were evaluated based on the variance analysis (ANOVA). According to the results of the test, the difference between total milk production, milk sales income and lactation times in the enterprises was found significant in terms of scales ($P < 0.05$) (Table 4).

Table 2. Distribution of cost factors in enterprises by scales.

	Enterprise Scales			
	Small	**Medium**	**Large**	**Total**
Cost Factors	**%**	**%**	**%**	**%**
Feed Costs	39.44	46.96	49.73	46.22
Veterinarian and Health Services Costs	4.62	4.93	5.95	5.19
Labour Costs	33.86	28.19	20.98	27.19
Electricity, Water Costs	1.08	1.06	1.00	1.04
Fuel Costs	6.75	5.04	5.26	5.44
Insurance Costs	0.27	0.52	1.71	0.85
Credit Interests	0.99	0.92	1.93	1.26
Inventory Value Loss	1.67	0.41	0.00	0.55
Building Equipment Depreciation Costs	2.90	3.23	3.80	3.33
Building Equipment Maintenance and Repair Costs	3.47	2.71	2.58	2.82
Live Fixture Depreciations	2.25	3.38	4.46	3.48
General Administrative Costs	2.66	2.64	2.59	2.63
TOTAL	100	100	100	100

Table 3. Distribution of sub-income factors in enterprises by scales.

	Enterprise Scales			
	Small	**Medium**	**Large**	**Total**
Sub-Income Factors	**%**	**%**	**%**	**%**
Goat Kids Sales	25.46	18.47	19.40	20.29
Fertilizer Sale	2.13	0.55	0.93	1.02
Inventory Value Increase	45.15	52.79	54.01	51.53
Breeding Animal Sales	9.65	5.67	9.13	7.66
Other Incomes	6.95	11.03	10.31	9.91
Cheese Sales	10.66	11.51	6.22	9.60
TOTAL	100	100	100	100

Table 4. Distribution of average data and statistical comparison on some variables between enterprise scales.

	Enterprise Scales					
Variables	**Small**	**Medium**	**Large**	***Total***	***F***	***P value***
1 L Milk Cost ($/kg)	0.42	0.41	0.38	0.41	0.244	*0.784*
Milk Yield per Animal (kg/day)**	2.10	2.07	1.70	1.95	1.495	*0.230*
Producer Milk Sales Price ($/kg)**	0.43	0.44	0.44	0.44	0.275	*0.760*
Total Milk Production (kg/year)**	15 565.00[a]	29 392.85[a]	56 050.00[b]	35 079	22.164	*0.000**
Absolute Profit ($/kg)	0.01	0.02	0.05	0.03	0.259	0.772
Proportional Profit (%)	3.30	5.04	11.91	6.68	0.258	0.773
Income of Milk Sales ($)**	6 833[a]	13 037[a]	24 963[b]	15 577	20.109	*0.000**
Duration of Lactation (day/year)**	210.75[a]	228.69[ab]	239.00[b]	228.15	3.820	*0.026**

*1$= 3.65 TL

**Reference: Gökdai A, Sakarya E (2020): Socio-economic structure and current problems of Saanen goat farms in Çanakkale province. Eurasian J Vet Sci, 36, 72-79.

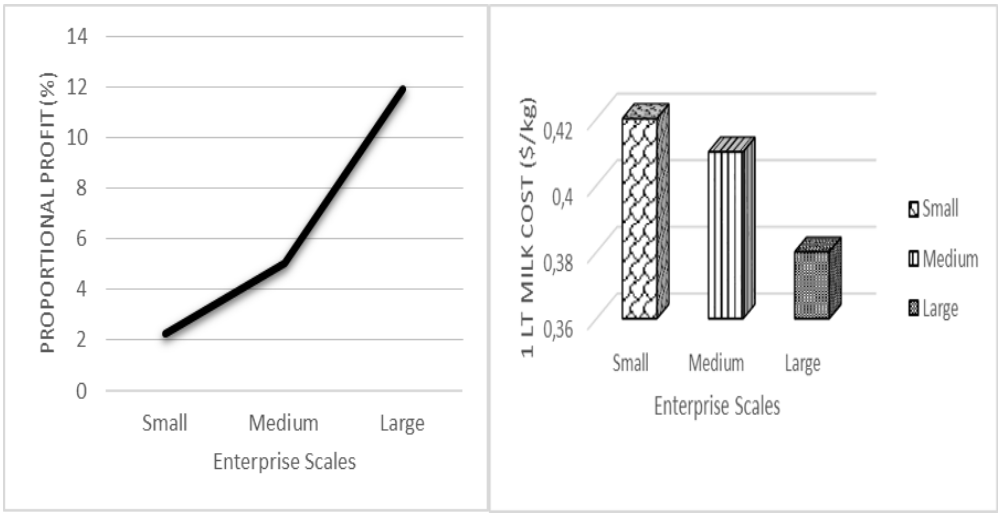

Figure 2. Milk production cost and profitability rates in enterprises.

Table 5. Coefficient of determination (R^2) regarding the last model created, Durbin-Watson value.

Model Summary[i]					
Model	**R**	**R^2**	**Adjusted R^2**	**Standard Error Estimation**	**Durbin-Watson**
Last	0.798[a]	0.636	0.615	34.505	1.739

a. (Constant), Other Costs, Producer Milk Sales Price, Fuel, 1 Lt Milk Cost, Feed Cost
b. Dependent Variable: Total Profit

Table 6. Coefficients in the last regression model.

Parameters	**Multiple Linear Regression Model**			**Collinearity Statistics**		
	B (Standardized)	**Standard Error**	**t Value**	**Sig.**	**Tolerance**	**VIF**
Constant	-	60.491	-1.255	0.213	-	-
Unit Cost	-0.626	4.677	-9.451	0.000	0.965	1.036
Sales Price	0.184	37.259	2.802	0.006	0.977	1.024
Feed Costs	0.208	0.072	2.652	0.010	0.684	1.462
Fuel Costs	0.204	0.815	2.970	0.004	0.900	1.111
Other Costs	0.223	0.341	2.957	0.004	0.742	1.348

Result of Regression Analysis: In regression model it was determined that there was no coefficient of 0.80 or more among the independent variables, and all the independent variables were included in the model**.** Durbin Watson value, which reveals whether there is any autocorrelation among the terms in the model was 1.739 within the 1.5-2.5 coefficients, which was the expected value range.

Table 5 shows that coefficient of determination (R^2) and adjusted R^2 values. Considering the adjusted R^2 value, 61.50% of the changes that may occur in the dependent variable were explained by the independent variables included in the model and 38.50% by the variables not included in the model.

When Table 6 is examined; unit cost, sales price, feed costs, fuel costs and other costs were found statistically significant (P <0.05). VIF values indicating whether there are multiple correlations in the model were expected to be below 10. The fact that all the VIF values in our model were below this value gives the conclusion that there were no multiple correlations.

The formula obtained with the dependent variable Y = Total Profit for the last model was given below.

$$\mathbf{Y= -0.626X_8 + 0.184X_9 +0.208X_1 + 0.204X_5+ 0.223X_7 + \varepsilon}$$

In total profit in enterprises, unit cost (X_8), sales price (X_9), feed costs (X_1), fuel costs (X_5) and other costs (X_7) was found to be statistically significant. If the equation is to be interpreted; providing the other expense items in the enterprises and the factors included in the analysis remain constant, when each independent variable is increased by 0.27 $, respectively, in the total profit; "Feed costs" (X_1) cause an increase of 0.06 $, "fuel costs" ($X_5$) cause an increase of 0.05 $, "other costs" ($X_7$) cause an increase of

0.06 $. It is understood from the equation that when the "unit cost" (X_8) included in the independent variables increases by 0.27 $, there will be a decrease of 0.17 $ in total profit, and when the "sales price" (X_9) increases by 0.27 $, there will be an increase of 0.05 $ in total profit.

Discussion and Conclusion

The cost factors, which have an important share in enterprises, differ in terms of scales. Feed costs with a ratio of 46.22% rank first among the cost elements that constitute the cost factors in enterprises. This was followed by 27.19% labour costs, 5.44% fuel costs and 5.19% veterinarian and health services costs, respectively. In a study conducted in goat enterprises with different scales in Hungary, they found that the most significant share among the cost factors that make up the cost in the enterprises were feed costs in the first place and labour cost in the second place (24).

In a study conducted in sheep enterprises in Muş province, it was revealed that as the scale increases in the enterprises, the unit costs decrease, sales income and profit increase. Similarly to our study, it was reported that if the enterprise scales increase, they can take economic advantage of it (21).

The unit cost of 1 Lt milk was 0.42 $ / kg in small-scale enterprises, 0.41 $ / kg in medium-scale enterprises and 0.38 $ / kg in large-scale enterprises. It was observed that the cost per unit decreases as the scale increases in the enterprises. Along with the increase in enterprise scales, specialization in the product and the use of technology makes it possible to benefit from economies of scale (12).

However, as the enterprise scale increases, the general administrative costs per unit decrease, as in our study; this causes minimization of unit costs and provides more economical management, decision and control tools. As the scale of enterprises increases, the amount of input that the enterprises purchase increases, which leads to an increase in its power in output marketing. Thus, an entity can make discount agreements at input prices, such as purchasing raw materials. This situation causes enterprises to become more economical in terms of unit cost (14).

It was observed that both absolute profit and proportional profit increase as enterprise scales increase in enterprises. These findings support large-scale enterprises with lower milk costs per unit. In a study conducted in goat enterprises in Pakistan's Kohistan region, it was found that the net present value is 29.65% in small-scale enterprises and 46% in large-scale enterprises (1). In a study conducted in dairy goat enterprises in Balıkesir, Çanakkale and İzmir provinces, absolute profit was calculated as 0.24 TL (0.05 $) / kg in small-scale enterprises, 0.57 TL (0.12 $) / kg in medium-scale enterprises and 0.79 TL (0.16 $) / kg in large-scale enterprises. Proportional profit was 18.18% in small-scale enterprises, 46.34% in medium-scale enterprises and 58.95% in large-scale enterprises (15). The increase in absolute profit and proportional profit ratio from small-scale enterprises to large-scale enterprises was similar to the results of our study.

When some milk production values and profitability rates in the enterprises are evaluated by scales; it was determined that the unit cost and daily milk yield per animal decreases as the scale increases. Total milk production and consequently, milk sales income increases as the scale increases, and this difference between the scales was statistically significant ($P < 0.05$). It was understood from the results that the absolute and proportional profitability was directly proportional to the scale of the enterprise.

In conclusion, feed costs were in the first place with the ratio of 46.22% in the distribution of the cost factors. As in other livestock sub-sectors, it is necessary to produce high quality and abundant roughage required for the minimization of feed costs, together with the solution of meadow-pasture shortage problem. On the other hand, as the enterprise scales increase, a decrease in the cost of 1 Lt of milk and an increase in absolute and proportional profits are remarkable. This reveals that large-scale enterprises are more advantageous in terms of profitability.

Besides, there are also some studies indicating that if the necessary technical information and equipment of the increased scale enterprises are not provided, and the enterprise works above its optimal capacity, some management activities can cause mistakes and the administration becomes more difficult; therefore the production and efficiency characteristics of the enterprises decrease (8, 10, 16). Here the important point is what the economic size should be in the classification of the enterprises, and it is an issue that needs to be discussed.

In the regression analysis, it was revealed that the effect of unit cost, sales price, feed costs, fuel costs and other cost factors among the factors affecting the total profit was statistically significant. Similar to our study, in a study examining the profitability of dairy plants by using regression analysis, it was also seen that price of raw milk and dairy products were among the factors that affect the profitability of the enterprise (2). In our study it was observed that feed, fuel and other costs increased by one unit, had a positive effect on the profitability of the enterprise contrary way to the expected. Considering that the data used is the primary data, it is necessary to manage cost factors in the most rational way by the enterprise, taking into account the shares of these independent variables in the total cost elements. In this way, in case the unit cost is reduced, its positive effect on the profitability of the enterprise can be seen.

However, it was inevitable that increasing the unit cost minimization and milk sales price and changing it to a stable structure will have a positive effect on the total profit of the enterprises. Based on this, if we take into account that enterprise owners do not have a right in determining the milk selling price by themselves, it is possible to say that providing unit cost minimization considering also the scale of the enterprise and precautions to be taken on milk price will have a positive effect on the profitability.

In order to increase goat milk production, some structural changes in dairy goat sector are needed in the medium and long term. Small scale, scattered and unorganized goat enterprises in the region, cause a problem in input supply and marketing, which lead to an increase in production costs. As a result, to improve productivity and increase efficiency and profitability, a model should be developed for the growth of small-scale and scattered enterprises by determining the economic herd size in the region.

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Ethical Statement

This study does not require the ethical statement.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Effects of replacing grain feed with rumen-protected fat on feedlot performance, ruminal parameters and blood metabolites in growing Merino lambs' diets during the hot season

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Abstract: In this study, the effects of rumen-protected fat (RPF) were evaluated as an energy source for partial replacement of grain feed in the diets of Merino male lambs during the hot season. Fourteen six months old Merino male lambs weighing average of 29.59 kg were randomly allocated into two groups: control diet (CON) and diets supplemented with 30 g/kg RPF. Live weight (LW), dry matter intake (DMI) and average daily gain (ADG) were recorded, and the feed conversion ratio (FCR) was then calculated. The ruminal pH, the ruminal ammonia nitrogen concentrations (NH_3-N) value, the molar proportion of volatile fatty acid (VFA), blood serum as well as hematological parameters were determined and protozoa numbers were counted. While the live weight and daily gain of the lambs were not influenced by dietary treatments, DMI was increased with RPF supplementation. The ruminal pH, VFA and ruminal NH_3-N concentrations were unchanged, but the protozoa numbers decreased significantly by RPF supplementation ($P<0.05$). RPF supplementation significantly affected some serum and hematological parameters in comparison to the control group. In conclusion, RPF may be added to concentrate mixture lamb feed at the level of 30 g/kg to enhance the diet energy density without negatively changing the animal's performance.

Keywords: By-pass fat, fattening, male lambs, rumen parameters.

Sıcak aylar boyunca Merinos ırkı kuzu rasyonlarına tane yem yerine ilave edilen rumenden korunmuş yağ ilavesinin besi performansı, rumen parametreleri ve kan metabolitleri üzerine etkileri

Özet: Bu çalışmada, sıcak sezon boyunca Merinos ırkı erkek kuzu rasyonlarına enerji kaynağı olarak tane yem yerine rumende korunmuş yağ ilavesinin etkileri değerlendirilmiştir. Ortalama 29,59 kg canlı ağırlığında 6 aylık yaşta, 14 adet Merinos ırkı erkek kuzular rastgele kontrol grubu ve diyete 30 g/kg rumende korunmuş yağ ilave edilen grup olarak ikiye ayrılmıştır. Canlı ağırlık, kuru madde tüketimi ve ortalama günlük canlı ağırlık artışı kayıt altına alınmış ve yemden yararlanma oranı hesaplanmıştır. Rumen pH değeri belirlenmiş, rumen amonyak-azot konsantrasyonları analiz edilmiş, protozoalar sayılmış ve kan serum ile hematolojik değerler belirlenmiştir. Korunmuş yağ ilavesi deneme gruplarında kuzuların canlı ağırlık ve günlük canlı ağırlık artışı önemsiz olarak etkilenirken, kuru madde tüketimi artmıştır. Korunmuş yağ ilavesi ile rumen pH değeri, uçucu yağ asitleri ve rumen amonyak-azotu konsantrasyonları değişmezken, protozoa sayısı önemli derecede azalmıştır. Kontrol grubu ile karşılaştırıldığında korunmuş yağ ilavesi bazı serum ve hematolojik parametrelerini önemli derecede etkilemiştir. Sonuç olarak kuzu konsantre yemlerine 30 g/kg korunmuş yağ hayvanın performansını negatif etkilemeksizin diyetin enerji değerini artırmak için rasyonlara ilave edilebilir.

Anahtar sözcükler: Besi performansı, korunmuş yağ, erkek kuzu, rumen parametreleri.

Introduction

Lambs fed on pasture have usually lower daily gain, and thus, they grow slower than those fed high-concentrate diets (9). However, in case of an excessive amount of available carbohydrates in the diet, rumination time and saliva production decrease, which further decrease ruminal pH (16) and increase risk for acidosis (22, 27). Ruminal acidosis leads to economic losses through declining (10, 27) dry matter intake (DMI). In such cases, the ruminal pH (< 5.8) decreases mainly due

to an increased accumulation of volatile fatty acids (VFAs) in the rumen (13). The higher dissociation and accumulation of VFAs increase the release of lipopolysaccharides (LPS) because of the death of bacteria, which adversely affect the animal's productivity and performance (13). Due to the negative effects of higher levels of readily fermented carbohydrates in the diet on animal performance (37) the energy balance of a diet could easily be compensated (31) by inclusion of rumen protected fat (RPF) or unprotected fat as a replacement for grain feeds in the rations of lambs (30). Several studies have been carried out to determine the optimal nutrition to improve animal performance, including fat supplementation (8, 23, 25). The use of unprotected fat by above 5% is proven to have some negative impacts on the microbial function of the rumen (30) such as decreased microbial activity of cellulolytic bacteria (7) resulting in decrease of the available nutrient digestibility (17) and rumen protozoal counts (8). However, RPF, also called "inert fat", passes the rumen directly and is digested in the abomasum and duodenum by enzymatic and chemical reactions (25) without causing any negative effects on the rumen's bacterial flora (4, 25). Supplementation of the calcium salts of by-pass oil improved ADG (36), as well as the efficiency of utilization of nutrients (26). This study was designed to increase the diet energy density by reducing the amount of grain feed (corn) in order to minimize the risk of acidosis in the lamb. The purpose of the study was to examine the feedlot performance, ruminal and blood parameters of Merinos male lambs fed on diet supplemented with RPF during the hot season.

Materials and Methods

Animal and feed management: The study was performed at the Agriculture, Livestock and Food Research Station of Mehmet Akif Ersoy University. Fourteen Merino male lambs, 6 months of age and at 29.59 of weight, were divided equally into two groups based on their live weights. Accompanying the vaccination program, the study lasted 105 days (from June until August), with the first 15 days constituting the adaptation period. The animals were housed in north-side closed feeding pens with dimensions of 3 × 3.5 m to protect them from north-east winds. They were fed every day in their pens at 09.00 and 17.00 h. The lamb concentrate mixture and oat hay in separate feeders and water were provided ad libitum. The ingredient composition of the nutrients in the diets used in the trial is presented in Table 1. In the rumen-protected fat (RPF) group, the amount of grain was decreased by approximately 22.14% in the concentrate mixture, and the remaining energy was provided by adding 30 g/kg of RPF. The diets were formulated to meet the nutrient requirements according to Nutritional Research Council (NRC) (34).

Growth trial: Before morning feeding, the lambs were weighed individually on two consecutive days biweekly to determine their LW. The ADG of each lamb was determined by dividing the live weight gain by the number of days on feed. The concentrate mixture and forage were given separately, and DMI was recorded daily. FCR was calculated as kg of DMI consumed divided by kg of LW gained per group.

Chemical composition: The feed samples were dried in a forced ventilation oven at 105°C for 24 h. The dried samples were ground to pass through a 1-mm sieve for estimation of the nutritional composition of the feeds as described by the Association of Official Analytical Chemists (AOAC) (2). The feed samples were analyzed based on the methodology of the AOAC for dry matter (DM), ash, ether extract (EE) and crude protein (CP) contents. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to the method described by Goering and Van Soest (15). Crude fiber (CF) content was analyzed by the procedure of Crampton and Maynard (11). ME content in the feedstuffs and experimental diet was calculated using EE, CP, CF and CA as determined by chemical analyses (1, 32). Digestible dry matter (DDM) was determined by using the ADF content of alfalfa silage. Then, DMI was measured by using the NDF content of alfalfa silage. Relative feed value (RFV) was calculated (34) by using DDM and DMI.

Rumen fermentation characteristics: Rumen fluid samples (50 mL) were collected by using an oral stomach tube from seven lambs of each group after 6 hours of feeding on d 45 and d 90 of the experimental period. After the rumen fluid samples were taken, the rumen pH value was immediately determined with a glass electrode pH meter from the filtrate. The filtrated rumen fluid samples were placed into plastic reagent bottles by acidification with sulfuric acid (50%) and frozen at -20°C until being analyzed to determine the ruminal ammonia nitrogen concentrations (NH_3-N) with indophenol methods by using a spectrophotometer (21). Afterwards, the samples of ruminal fluids were squeezed through layers of cheesecloth with a mesh size of 250 µm. Fifteen mL of the rumen-filtrated samples were preserved into plastic reagent bottles by acidification with 1 mL of 25% (w/v) meta-phosphoric acid and stored at -20°C until being analyzed to determine the molar proportion of VFA concentration [acetate (C2), propionate (C3), butyrate (C4)] by using gas chromatography (21). Rumen protozoa numbers were identified according to the method of Ogimoto and Imai (29) using a Fuchs-Rosenthal chamber.

Blood samples: Roughly 10 mL of blood samples was drawn via jugular vein puncture into EDTA tubes and vacutainer blood tubes without an anticoagulant on d 45 and 90. The blood samples taken into the vacutainer blood tubes without an anticoagulant were centrifuged at 1500 rpm for 15 minutes at room temperature to separate the

serum and then stored at -80°C until analysis. Serum parameters were determined by an enzymatic and colorimetric procedure using an auto analyzer. The blood in the EDTA-containing tubes was analyzed for hematological parameters by using automated equipment (35).

Statistical analyses: The statistical analyses were conducted with SPSS (Statistical Package for the Social Sciences; Inc., Chicago, IL, USA). The data were analyzed by independent-samples t-test to determine the effects of RPF addition on the feedlot performance, ruminal parameters and blood metabolites in the Merino male lambs. The level of significance was taken as $P<0.05$ (12).

Results

The chemical compositions of the feedstuffs and experimental rations are shown in Table 1. The crude protein contents were 17.06% in the CON group and 16.74% in the RPF group. The metabolic energies of the CON and RPF groups were found to be 3245.3 and 3231.5 kcal/kg, respectively. Supplementation of RPF to the lamb diet had no significant effect ($P>0.05$) on LW or ADG during the experiment. As presented in Table 2, RPF supplementation numerically increased ($P>0.05$) the overall mean value of ADG by 0.01 kg/d. The oat hay intake and roughage: concentrated value was lower ($P<0.05$) in the RPF group than the CON group during the study except for weeks between 0-2. The concentrated feed intake was similar ($P>0.05$) in both groups (Table 3). The total dry matter intake (TDMI) was significantly lower ($P<0.05$) in the RPF group after sixth week of beginning, while no significant differences ($P>0.05$) were observed for FCR (Figure 1). There were no significant differences ($P>0.05$) between the groups in terms of the rumen pH, NH_3-N, C2, C3 and C4 concentration on all treatment days (Table 4). The rumen pH value was numerically decreased ($P>0.05$) in the RPF group on d 45, but it increased on d 90. Addition of RPF reduced ($P>0.05$) the concentration of NH_3-N on all treatment days. The C2 (mM) and C3 (mM) concentrations were higher ($P>0.05$) in the RPF group than the CON group at d 45 but lower at d 90. There was an increase in the C4 concentrations in CON in comparison to that of RPF. The protozoa count was significantly reduced ($P<0.05$) by RPF addition on d 90, but it was unaffected on d 45 (Table 4). The effect of RPF was insignificant ($P>0.05$) on the triglyceride, AST, ALT, glucose and HDL-cholesterol levels. The concentrations of LDL-cholesterol were significantly increased ($P<0.05$) by adding RPF in comparison to the CON group (Table 5). The HDL-cholesterol concentration was numerically lower ($P>0.05$) in the CON group in comparison to the RPF group. The glucose concentration was insignificantly lower ($P>0.05$) in the CON group in comparison to RPF at d 45 but higher at d 90 (Table 5). For all treatment days, there was no significant effect ($P>0.05$) of RPF on the blood hematological parameters except the PLT and PCT values which were higher ($P<0.05$) in the RPF group in comparison to the CON group at d 90 (Table 6).

Table 1. Ingredient composition and analyzed content of the nutrients in the diets used in the trial.

Ingredients (g kg^{-1})	CON	RPF	Nutritional content (%)	CON	RPF
Barley	100	135	Dry matter	90.84	93.47
Corn	600	410	Ash	6.00	7.64
Wheat bran	90	230	Organic matter	94.00	92.36
Sunflower meal	40	45	Crude fiber	4.18	6.00
Soybean meal	144	124	Crude protein	17.06	16.74
Rumen protected fat	-	30	Ether extract	2.20	3.96
Limestone	21	21	Neutral detergent fiber	16.22	20.85
DCP	2	2	Acid detergent fiber	6.68	7.99
Salt	2	2	Hemi-cellulose	9.54	12.84
Premix[1]	1	1	Non-fiber carbohydrate	49.36	44.28
			Nitrogen free extract	61.39	59.14
			ME* (kcal/kg)	3245.3	3231.5

Oat Hay**				
ME_{ADF} (kcal/kg)	**ME_{NDF} (kcal/kg)**	**DDM%**	**DMI%**	**RFV%**
2061.7	2097.9	57.35	1.86	83.01

CON: Control group, RPF: Rumen-protected fat group; [1]Each kilogram of vitamin-mineral mix contains 1,000,000 IU vit A, 200,000 IU Vit D, 1,800 mg vit E, 8,400 mg Zn; *Metabolic energy was calculated by TSI; ** ME_{ADF}, and ME_{NDF} were calculated by Alderman (1); DDM: Dry digestible matter, DMI: Dry matter intake; RFV: Relative feed value. [DDM% = 88.9 - (0.779 × ADF%)], [DMI%=120/NDF%)], [RFV = DDM% × DMI% × 0.775]. Non-fiber carbohydrate= [OM%– (NDF%+CP%+EE%)], Nitrogen free extract= [DM%- (CP%+CF%+Ash%+EE%)].

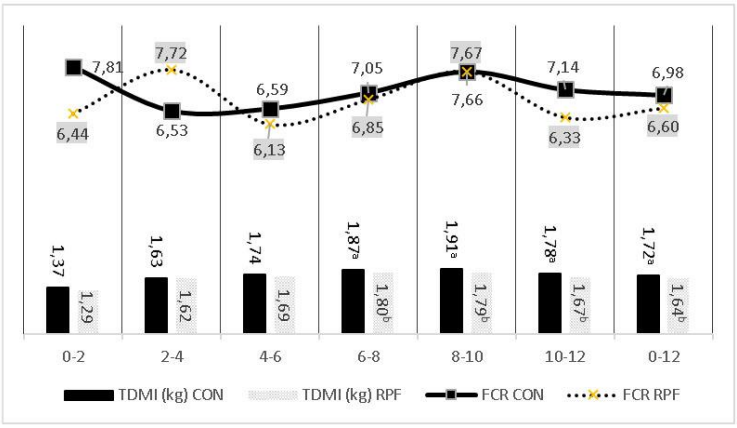

Figure 1. The total dry matter intake (TDMI) (kg/lamb) and feed conversion ratio (kg DM/ kg live weight) of lamb fed on diet supplemented (RPF) or not (CON) with rumen-protected fat.
TDMI: The total dry matter intake
FCR: Feed conversion ratio
CON: Control group
RPF: Rumen-protected fat group

Table 2. The live weight and average daily gain of lamb fed on diet supplemented (RPF) or not (CON) with rumen-protected fat (Mean±SEM).

Items	**Live weight (kg)**			**Average daily gain (kg/d)**			
Weeks	**CON**	**RPF**	**P**	**Weeks**	**CON**	**RPF**	**P**
Initial live weight	29.98±3.39	29.20±3.24	0.67	0-2	0.20±0.11	0.30±0.06	0.06
2nd	32.81±4.17	33.46±3.90	0.76	2-4	0.23±0.12	0.21±0.03	0.66
4th	36.10±4.31	36.48±3.80	0.86	4-6	0.29±0.10	0.28±0.05	0.89
6th	40.20±3.42	40.49±4.11	0.89	6-8	0.24±0.12	0.27±0.04	0.62
8th	43.66±2.76	44.29±3.78	0.69	8-10	0.25±0.02	0.22±0.06	0.27
10th	47.20±2.94	47.40±3.44	0.90	10-12	0.26±0.03	0.25±0.04	0.87
12th	50.75±3.16	50.91±3.39	0.93	0-12	0.25 ±0.02	0.26±0.01	0.27

CON: Control group, RPF: Rumen-protected fat group.

Table 3. The dry matter intake (g/lamb) and feed conversion ratio (g DM/ g live weight) of lamb fed on diet supplemented (RPF) or not (CON) with rumen-protected fat (Mean±SEM).

Week	**Groups**	**Oat hay intake (kg, DM)**	**Concentrated Feed intake (kg, DM)**	**Roughage: Concentrated**
	CON	0.38±0.03	0.98±0.07	0.39±0.13
0-2	RPF	0.35±0.04	0.94±0.04	0.37±0.12
	P	0.51	0.62	0.54
	CON	0.48±0.05a	1.15±0.09	0.41±0.01a
2-4	RPF	0.40±0.04b	1.21±0.14	0.34±0.02b
	P	0.02	0.35	0.01
	CON	0.50±0.02a	1.23±0.01	0.40±0.02a
4-6	RPF	0.45±0.01b	1.23±0.01	0.36±0.01b
	P	0.04	0.79	0.049
	CON	0.61±0.01a	1.27±0.01	0.47±0.01a
6-8	RPF	0.54± 0.02b	1.26±0.01	0.42±0.02b
	P	0.001	0.06	0.001
	CON	0.60±0.01a	1.31±0.01	0.45±0.01a
8-10	RPF	0.47±0.02b	1.31±0.01	0.36±0.02b
	P	0.001	0.83	0.001
	CON	0.53±0.02a	1.22±0.01	0.46±0.01a
10-12	RPF	0.41±0.01b	1.26±0.03	0.33±0.02b
	P	0.001	0.27	0.001
	CON	0.52±0.01	1.19±0.01	0.43±0.04
0-12	RPF	0.44±0.07	1.21±0.13	0.36±0.04
	P	0.001	0.65	0.001

CON: Control group, RPF: Rumen-protected fat group
a,b; Means with different superscripts in the same column are significantly different (P<0.05).

Table 4. The rumen fermentation characteristic of lamb fed on diet supplemented (RPF) or not (CON) with rumen-protected fat (Mean±SEM).

Items	Day 45			Day 90		
	CON	RPF	P	CON	RPF	P
pH value	7.09±0.13	7.05±0.08	0.45	6.55±0.21	6.58±0.13	0.77
NH_3-N (mg/100ml)	4.73±0.04	4.68±0.07	0.55	4.87±0.04	4.79±0.01	0.07
C2 (mM)	15.42±3.48	17.75±4.17	0.64	29.59±2.08	25.78±5.4	0.47
C3 (mM)	11.66±4.22	12.40±5.30	0.91	29.66±2.41	25.75±3.6	0.25
C4 (mM)	4.42±1.59	3.61±0.55	0.63	11.19±2.52	10.44±1.36	0.77
Protozoa count (10^4 cell/mL)	172±14.12	156±16.31	0.71	170.35±16.35[a]	91.78±16.91[b]	0.005

CON: Control group, RPF: Rumen-protected fat group
[a,b;] Means with different superscripts in the same row are significantly different (P<0.05).

Table 5. The blood biochemical parameters of lamb fed on diet supplemented (RPF) or not (CON) with rumen-protected fat (Mean±SEM).

Items	Day 45			Day 90		
	CON	RPF	P	CON	RPF	P
Cholesterol (mg/dl)	56.87±3.50[a]	68.00±3.85[b]	0.04	60.85±5.42	68.28±3.92	0.28
Triglyceride (mg/dl)	30.00±1.37	35.57±3.53	0.18	24.42±3.77	22.85±2.24	0.73
AST (U/L)	117.11±6.86	111.1±5.76	0.51	119.11±10.04	111.82±3.74	0.51
ALT (U/L)	21.15±1.39	17.28±1.99	0.13	19.42±1.71	19.14±1.96	0.91
Glucose (mg/dl)	83.72±6.28	99.72±6.58	0.10	79.57±2.83	75.42±1.78	0.24
HDL (mg/dl)	31.28±1.88	32.71±1.75	0.59	33.57±2.33	36.14±2.32	0.45
LDL (mg/dl)	15.57±0.57[a]	18.28±1.16[b]	0.04	19.57±0.99[a]	23.28±0.89[b]	0.02

CON: Control group, RPF: Rumen-protected fat group, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, HDL: High-density lipoprotein, LDL: Low-density lipoprotein
[a,b;] Means with different superscripts in the same row are significantly different (P<0.05).

Table 6. The blood hematological parameters of lamb fed on diet supplemented (RPF) or not (CON) with rumen-protected fat (Mean±SEM).

Items	Day 45			Day 90		
	CON	RPF	P	CON	RPF	P
WBC ($x10^3$/µL)	8.92±1.28	11.17±1.21	0.23	10.60±1.75	12.63±1.73	0.43
LYM ($x10^3$/µL)	5.09±1.20	4.69±0.55	0.76	5.37±1.38	4.84±0.51	0.72
MID ($x10^3$/µL)	0.04±0.01	0.05±0.01	0.25	0.05±0.01	0.06±0.01	0.38
GRA ($x10^3$/µL)	3.78±0.70	6.42±0.79	0.03	5.18±0.63	7.32±1.02	0.11
RBC ($x10^3$/µL)	9.91±0.61	10.22±0.33	0.66	10.71±0.67	11.22±0.38	0.53
HGB (g/dL)	11.14±0.69	10.54±0.43	0.47	11.60±0.41	11.10±0.51	0.48
HCT (%)	30.53±1.58	29.65±0.82	0.63	33.81±1.26	32.36±1.68	0.51
MCV, fL	30.14±0.70	30.00±0.95	0.90	30.40±0.60	30.20±1.15	0.88
MCH, pg	10.62±0.25	25.20±0.35	0.32	10.44±0.19	10.34±0.32	0.79
MCHC (g/dL)	35.45±0.53	36.41±0.63	0.27	34.42±0.52	34.30±0.85	0.90
RDW (%)	25.95±0.48	25.94±0.78	0.98	24.40±0.47	26.04±0.87	0.13
PLT ($x10^3$/ µL)	655±96.7	604±33.5	0.63	525±86.5[b]	755±56.0[a]	0.04
PCT	0.39±0.05	0.37±0.01	0.71	0.31±0.04a	0.46±0.04a	0.05
MPV,fL	5.95±0.06	6.15±0.15	0.23	6.14±0.18	6.10±0.15	0.87

CON: Control group, RPF: Rumen-protected fat group, WBC: White blood cell, LYM: Lymphocyte, MID: Monocyte, GRA: Granulocyte, RBC: Red blood cell, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, RDWc: Red blood cell distribution Width, PLT: Platelet, PCT: Platelet crit, MPV: Mean Platelet Volume [a,b]; Means with different superscripts in the same row are significantly different (P<0.05).

Discussion and Conclusion

The LW of lambs in the CON group and RPF-supplemented group were 50.75 and 50.91 at the end of the experiment, respectively. The increase in the ADG by 0.01 kg/day in the RPF-supplemented diet may be associated with the intake of concentrated feed, which consists of 1.21 kg of DM. Supplementation of RPF to the lamb diet had no significant effect on ADG during the experiment. The results of this study were in accordance with Manso et al. (23), who showed insignificant increases in ADG and LW. Likewise, Bhatt et al. (6) reported that final live weight and ADG were significantly higher in the Ca-soap group in comparison to the control. Oat hay intake was lower (approximately 15.39%) and concentrated feed intake was higher (1.61%) observed in the RPF group, which suggested that increased energy density of the ration limits the thermic effects of feeding. The feed intake could be more closely associated with heat stress. Heat increment is increased by forage intake more than concentrate, and it is generally thought to be closely related to the metabolism of acetate (14). As expected, the decrease in the acetate by 13.18% in the RPF-supplemented diet may be associated with the heat increment. Conversely, neither fat nor concentrate addition changed DMI when cows were exposed to hot weather (7, 24). Bhatt et al. (6) stated that decreased hay intake might be attributed to an increase in the energy density of diet due to the chemo-static regulation of voluntary intake. They (6) also reported decreased roughage intake and increased concentrate intake with addition of Ca-soap fat in the diets of Malpura lambs. Addition of RPF did not affect the ruminal pH. The findings of this study were in agreement with results of previous studies conducted on cattle (20) and lambs (6). A significant increase in ruminal pH with RPF supplementation could be mainly associated with the maintenance of better rumen environment (7). The greater ruminal pH in RPF-fed lambs compared to control-fed lambs might provide greater production of buffering agents (i.e., saliva) resulting from an increase in rumination time during the hot season (24). A partial degradation of RPF might likely have contributed to the buffer capacity of ruminal pH, which prevents decline in ruminal pH (7). Bhatt et al. (6), observed a higher ruminal pH upon addition of RPF to the diets of lambs. The concentration of NH_3-N was lower ($P>0.05$) in the RPF group than the CON group at d 45 and d 90. In comparison to control group, the NH_3-N concentration in the ruminal fluid of the lambs fed with RPF in this study was found to be about 4.79 mmol/L at d 90. The decrease in the NH_3-N concentration in the ruminal fluid could be explained by the increase in the amino acid flow from the stomach to the intestinal tract due to utilization and absorption, which was associated with defaunation of protozoal counts (19). A low protozoal count was found in this study, further supported this condition. This may be explained by the greater proliferation of bacteria (4). The findings of this study proved that RPF supplementation numerically decreased the molar proportion of C2, C3 and C4, these results agreed with the data published by Bhatt et al. (6). To some extent, this explains that the increase in the molar proportion of C3 in the CON group was associated with the lambs fed the high-cereal diet (3) and thus increased the concentration of glucose due to the gluconeogenic effect. In this study, the increase in the molar concentration of C2 in the CON group in comparison to the RPF group was associated with an increase in the oat hay intake. This was an inconsistent with the results of Kang et al. (20), who revealed that RPF increased C2 and C3. Bettero et al. (5) achieved a similar acetate concentration in sheep by adding rumen protected fat to their diets. Similar to this study, Bhatt et al. (6) also showed a lower protozoon count with RPF supplementation in lamb diets. This could be due to the breakdown of symbiotic rumen protozoa-methanogenic bacteria interaction and subsequent interspecies H_2 transfer resulting in limitation of H_2 production during fermentation, which was associated with the higher pH of the ruminal fluid (6, 19). At d 45 of the experiment, the lambs fed the RPF diet had higher cholesterol concentrations than those fed the control diet ($P<0.05$). Similar findings were emphasized by Bhatt et al. (6), who also showed increased cholesterol concentrations using RPF in the basal diets of lambs. The same authors stated that cholesterol concentrations might be increased due to stimulation in its synthesis by supplementation of RPF. Similar observations of an increase in cholesterol concentrations through feeding of RPF were also reported by Tyagi et al. (33) and Wadhwa et al. (36). The lambs fed RPF had insignificantly higher glucose concentrations, as well as lower concentrations of AST and ALT than those in the control group at d 45. In growing diets, RPF may be used as a substitute for grain feeds to ensure that the energy density in lamb rations does not cause adverse impacts on the glucose concentration (18). Tyagi et al. (33) also reported no significant impact of RPF on glucose levels. The hematocrit level slightly increased during the experiment. Vieira et al. (35) stated that a low pH causes splenic contraction because of the action of epinephrine resulting in increased hematocrit.

In conclusion, a review of the literature showed that the statistically significant or insignificant differences were mostly due to individual differences in the animals such as live weight, sex and age of the animal or forage type and ingredients of the concentrate mixture lamb feed. The current results of the study indicated that dietary supplementation of RPF at 30 g/kg slightly increased the LW and ADG. In comparison to CON, addition of RPF

did not affect the ruminal pH value, NH_3-N concentration, molar proportion of C2, C3 and C4, whereas it significantly reduced the forage intake and the rumen protozoa numbers. RPF supplementation affected the serum blood parameters such as cholesterol and LDL cholesterol. It is concluded that RPF is important especially for forage intake. Therefore, further research is required to understand the influence of different rates of RPF on male lamb nutrition.

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Ethical Statement

All procedures were warranted by the Ethics Committee for Animal Use of the Mehmet Akif Ersoy University (Protocol number: 26.05.2015/132).

Conflict of Interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript

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Bioactive components, antibacterial and antiradical properties of home-made apple and grape vinegar

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Abstract: The present study aimed to investigate major volatile compounds, organic acid, phenolic and mineral contents, and antiradical and antimicrobial properties of home-made apple/grape vinegar. Grape vinegar showed higher total acidity, organic acid content, total phenolic content (TPC), antiradical activity and lower minimum inhibitory concentration (MIC) values compared to apple vinegar. While acetic and tartaric acids were the most abundant organic acids in grape vinegar, acetic and succinic acids were the most abundant organic acids in apple vinegar. The most abundant phenolic compound was gallic acid in both grape and apple vinegar. A total of 18 and 9 volatile compounds were determined in grape vinegar samples and apple vinegar samples, respectively. The most abundant volatile compounds were acetic acid and acetoin in grape vinegar, acetic acid, ethyl acetate and 2,4,5-trimethyl-1,3-dioxolane in apple vinegar. K, Ca and Na were common minerals in both vinegar and more in the grape vinegar compared to apple vinegar. Although MIC value for grape vinegar was at 6.25% with minimum bactericidal concentration (MBC) values ranged from 6.25% to 12.50%, MIC value for apple vinegar was at 12.50% with MBC values ranged from 12.50% to 25.00% for all test bacteria. The antiradical and antibacterial activities of the vinegar samples were correlated with their TPC and organic acid contents.

Keywords: Antibacterial, antioxidant, apple, grape, vinegar.

Ev yapımı elma ve üzüm sirkelerinin kimyasal, antibakteriyel ve antiradikal özelliklerinin araştırılması

Özet: Bu çalışmada, ev yapımı elma ve üzüm sirkelerinin başlıca uçucu bileşikleri, organik asit, fenolik ve mineral içerikleri ile antiradikal ve antimikrobiyal özelliklerinin araştırılması amaçlanmıştır. Üzüm sirkesinin elma sirkesine göre daha yüksek toplam asitlik, organik asit içeriği, toplam fenolik içerik (TFİ) ve antiradikal aktiviteye sahip olduğu, aynı zamanda daha düşük minimum inhibitör konsantrasyon (MİK) değerleri gösterdiği tespit edildi. Üzüm sirkesinde, asetik ve tartarik asitler en çok bulunan organik asitti. Elma sirkesinde ise en çok bulunan organik asitler asetik ve süksinik asitlerdi. Her iki sirke çeşidinde de en fazla bulunan fenolik bileşik gallik asitti. Üzüm ve elma sirkelerinin sırasıyla 18 ve 9 adet uçucu bileşik içerdiği belirlendi. Üzüm sirkesinde asetik asit ve asetoin en çok bulunan uçucu bileşikler iken; asetik asit, etil asetat, 2,4,5-trimetil-1,3-dioksolan elma sirkesinde en fazla bulunan uçucu bileşiklerdi. K, Ca ve Na, her iki sirkede en yaygın bulunan minerallerdi. Aynı minerallerin, üzüm sirkesinde elma sirkesine kıyasla daha fazla oranda bulundukları tespit edildi. Üzüm sirkesinde test edilen bakterilere karşı MİK değerlerinin %6,25 olduğu, minimum bakterisidal konsantrasyon (MBK) değerlerinin %6,25 ile %12,50 arasında değiştiği görüldü. Elma sirkesinin test edilen bakterilere karşı MİK değerinin %12,50 olduğu, MBK değerlerinin ise tüm test bakterileri için %12,50 ile %25,00 arasında değiştiği görüldü. Yapılan çalışmada sirkelerin sahip olduğu antiradikal ve antibakteriyel aktivitelerinin, TFİ ve organik asit içerikleri ile ilişkili olduğu sonucuna varıldı.

Anahtar sözcükler: Antibakteriyel, antioksidan, elma, sirke, üzüm.

Introduction

Vinegar is a fermented product made by acetic acid bacteria that convert ethyl alcohol into acetic acid by oxidation. Vinegar can be made from fruits, cereals and vegetable and used as food supplement, tonic and nutraceutical (35, 49). Vinegar is produced from a double fermentation of any fermentable sugary substrates, and its organoleptic and chemical properties can be changed according to the type of raw materials used and fermentation methods. First step is alcoholic fermentation which is the conversion of fermentable sugars into ethanol mainly by yeast. Second step is acetic acid fermentation in which ethanol is oxidized to acetic acid aerobically by acetic acid bacteria (30, 43). In general, two different methods, traditional (slow) and submerged (quick), are used for vinegar production. Vinegar produced by slow method are high-quality in acidity and contents of aroma components but rather expensive, while vinegar produced by submerged method are quick but cheaper, which makes it the one that is most employed (14).

Foodborne pathogens (mainly bacteria) are the major cause of the diseases and affecting food safety and cause human illness worldwide (10). Also, some bacteria can cause food spoilage resulted in objectionable by-products in the food and altering the food's nutritional value and sensory properties such as smell, texture and appearance (20). Natural antimicrobial agents have advantages over antibiotics in terms of their antimicrobial resistance (41). The antimicrobial properties of vinegar not only make it the best alternative for antibiotics, but also make them a very useful product for cleaning fruits and vegetables, sanitizing surfaces and preventing the spoilage (35). In addition to its antimicrobial activity, several studies have indicated that vinegar has antimicrobial, antioxidant, antidiabetic, anti-inflammatory, antihypertensive, immune stimulatory and anticancer activities (13, 25, 34, 42, 49). These therapeutic activities are attributed to the presence of bioactive compounds including organic acids, phenolics, flavonoids, essential amino acids, vitamins and minerals in vinegar (8, 16). Hence, the aim of the present study was to evaluate the physicochemical parameters, major volatile compounds and mineral contents, antioxidant and antimicrobial properties of apple and grape vinegar produced with traditional spontaneous fermentation.

Materials and Methods

***Vinegar production*:** Vinegar samples produced traditionally from apples and grapes by adding Lavender honey via spontaneous fermentation were used in this study. The "Red delicious" apples, "Uluğbey Karası" grape fruits and Lavender honey were purchased from Isparta province of Turkey. The fruits were washed in clean water to remove residues, and the apples were shredded. The procedure of vinegar production is given on the Figure 1. Vinegar samples were produced using 10 L polypropylene food containers in Food Hygiene and Technology Laboratory (Burdur, Turkey). The samples were stored in 1 L polyethylene terephthalate jars and kept in the dark at 4ºC until required. The vinegar samples were filtered through a 0.45-µm membrane filter before being used in the tests. Vinegar production for each fruit was performed in triplicate.

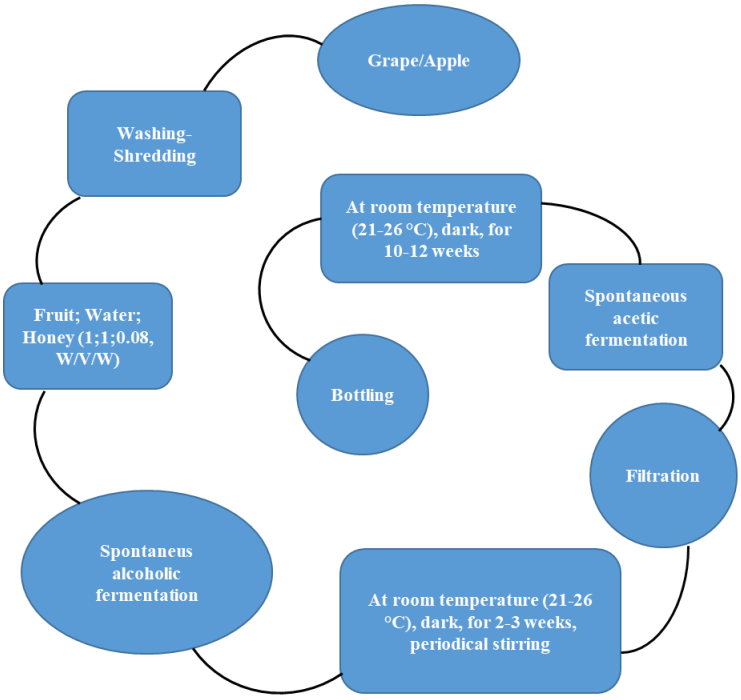

Figure 1. Vinegar production scheme.

***Physicochemical properties*:** pH values of the vinegar samples were measured with a pH meter (WTW Lab-pH Meter inoLab® pH 7110) at 25ºC ± 2ºC. Total acidity quantification of the vinegar samples was performed by titration method. Briefly, a 20 mL distilled water was mixed with 10 mL of vinegar and the diluted vinegar solution was titrated with 0.10 M NaOH. The results were expressed as acetic acid equivalent (g acetic acid/L vinegar sample) (1).

***Total phenolic content*:** Total phenolic content (TPC) of the apple and grape vinegar samples was measured by the Folin-Ciocalteu method (40). Before appropriately diluted, vinegar samples were filtered with 0.45 mm filter. The filtrate (4 mL) was mixed with Folin Ciocelteau's phenol reagent (2 mL) and Na_2CO_3 (7%, 1.6 mL). The mixture was left for 90 min at room temperature, and the absorbance was measured at 760 nm by a spectrophotometer (Thermo-MultiScan GO, ThermoScientific™ Multiskan™ GO Microplate Spectrophotometer). The standard curve was prepared using Gallic acid (methanol: water [1/99, v/v] in the range of 0.00625 - 0.1 mg/mL [$y = 6.5097x – 0.0086$] [$R^2 = 0.9956$]). Total phenolic values were expressed as mg Gallic acid equivalent (GAE)/L.

Antiradical activity: The antiradical activity of the apple and grape vinegar samples was determined as free 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity (35, 39). 0.1 mL of the filtrate which was obtained by filtrating vinegar samples using 0.45 mm filter and 5 mL of 0.1 mM DPPH (Sigma-Aldrich, St. Louis, USA) solutions were mixed vigorously with vortex. The mixture incubated for 13 min at 27ºC in dark conditions. After incubation, absorbance of the final mixture was calculated at 517 nm using a spectrophotometer.

Antiradical activity (ARA, %) was calculated as below:

ARA (%) = [(ARC- ARS)/ARC] *100

ARC indicates the absorbance of control (DPPH solution) and ARS indicates the absorbance of the sample.

***Volatile compounds*:** Volatile compounds (VC) of the vinegar samples were determined using a Gas Chromatography-Mass Spectrometry GC-MS. The chromatographic analysis was performed on a Shimadzu GC-2010 Plus equipped with a mass spectrometer selective detector Shimadzu GCMS-QP2010 SE (Shimadzu, Kyoto, Japan). A three mL of vinegar sample was placed into SPME vial (Supelco 27159 15 mL clear PTFE/Siliconesepta Cap). The vial was kept for 15 minutes in a hotplate magnetic stirrer (H4000- HSE, Benchmark Scientific Inc. USA) at 45 ºC for 15 minutes, and then Fused silica SPME fiber (carboxen/ polydimethylsiloxane [CAR/PDMS]) was exposed to the headspace for 30 minutes. The desorption of absorbed volatile compounds was performed at 250 °C for 3 minutes when the fiber was inserted into the injection port and they were injected to GC-MS in the splitless mode. The GC-MS conditions and settings are shown in Table 1. The volatile compounds were compared their MS fragments with a library of mass spectra (Wiley, NIST, Tutor, FFNSC) for identification. The amount of each volatile compound was expressed as a percentage. The percentage of each compound was calculated by dividing the area of its peak to the total area under all of the peaks.

***Organic acid analysis*:** A Shimadzu HPLC system equipped with an isocratic pump (LC-10AT), a UV-VIS detector (SPD-10A set 210 nm), a column oven (CTO-10AS), a degasser (DGU-12AS), a system controller (LC-20AT) was used to determine the main organic acids in vinegar samples. Analysis was performed by the modified method described by Alhendawi et al. (2) and Krapez et al. (28). Briefly, the Supelco solid phase extraction (SPE) cartridges were conditioned with 3 mL of methanol and washed with 10 mL of distilled water. Five mL of vinegar sample were transferred to the 15-mL tubes and 2% H_3PO_4 solution was added to the tubes and the mixture was homogenized and filtered through a filter paper. One mL of filtrate was diluted with 1 mL of extraction solution (0.01 M KH_2PO_4 solution, pH adjusted to 8.00). One mL of this solution was passed through the cartridge, and the eluate was placed into a tube. The cartridge was washed with 1 mL of extraction solution. Both eluates were combined, and the aliquots of 20 µL from the eluate was injected into a HPLC system. The HPLC instrumental conditions are shown in Table 1.

Phenolic compounds: Analysis of vinegar sample was carried out by high performance liquid chromatography (HPLC) using a chromatograph equipped with an Agilent Eclipse XDB-C18 (250x4.60 mm) 5-micron column and a diode array detector (SPD-M10A, Shimadzu). The standard solutions were prepared using methanol to dissolve the chemicals to reach concentrations ranging from 0.7 to 500.0 µg/mL for gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, p-coumaric acid. The chromatographic conditions for vinegar samples were: flow rate: 0.8 mL/min; injection volume: 20 µL; column temperature: 30ºC. Methanol and acetic acid (3%) solvents were used as mobile phase (12).

***Mineral analysis*:** The concentration of mineral elements including Copper (Cu), Calcium (Ca), Chromium (Cr), Magnesium (Mg), Phosphorus (P), Potassium (K) and Sodium (Na) in vinegar samples was measured with inductively coupled plasma-optical emission spectrometry (ICP-OES, Perkin Elmer OPTIMA 5300 DV) according to EPA 6010D method (46). The ICP-OES instrumental conditions are shown in Table 1.

Table 1. GC-MS, HPLC and ICP-OES conditions and settings.

	Conditions	Setting
GC/MS	GC	Shimadzu GC- 2010 Plus
	MS	Shimadzu GCMS-QP2010 SE
	Detector and injector port temperature	250°C
	Column flow	1.61 min/mL
	Ionization method	Electron ionization (EI), 70 eV
	Carrier gas	Helium
	Column	Restek Rxi-5Sil MS (30 m * 0.25 mm, 0.25 um (Restek, 13623)
	Oven temperature	Held at 40°C for 2 mins, raised by 4°C/min to 250°C and held for 5 min at 250°C
	Used libraries	Wiley, NIST, Tutor, FFNSC
	SPME conditions	Sample was kept in the SPME vial for 15 min at 45ºC and then the fiber (Fused silica SPME fiber (CAR/PDMS) (Supelco, 57318) exposed to the headspace for 30 min. Following, the desorption was performed in 250ºC.
	SPME vial	Supelco 27159 15 mL clear PTFE /Siliconesepta Cap
HPLC	Instrument	Shimadzu HPLC system
	Detector	SPD-10Avp UV-VIS detector (210 nm)
	System controller	LC- 20AT prominence
	Auto sampler	SIL–20AC prominence
	Pump	LC- 20AT prominence
	Degasser	DGU- 20A5 prominence
	Column oven	Shimadzu CTO-10ASvp
	Column	Teknokroma Tracer Extrasil ODS-2, 250 mm × 4.6 mm id, 5 μm (TR-016059)
	Column temperature	30°C
	Mobil phase	H3PO4/H2O (pH: 2.2)
	Injection volume	20 μL
	Flow rate	0.8 mL/min
ICP-OES	Plasma gas flow	15 L/min
	Auxiliary gas flow	0.2 L/min
	Nebulizer gas flow	0.6 L/min
	Power	1450 watt
	Torch cassette position	-3
	Pump speed	1.5 mL/min
	Purge	Normal
	Resolution	Normal
	Integration time	10 seconds minimum/20 seconds maximum
	Read delay	60 sec

***Minimum inhibitory concentration (MIC)*:** The MIC and MBC of vinegar samples against 11 microorganisms including *Staphylococcus aureus* (ATCC 25923), Methicillin-Resistant *S. aureus* (ATCC 43300) (MRSA), *Bacillus cereus* (ATCC 33019), *Listeria monocytogenes* (Refik Saydam Laboratory Culture Collection, Turkey, RSKK 472), *L. monocytogenes* (RSKK 02028), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas fluorescens* (ATCC 13525), *Escherichia coli* O157:H7 (ATCC 35150), *Salmonella* Enteritidis *(*ATCC 13076), *Salmonella* Enteritidis Phage Type 4 (NCTC 13349) and *S.* Typhimurium (ATCC 14088) were determined. The MIC values of the vinegar samples was determined by using microdilution method in 96-well microplates according to the CLSI guidelines (17). The bacterial strains were cultivated in Mueller-Hinton agar (MERCK 105437) and incubated for 18-24 h at 37°C for *S. aureus*, *B. cereus*, *E. coli* O157:H7 and *Salmonella* strains, or at 30°C for *P. fluorescens* for growth. Each bacterial cell was transferred into 0.9% sterile saline buffer and adjusted to 0.5 McFarland scale which represents a concentration of approximately 1.5 x 10^8 CFU/mL. The two-fold serial dilutions of vinegar samples (50%, 25%, 12.5%, 6.75%, 3.12%, 1.56% and 0.78%) were prepared in Mueller-Hinton broth (MH, MERCK 110293) and dispensed into wells of the microplate. 20 μL of the bacterial culture was inoculated into each well. Three control tubes were maintained for each test batch (media control, organism control and extract control). After incubating the plates at 30°C for *P. fluorescens* and at 37°C for the other bacterial strains for 24 h, microbial growth (turbidity) was determined at 600 nm using a

microplate reader (Epoch, BioTek, USA). The lowest concentration of the vinegar where no visible microbial growth was selected as the MIC value.

***Minimum bactericidal concentration (MBC)*:** The MBC was determined by subculturing the suspension (10 µL) from each well in the plate on Mueller-Hinton agar. The plates were incubated at 37°C for *S. aureus*, *B. cereus*, *E. coli* O157:H7 and *Salmonella* strains, or at 30°C for *P. fluorescens* strains for 24 h. MCB values were identified by determining the lowest concentration of vinegar samples that completely killed the growth of culture (3).

Results

The pH values of apple vinegar and grape vinegar were 3.03±0.01 and 2.94±0.01, respectively. The total acidity for grape vinegar (2.43 g/100 mL) was higher than apple vinegar (0.99 g/100 mL). The pH values of the vinegar samples were in good correlation with the total acidity (Table 2).

The concentrations of phenolic compounds present in grape vinegar and apple vinegar were 26.7 µg/mL and 6.83 µg/mL, respectively. Gallic acid was found to be the major phenolic acid in the vinegar. Although the vinegar had low p-coumaric acid concentrations, chlorogenic acid was not detected in apple vinegar. The TPC values of grape vinegar and apple vinegar were 498.36 mg GAE/L and 209.10 mg GAE/L respectively. TPC values belonging to different dilutions (1/1; 1/2; 1/4; 1/8) of grape and apple vinegar were given in Table 2. A proportional decrease depending on the dilution was observed. Antiradical activity of grape vinegar and apple vinegar measured 30.14% and 16.44% and were indicated as DPPH free radical scavenging abilities (Table 2).

The results of major organic acids, phenolic acids and mineral compounds of the vinegar samples are shown in Table 3. The main organic acid in grape and apple vinegar was acetic acid, accounting for 79.9% and 84.2% of the organic acid present in grape vinegar and apple vinegar, respectively. The level of organic acids (22283.8 µg/g) in grape vinegar was higher than apple vinegar (9003.8 µg/g). While tartaric acid (3769.4 µg/g) was the second most abundant organic acid, lactic acid was not detected in grape vinegar. Whereas citric acid content in grape vinegar was low, being 0.3%, malic acid content in apple vinegar was low, being 1.1%.

Table 2. Physicochemical properties, total phenolic contents and antiradical activity of grape and apple vinegars.

Parameters	Dilutions	Apple vinegar	Grape vinegar
pH	-	3.03±0.23	2.94±0.11
Total Acidity (g/100 mL)	-	0.99±0.05	2.43±0.14
TPC (mg GAE/L)	100%	209.10±5.97	498.36±7.55
	50%	117.11±3.07	322.15±5.07
	25%	79.49±2.29	153.60±2.25
	12.5%	34.42±1.02	81.58±1.05
DPPH (%)	100%	16.44±0.12	30.14±0.75
	10%	10.40±0.66	9.87±0.10

Values are expressed as mean±SD. TPC: Total phenolic content, DPPH: 2.2-diphenyl-1-picrylhydrazyl.

Table 3. Major organic acids, phenolic acids and mineral compounds of the vinegars.

Groups	Compounds	Grape vinegar	Apple vinegar
Organic acids (µg/g)	Tartaric	3769.4 (16.9%)	110.8 (1.2%)
	Malic	323.8 (1.4%)	101.8 (1.1%)
	Lactic	ND*	467.9 (5.2%)
	Acetic	17815.8 (79.9%)	7584.4 (84.2%)
	Citric	67 (0.3%)	167.1 (1.8%)
	Succinic	307.7 (1.3%)	571.8 (6.3)
Phenolic compounds (µg/mL)	Gallic acid	10.8	4.5
	Protocatechuic acid	2.4	0.7
	Catechin	4.4	1.3
	Chlorogenic acid	3.1	*
	Caffeic Acid	5.2	0.3
	P-coumaric acid	0.8	0.03
Mineral content (mg/g)	Cu	<0.006 µg/g	<0.006 µg/g
	Mg	5.696±0.4846	5.389±0.2275
	Cr	<0.005 µg/g	<0.005 µg/g
	Ca	30.04±0.522	6.715±0.2967
	K	153.8±5.22	131.2±4.29
	Na	21.85±1.502	15.37±0.734
	P	10.90±0.087	6.901±0.1194

*ND: Non-Detected.

K, Ca, Na, P and Mg were the most abundant minerals found in the vinegar (Table 3). Grape vinegar was richer than apple vinegar in terms of the amounts of these minerals. The concentrations of Cu and Cr in the vinegar were <0.006 and <0.005 µg/g, respectively.

Volatile compounds of the vinegars were presented in Table 4. A total of 9 and 18 volatile compounds were found in apple and grape vinegar, respectively. While acetic acid and acetoin were the most abundant in apple vinegar, acetic acid, ethyl acetate and 2,4,5-trimethyl-1,3-dioxolane were the most abundant in grape vinegar. Acetic acid constituted 67.50% and 62.37% of apple and grape vinegar, respectively.

Table 5 and Figure 2 show the antibacterial activity of the vinegar against test bacteria. The MIC value of the vinegar was determined against eleven microorganisms (six Gr (+) and five Gr (-) bacteria). Although MIC value for grape vinegar was at 6.25% with MBC values ranged from 6.25% to 12.5%, MIC value for apple vinegar was at 12.5% with MBC values ranged from 12.5% to 25% for all test bacteria. Both grape (6.25%) and apple vinegar (12.5%) had similar MIC and MBC values on *B. cereus*, *S.* Enteritidis, *S.* Enteritidis PT4 and *S.* Typhimurium. The MBC values of grape vinegar and apple vinegar for *L. monocytogenes*, *E. faecalis*, MRSA, *S. aureus*, *P. fluorescens* and *E. coli* O157:H7 were twice the MIC values.

Table 4. The volatile compounds of traditional apple and grape vinegars.

Compounds	Apple vinegar (%)	Grape vinegar (%)
2,3-Butanedione	1.88	ND*
2-Methylbutanal	5.97	ND*
Acetic acid	67.50	62.37
Acetoin	22.72	0.84
3-Methyl-1-butanol	0.41	0.65
2-Methyl-1-butanol	0.36	ND*
6-Methyl-5-hepten-2-one	0.19	ND*
n-Hexanoic acid	0.27	ND*
Phenylethyl Alcohol	0.71	ND*
Acetaldehyde	ND*	0.66
Ethyl alcohol	ND*	0.40
Butan-3-Enoic Acid Methyl Ester	ND*	0.35
Methyl acetate	ND*	1.02
Ethyl Acetate	ND*	27.72
3-Methylbutanal	ND*	0.10
2,4,5-trimethyl-1,3-dioxolane	ND*	20.06
1-Butanol, 2-methyl-, (.+/-.)	ND*	0.83
Isobutyrate <ethyl->	ND*	0.14
Isobutyl acetate	ND*	1.08
Furfural	ND*	0.23
Isovalerate <ethyl->	ND*	0.30
iso-Valeric Acid	ND*	0.38
Isoamyl acetate	ND*	1.46
11-Butanol, 2-methyl-, acetate	ND*	1.39

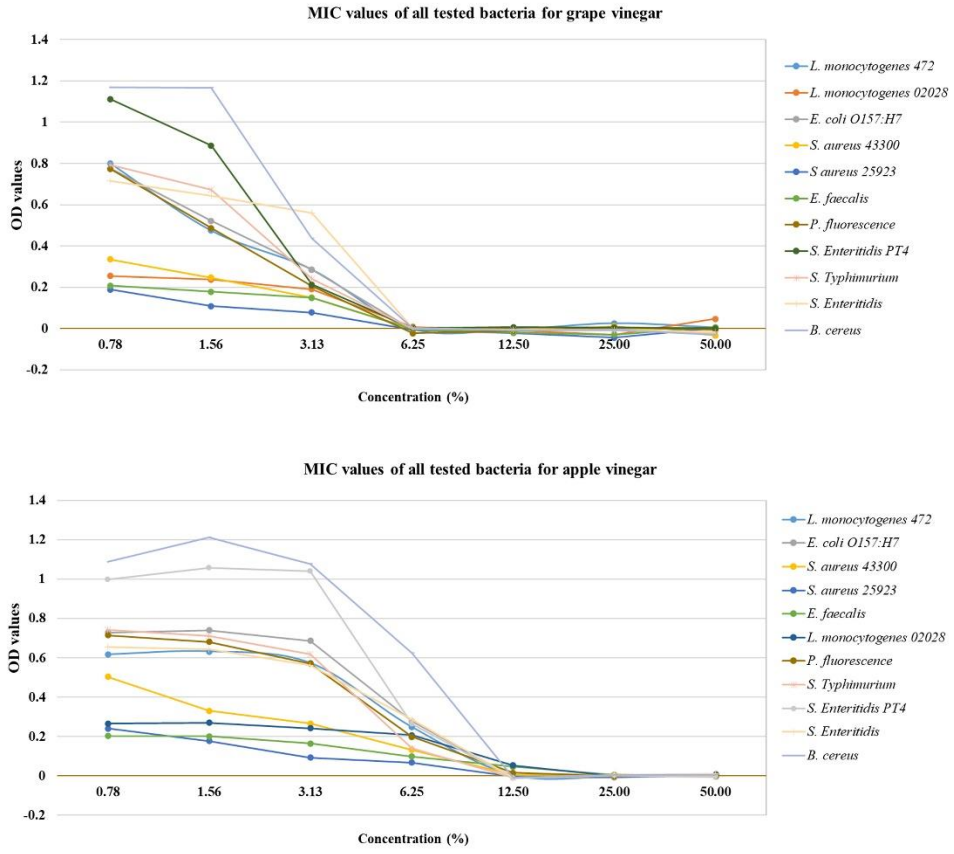

Figure 2. MIC values of grape and apple vinegars for food pathogens.

Table 5. Antimicrobial activity of home-made apple and grape vinegar against some pathogens.

	Strains	Grape vinegar		Apple vinegar	
		MIC (%)	MBC (%)	MIC (%)	MBC (%)
Gr(+)	*B. cereus* ATCC 33019	6.25	6.25	12.5	12.5
	L. monocytogenes RSK 472	6.25	12.5	12.5	25
	L. monocytogenes RSK 02028	6.25	12.5	12.5	25
	E. faecalis ATCC 29212	6.25	12.5	12.5	25
	S. aureus ATCC 43300	6.25	12.5	12.5	25
	MRSA ATCC 25923	6.25	12.5	12.5	25
Gr(-)	*S.* Enteritidis ATCC 13076	6.25	6.25	12.5	12.5
	S. Enteritidis PT4 NCTC 13349	6.25	6.25	12.5	12.5
	S. Typhimurium ATCC 14088	6.25	6.25	12.5	12.5
	P. fluorescens ATCC 13525	6.25	12.5	12.5	25
	E. coli O157:H7 ATCC 35150	6.25	12.5	12.5	25

MRSA: Methicillin-resistant *Staphylococcus aureus*.

Discussion and Conclusion

The search for potential alternatives to antibiotic has become more important due to the increasing occurrence of antimicrobial resistance among bacteria (41). Plants or plant extracts may contain active ingredients having antibacterial properties (27, 45). Vinegar is fermented traditional plant-based products and have several functional therapeutic properties such as antimicrobial, antioxidant due to the presence of active substances from plants. A wide variety of different vinegars are produced from raw materials of different agricultural origin containing starch and sugars around the world (35, 49).

Depending on its origin and production methods, pH values of vinegar may differ. In this study, pH values in grape and apple vinegar varied from 2.36 to 3.27 which were in line with previous studies (1, 18). pH value was lower in grape vinegar than in apple vinegar. Total acidity is an important indicator for assessing the quality of vinegar. Although commercial vinegar should comply with the national standard, it is not always possible to obtain standard acidity values for home-made vinegar. In the current study, the vinegar samples did not comply with regulatory limits for total acidity (total acidity ≥40g/L) (44).

In general, our findings showed that K, Ca and Na were the most abundant minerals present in both vinegar, and grape vinegar was rich in the minerals ranked as K, Ca, Na, Mg and P in a descending order, respectively, compared to apple vinegar. The results were consistent with previous study (1,35). Cu level of <0.006 µg/g in the vinegar samples was in conformity with the maximum limit (for Cu+Zn), which was 10 mg/L, approved by Turkish Food Codex (4).

Phenolic compounds in vinegar mainly derived from raw material used in the preparation of the vinegar and are the major ingredients for the antioxidant activities of the vinegar. The phenolic compounds contain one or more hydroxyl groups attached directly to the aromatic ring and are acknowledged as strong antioxidants playing an important role in pharmacological properties such as antibacterial (29, 50). Many studies demonstrated that presence of phenolic compounds in vinegar promoted their antibacterial activity (7, 37). Production methods and raw material used in the preparation of vinegar may result in differences in the phenolic composition of the vinegar (8, 12). In addition, it has been reported that vinegar made from Uluğbey Karası grapes exhibited high antioxidant activity due to its phenolic substances and red delicious apple had high phenolic compounds compared to other kinds of apples (12, 15).

The findings of this study showed that the TPC value for the grape vinegar were near 2.5 times higher than the apple vinegar. Similarly, the concentrations of all phenolic compounds were higher in the grape vinegar compared to the apple vinegar. Ozturk et al. (35) measured TPC and antioxidant activity of traditional home-made vinegar collected from different regions of Turkey. They obtain variable result in terms of TPC and antiradical activity levels. Although the TPC and antiradical activities measured in grape and apple vinegar samples were in a wide range, similar to our study, in general they found higher TPC and antiradical activity in grape vinegar than apple vinegar. These differences may be result of different type and composition of the raw material of vinegar.

Kelebek et al. (26) analyzed three batches of eight apple vinegar samples and eight grape vinegar samples of different brands, produced with biological fermentation. The grape vinegar was produced from grapes obtained from the Aegean region, while the apple vinegar was produced from apples obtained from Central Anatolia Region. The concentration of gallic acid ranged in grape and apple vinegar from 7.45-21.84 and 0.47-2.57 mg/L,

respectively. We measured gallic acid values 10.8 mg/L in grape vinegar and 4.5 mg/L in apple vinegar. The gallic acid content in grape vinegar depends on the origin of the wine and on the enological techniques to which it has been subjected. Their result also showed that antioxidant activity of grape vinegar changed between 5.39 and 14.43 and the antioxidant activity of apple vinegar changed between 2.65 and 14.69 (mMTrolox/L). Contrary to their findings, we measured higher antiradical activity in grape vinegar than apple vinegar. Liu et al. (31) determined the antioxidant activities, TPC of 23 commonly-consumed fruit vinegar. The TPC values of fruit vinegar ranged from 29.64 to 3216.60 mg GAE/L. The fruit vinegar with the highest TPC values were balsamic vinegar (3216.60 mg GAE/L). Although TPC values were higher than our results, similarly, they also report that red wine vinegar had higher (ranged between 993.51- 654.95 mg GAE/L) TPC values than apple vinegar (495.52 mg GAE/L).

The results of this study indicated that grape vinegar displayed significantly higher TPC values and antiradical activity compared to apple vinegar. In addition, TPC values and antiradical activity were found to be higher in 1/1 and 1/2 diluted grape vinegar than in 1/1 diluted apple vinegar, probably due to the phenolic contents, as we measured gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, p-coumaric acid values much higher in grape vinegar. In this study, the effects of dilution of vinegar on antiradical activity and TPC were also examined. TPC values decreased in both grape and apple vinegar in proportion to dilution (Table 2). Antiradical activity also decreased depending on the dilution (Table 2). Interestingly, antiradical activity did not reduce at the same rate of the dilution. While there was a 36% reduction of antiradical activity of apple vinegar, it was measured as approximately 67.2% in the grape vinegar in 1/10 dilution. The changes in antiradical activity depending on dilution of samples were similar to the results of the study conducted by Aydin and Gokisik (5). Many studies indicated that the antioxidant capacity of vinegar was highly correlated with their phenolic contents being affected by the raw materials and manufacturing processes (6, 47). Similarly, a correlation was found between phenolic content and antiradical activity in this study.

Organic acids, volatile compounds and other fermentation products in vinegar play a role on its organoleptic and antimicrobial properties (13, 43). Organic acids are used in food industry applications to control pathogenic bacteria (36). All test microorganisms were found sensitive to both vinegar samples and grape vinegar has exhibited higher antibacterial activity than apple vinegar. Amongst the tested bacteria, While *Salmonella* spp. and *B. cereus* were more sensitive to both grape vinegar and apple vinegar, *S. aureus*, MRSA, *L. monocytogenes*, *E. faecalis*, *P. fluorescens* and *E. coli* O157:H7 were less sensitive to both grape vinegar and apple vinegar. A large number of studies indicated that home-made vinegar had antibacterial activity on a wide range of food pathogens (19, 23, 24, 35, 38). Vinegar is rich in polyphenols such as gallic, protocatechuic, chlorogenic, caffeic acids and organic acids such as citric, malic, tartaric, lactic, acetic and succinic acids, which are responsible for antimicrobial activity (31, 48). Organic acids act on bacteria via destruction of the outer membrane of bacteria, inhibition of macromolecular synthesis and increase in intracellular osmotic pressure (16). Polyphenols show antibacterial activity by alteration in the permeability of the bacterial cell wall, in various intracellular functions and the cell wall rigidity (11). In this study, the high antimicrobial activity of grape vinegar may be related to having high level of organic acid and phenolic compounds in grape vinegar compared to apple vinegar. Antibacterial potential of the vinegar depends on the amount of the organic acid and phenolic compounds and the findings of this study are consistent with previous studies (23, 24, 35, 38). Bioactive properties of the vinegar can change according to the type and composition of the raw material. The differences in the antibacterial activity between apple and grape vinegar were attributed to their different organic acids and other compositions present in the vinegar.

In this study, the honey, which is a monofloral honey produced in the lavender fields in Isparta province, was included before the initial fermentation step (Figure 1). The honey is rich in antioxidants including phenolic acids, organic acids and flavonoids that exhibit antioxidant activity (9). Honey also exhibits antibacterial activity against a large diversity of bacteria due to its high sugar content and low pH level (3, 32). In the current study, the honey added in the vinegar production process may have contributed to the antiradical and antibacterial activity of the vinegar due to its components such as sugars, flavonoid, phenolic acid and organic acid.

The antibacterial activity of the vinegar varies for each bacterial species, and the activity depends on the raw material used for vinegar production. In this study, home-made grape vinegar contained higher amounts of organic acids and phenolic compounds than apple vinegar, therefore it had a stronger antibacterial effect on food pathogens. The antibacterial activity was in a positive correlation with the concentration of phenolic compounds and organic acids. The vinegar samples had high antiradical activity; grape vinegar showed stronger activity than apple vinegar. Antiradical capacity of the vinegar was related with their phenolic, volatile and organic acid contents. Our findings confirmed that home-made vinegar had significant bacteriostatic and bactericidal activities on several pathogenic bacteria.

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Ethical Statement

This study does not present any ethical concerns.

Conflicts of Interest

The authors declare no conflict of interest.

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The evaluation of the therapeutic potential of hesperetin on diethylnitrosamine and phenobarbital induced liver injury in rats

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Abstract: Nitrite and amine reactions can occur rapidly and produce nitrosamines, in-vivo. Diethylnitrosamine (DEN) and phenobarbital (PB) are readily inducing liver injury and hesperetin (HES), as a flavonoid found in citrus fruits, have the potential to compensate for their harmful effects. In this study, the therapeutic effects of HES were evaluated in DEN and PB mediated liver defect. Adult male Sprague-Dawley rats were split into 5 groups (n=10): Control, DEN, DEN+PB, HES, and DEN+PB+HES. 150 mg/kg DEN was applied intraperitoneally to DEN groups. Fifteen days after the DEN application 500ppm of PB was given in drinking water. HES were administered at 50 mg/kg dose orally for 8 weeks. Blood and liver malondialdehyde (MDA), glutathione (GSH) levels, and catalase (CAT), superoxide dismutase (SOD) activities were measured spectrophotometrically. Moreover, histologic examination of liver sections and apoptosis were determined with hematoxylin-eosin and TUNEL methods, respectively. DEN-PB application was found to increase blood and liver MDA levels and liver CAT activity, oppositely, decreased blood and liver SOD activity, GSH levels, and blood CAT activity. HES was found to have a positive impact on oxidative stress parameters by decreasing liver and blood MDA activity, increasing blood CAT and SOD activity together with liver GSH levels and SOD activity. Whereas DEN and PB application increased all histopathological findings and TUNEL positive cells, HES administration decreased these findings which might be important for the protection of liver cell structure from cell damage. These results suggest that HES administration could be an alternative therapeutic approach to liver damage.

Keywords: Apoptosis, diethylnitrosamine, hesperetin, oxidative stress, phenobarbital.

Hesperetin'in, sıçanlarda dietilnitrozamin ve fenobarbital kaynaklı karaciğer hasarı üzerindeki terapötik potansiyelinin değerlendirilmesi

Özet: Nitrit ve amin reaksiyonları hızla meydana gelir ve in-vivo olarak nitrozaminler üretir. Dietilnitrosamin ve fenobarbital kolayca karaciğer hasarına neden olur ve turunçgillerde bulunan bir flavonoid olan Hesperetin (HES) bu zararlı etkileri telafi etme potansiyeline sahiptir. Bu çalışmada, HES'in tedavi edici etkileri DEN ve PB ile oluşturulan karaciğer hasarında değerlendirildi. Yetişkin erkek Sprague-Dawley sıçanlar 5 gruba (n=10) ayrıldı: Kontrol, DEN, DEN+PB, HES ve DEN+PB+HES. DEN gruplarına 150 mg/kg DEN intraperitoneal olarak uygulandı. DEN uygulamasından 15 gün sonra DEN+FB grubu ve DEN+FB+HES gruplarına, içme suyu içerisinde 500 ppm FB verildi. HES ve DEN+PB+HES grupları, 8 haftalık deney sırasında 50 mg/kg HES oral olarak uygulandı. Kan ve karaciğer MDA, GSH seviyeleri ve CAT, SOD aktiviteleri spektrofotometrik olarak ölçüldü. Ayrıca karaciğer kesitlerinin histolojik incelemesi ve apoptoz sırasıyla Hematoksilen-eozin ve TÜNEL yöntemleriyle belirlendi. DEN ve PB uygulamasının, karaciğer CAT aktivitesi ile birlikte kan ve karaciğer MDA seviyelerini arttırdığı, bunun aksine, kan ve karaciğer SOD aktivitesini, GSH seviyelerini ve kan CAT aktivitesini azalttığı belirlendi. HES'in oksidatif stres parametreleri üzerine, hem karaciğer hem de kan MDA aktivitesini azaltarak, ayrıca karaciğer glutatyon seviyeleri ve SOD aktivitesi ile birlikte kan katalaz ve SOD aktivitesini artırarak olumlu bir etkisi olduğu bulunmuştur. DEN ve PB uygulaması tüm histopatolojik bulguları ve tünel pozitif hücreleri artırırken, bunun tersine HES uygulaması, karaciğer hücre yapısının hücre hasarından korunması için önemli olabilecek bu bulguları azaltmıştır. Bu sonuçlar, HES uygulamasının karaciğer hasarına karşı alternatif bir terapi yaklaşımı olabileceğini düşündürmektedir.

Anahtar sözcükler: Apopitoz, dietilnitrozamin, hesperetin, oksidatif stres, fenobarbital.

Introduction

Nitrosamines may present in cigarettes, alcoholic beverages, smoked seafood, processed meat products, and generated in foods by a reaction that occurs between amines and nitrite (7, 13, 26, 27, 29). Nitrite is used to preserve processed meat products from the generation of clostridium toxins (26). For this reason, processed meat products and other products as well, may contain high nitrosamine content which can be hazardous due to the ROS forming capacity (26). Moreover, DEN, which is a nitrosamine compound, has been indicated to be produced in stomach due to the activity of some bacteria in acidic condition (26, 35). The animal experiments revealed that DEN application cause liver and other organ damages (29, 30, 35). Histologic evaluation of animals treated with DEN was demonstrated to cause inflammatory cell infiltration, bi-nucleated cells, and sinusoidal dilatation in the liver (33, 35). These features are the reason for using DEN to initiate experimental liver damage (7, 36). PB, on the other hand, promotes liver cell proliferation and tumor formation following DEN administration and together with DEN are widely used agents in liver toxicity and tumor studies, as initiators and promoters (38, 45). Oxidative stress is regarded as one of the leading cause of the majority of the liver disorders (5). ROS forming capacity of DEN can be detrimental due to the accumulation of ROS in liver cells, which further might stimulate cell death (27, 30, 36). ROS accumulation in the liver cell causes a deficiency of antioxidant such as GSH (30). Disrupted balance between oxidant and antioxidant result with oxidative stress (42, 47). Counteracting oxidative stress via potential antioxidant may have beneficial effects on DEN and PB induced liver damage (40).

Nitrosamine exposure either directly with consumed food or indirectly via the reaction occurs in body indicates that avoiding intake of nitrosamines is highly challenging. Instead of avoiding its intake, reducing its strength may be more effective. Growing attention has been paid to evaluate the effects of the phytochemicals as therapeutic agents (13). Flavonoids attract the attention of scientists due to major antioxidant capacity (10). HES is one of the most important flavonoids that exists in citrus fruits, possesses antioxidant, antiapoptotic, and anticancer properties (10, 16, 25, 32). Lipophilic feature of HES provide facile entrance to the cells, and just as other flavonoids, HES presents in blood as sulfate conjugate (14). All these features may be important for contributing antioxidant potential of HES. In this concept, the present study purposed to evaluate the therapeutic effects of HES on DEN-PB induced liver defect in rats.

Materials and Methods

Chemicals and reagents: DEN (CAS No: 55-18-5), PB (CAS No: 50-06-6), Trichloroacetic acid (CAS No: 76-03-9), 2-Thiobarbituric acid (CAS No: 504-17-6), 5,5′-Dithiobis-2-nitrobenzoic acid (CAS No: 69-78-3), nitrobluetetrazolium (NBT) (CAS No: 298-83-9), Hydrogen Peroxide (CAS No: 7722-84-1) were purchased from Sigma-Aldrich (St . Louis MO, USA).

Experimental design: Fifty adult male approximately 220 ± 20 g weight, Sprague-Dawley rats were utilized in this study. Standard pellet diet and tap water were ad libitum given to the animals. Subjects were divided into 5 groups as control, DEN, HES, DEN+PB, and DEN+PB+HES group. The control group was provided with standard diet and received tap water for 8 weeks. DEN, DEN+PB and DEN+PB+HES groups were intraperitoneally (i.p.) injected with 150 mg/kg DEN (21). Subsequently, DEN+PB and DEN+PB+HES groups were given 500 ppm PB in drinking water for 6 weeks in pursuit of the DEN application (20). DEN+PB+HES and HES groups received HES at a dose of 50 mg/kg/day by oral gavage during 8 weeks experimental procedure (2). Following the 8 weeks experimental period, blood samples were taken from the heart under the anesthesia subsequently the rats were euthanized and liver samples were collected.

Blood sample preparation: Blood samples were taken into ethylene diamine tetraacetic acid containing sample tubes (two tubes for each subject). The first blood sample containing tube was kept for whole blood analyses. The second was centrifuged for 5 minutes at 3500 rpm (Nuve NF800R); plasma was taken and maintained at -80^{o}C until analyses. The red blood cell suspension in the bottom of the second tube were washed thrice with 0.9% NaCl and kept at -80^{o}C before used. MDA and GSH levels together with CAT and SOD activities were evaluated spectrophotometrically.

Liver sample preparation: Liver tissues were cleaned in 0.9% NaCl for biochemical analysis and kept at -80^{o}C. Tissue homogenization was made in Potter-elvehjem homogenizer by using 1.15% KCl containing buffer to have 1/10 (weight/volume) final volume of whole homogenate. The whole homogenate were centrifuged at 3500 rpm for 15 minutes to analyze MDA, GSH levels and CAT, SOD activities. MDA and GSH levels also CAT and SOD activities were evaluated spectrophotometrically in liver tissues.

Liver and blood sample analyzes: MDA were assayed in the taken plasma and homogenate, according to the modified method of Placer et al. (28). This method is based on the adduction of thiobarbituric acid with MDA which is one of the aldehyde products of lipid peroxidation and the formation of pink complex (28). The absorbance values of MDA (plasma and liver) were determined according to the pink complex generated. The MDA levels were read at 532 nm and expressed as nmol/mL and nmol/g respectively in plasma and liver tissues.

GSH levels were determined in the whole blood which was diluted with purified water before the analyzes and also in liver tissue supernatant according to the method described by Chavan et al. (9). Briefly, GSH levels were determined with the reaction which cause the formation of the yellow color following the addition of 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB). Subsequently, yellow color formed in the samples was read at 412 nm. GSH levels were expressed as µmol/g Hb and µmol/g protein respectively in the whole blood and liver tissues.

The liver tissues supernatant and washed erythrocytes were diluted with deionized water and used for SOD activity determinations. SOD activity was measured according to a reaction based on blue color formation which occurs as a result of the conversion of nitrobluetetrazolium (NBT) to formazan with the activity of xanthine-xanthine oxidase system. The exhibited blue color was measured at 560 nm. SOD enzyme activity was expressed as U/g Hb and U/g protein respectively in diluted erythrocytes and liver tissues (39).

Washed erythrocytes were diluted with deionized water first and then were diluted with phosphate buffer (50mM, pH:7) for CAT activity determinations. Liver tissues supernatant were diluted with phosphate buffer (50mM, pH:7) for CAT activity determinations. CAT activity was measured in diluted erythrocytes and liver tissues supernatant according to Aebi method (1). Aebi method is based on the consumption of H_2O_2 by CAT enzyme activity at 240 nm wavelength. CAT enzyme activity was expressed as katal (k)/g Hb and k/g protein respectively in diluted erythrocytes and liver tissues.

Histological evaluation: The liver tissue samples were fixed in 10% formaldehyde for about 24 hours for histological analysis. Upon fixation was done, the tissues were embedded in paraffin blocks and the blocks were cut into 5-6 µm thickness. The tissue sections were stained with hematoxylin eosin (H&E) (11). Four parameters of liver injury 1-double nucleated hepatocyte numbers, 2-congestion, 3-inflammatory cell infiltration and 4-sinusoidal dilation the severity were semi-quantitatively evaluated. These parameters were scored as 0= absent, 1= weak, 2= moderate and 3= strong for each parameter (18).

TUNEL assay: The tissues were fixed in 4% paraformaldehyde and embedded in paraffin, then cut into serial 5-6 µm-thick sections and placed on poly-L-lysine coated glass slides. ApopTag Plus Peroxidase, Apoptosis Detection Kit (S7101) was used for the determination of apoptotic cells following the manufacturer's guideline. Sections were visualized with 3, 3-diaminobenzidine tetra hydrochloride (DAB) and counterstained with Harris hematoxylin. TUNEL positive cells and total cells were counted from number of randomly selected fields at 200x magnification from each section under light microscopy (Novel N 800 M).

In each microscopic field of view, 500 cells were counted in 20 randomly selected fields. Counted brown and blue nuclei stained cell number used to calculate apoptotic index and this index was given as the ratio of the TUNEL – positive (brown stained nuclei cells) cell number to the total cell number (brown and blue stained cells) (18).

Statistical analyses: Descriptive statistics presented as mean ± standard error for continuous variable. The homogeneity assumption was checked with Levene test and the normality assumption was tested with Shapiro-Wilks test and the coefficient of variation. As all of the continuous dependent variables are violated the homogeneity and normality assumption, Kruskal Wallis test was performed in order to indicate the difference among the five experimental groups. In case any statistical difference, Mann-Whitney U test with Bonferroni correction were used as post-hoc tests. Values less than 0.05 are considered to be statistically significant. All statistical analyses were performed by using IBM SPSS Statistics for Windows, Version 22.0.

Results

Compared to control, DEN and PB application remarkably increased blood and liver MDA levels ($P<0.05$). While blood CAT activity decreased in DEN+PB group, liver CAT activity increased significantly. DEN administration followed by PB treatment resulted with decreased GSH levels both in blood and liver. Blood SOD activity decreased approximately two fold, while liver SOD activity statistically remained unchanged (Table 1, 2).

Compared to DEN+PB group, HES administration decreased blood and liver MDA levels significantly in DEN+PB+HES groups ($P<0.05$). While blood CAT activity increased significantly in DEN+PB+HES group ($P<0.01$), the increase in liver CAT activity was not statistically significant ($P>0.05$). Although, blood GSH levels remained unchanged in DEN+PB+HES group, liver GSH levels increased significantly ($P<0.05$). Both blood and liver SOD activity increased in DEN+PB+HES group ($P<0.05$, Table 1, 2). Hepatocytes in liver of control group were normal in histological appearance (Figure 1A).

DEN administration caused sinusoidal dilatation and an increasing number of double nucleated hepatocytes in the periportal area. Additionally, in the same area pronounced inflammatory cells together with mild congestion have been observed (Figure 1B). Compared to the DEN group, PB addition turned out to increase all histopathological findings and the number of large nucleated hepatocytes in the DEN+PB group (Figure 1C). In the DEN+PB+HES group a decrease was observed in all histopathologic changes especially in terms of sinusoidal changes and congestion (Figure 1D and E). Histological changes were not observed in liver of HES

group compared to that of control group (Figure 1F). Histological scores were shown in Table 3.

TUNEL examination revealed that the number of TUNEL positive cells significantly increased in the DEN and DEN+PB groups compared to the control group (Figure 2). Moreover, a significant decrease was observed in the TUNEL positive cell number of DEN+PB+HES group compared to DEN+PB group (P<0.05, Table 4).

Table 1. Effects of Hesperetin on blood/erythrocytes/plasma antioxidants enzymes, glutathione (GSH) and malondialdehyde (MDA) levels in DEN+PB induced liver damage in rats.

Items	Groups				
	Control	**DEN**	**DEN+PB**	**HES**	**DEN+PB+HES**
MDA (nmol/ml plasma)	10.39±0.45^{b}	10.11±0.69^{b}	12.51±0.73^{a}	5.79±0.13^{c}	10.40±0.13^{b}
CAT (k/g Hb)	66.43±8.23^{a}	69.24±5.57^{a}	40.75±3.89^{b}	67.54±7.52^{a}	77.41±3.58^{a}
GSH (µmol/g Hb)	62.77±4.69^{b}	40.84±3.80^{c}	45.20±3.62^{c}	76.14±4.64^{a}	47.77±4.32^{bc}
SOD (U/g Hb)	94.11±14.17^{a}	78.50±7.42^{ab}	56.85±4.66^{b}	95.13±3.15^{a}	108.34±5.61^{a}

* The data presented as means and standard error.
* $^{a,b,c:}$ Values with different letters in the rows indicates statistically difference between groups (P<0.05).

Table 2. Effects of Hesperetin on liver antioxidants enzymes, glutathione (GSH) and malondialdehyde (MDA) levels in DEN+PB induced liver damage in rats.

Items	Groups				
	Control	**DEN**	**DEN+PB**	**HES**	**DEN+PB+HES**
Liver MDA (nmol/g tissue)	21.06±0.42^{d}	29.44±0.68^{b}	64.06±1.2^{a}	25.63±0.46^{c}	26.94±1.33^{bc}
Liver CAT (k/g protein)	175.21±5.45^{cd}	148.32±4.29^{d}	196.17±2.51^{bc}	277.56±15.08^{a}	210.65±3.51^{b}
Liver GSH (µmol/g protein)	627.82±10.23^{c}	544.61±19.07^{d}	485.54±7.90^{d}	1185.23±12.47^{b}	1045.77±24.78^{a}
Liver SOD (U/g protein)	117.50±2.98^{b}	107.39±3.33^{b}	112.23±5.45^{b}	151.54±2.17^{a}	146.14±1.11^{a}

* The data presented as means and standard error.
* $^{a,b,c:}$ Values with different letters in the rows indicates statistically difference between groups (P<0.05).

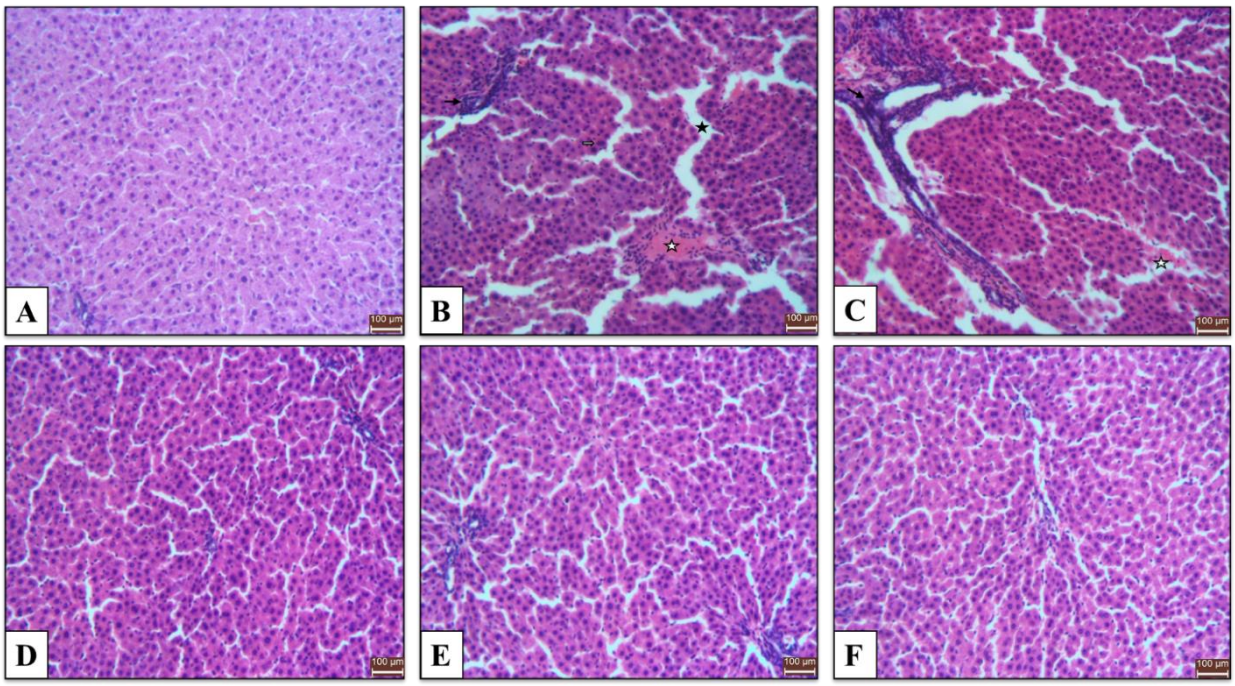

Figure 1. Hesperetin Effect on Liver Histopathology of diethylnitrosamine and phenobarbital induced damage in rats.
A: Control group. Normal hepatocytes and sinusoids.
B: DEN group. Sinusoidal dilation (black star), inflammatory cell infiltration in periportal area (black arrow), congestion (white star) and double nucleated hepatocytes (white arrow).
C: DEN + PB group. Inflammatory cell infiltration in periportal area (black arrow) and congestion (white star).
D and E: DEN + PB + HES group. All histopathological changes, especially sinusoidal dilatation and congestion, were decreased.
F: The HES group. Normal hepatocytes and sinusoids. H & E x200.

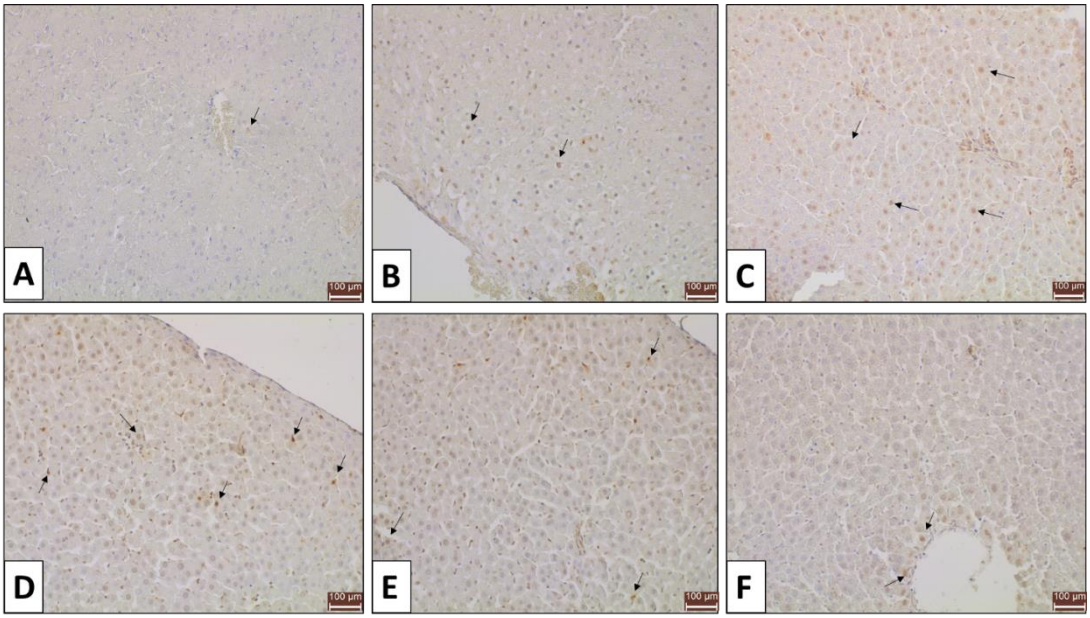

Figure 2. Hesperetin effect on Liver Apoptosis Index of diethylnitrosamine and phenobarbital induced damage in rats. Comparison of TUNEL positivity (brown nucleus) among groups.
Control (A) DEN (B) DEN+PB (C, D) DEN+PB+ HES (E) HES (F)

Table 3. Effects of Hesperetin on Liver Histopathology of diethylnitrosamine and phenobarbital induced damage in rats.

Groups	Increase of Double Nucleated Hepatocyte Numbers	Congestion	Inflammatory Cell Infiltration	Sinusoidal Dilation
Control	0.40±0.54[b]	0.40±0.54[b]	0.20±0.44[b]	0.20±0.44[b]
DEN	2.00±0.70[a]	2.00±0.00[a]	1.80±0.44[a]	1.60±0.54[a]
DEN+PB	3.00±0.00[a]	2.80±0.44[a]	2.80±0.44[a]	2.60±0.54[a]
DEN+PB+HES	1.00±0.70[b]	1.00±0.70[b]	1.00±0.70[b]	0.40±0.54[b]
HES	0.60±0.54[b]	0.60±0.54[b]	0.40±0.54[b]	0.40±0.54[b]

* Values are means ± standard deviation. [a] P<0.05 Control vs. all group. [b] P<0.05 DEN+FB vs. all group.

Table 4. Effects of Hesperetin on liver apoptotic index (%) in DEN+PB induced liver damage in rats.

Item	Groups				
	Control	**DEN**	**DEN + PB**	**HES**	**DEN+PB+HES**
Apoptotic Index (%)	4.8 ± 2.73	18.4 ± 3.12[ab]	25.2 ± 3.15[a]	7.2 ± 1.55[ab]	9.83 ± 2.84[ab]

* Values are means ± standard deviation. [a] P<0.05 Control vs. all group. [b] P<0.05 DEN+FB vs. all group.

Discussion and Conclusion

PB promotion after a sub chronic DEN administration is a widely used model in the liver carcinogenesis studies (43, 45). DEN, PB, and their metabolites cause toxicity by inducing the production of ROS, which in return lead to liver and erythrocyte peroxidation (45). Moreover, DEN and PB exposure have been shown to increase lipid peroxidation in rats (35, 43). Consistently with the previous studies, enhanced levels of MDA have been observed in the current study, after DEN and PB application. High MDA levels are widely regarded as an indicator of lipid peroxidation, which occurs due to the surplus generation of ROS (35). Decreased MDA levels after HES administration in the present study both in liver and erythrocytes may be related to the antioxidant properties of HES. A large body of evidence proposes that flavonoids participate in the detoxification process of ROS related damage (10, 16). Revealed properties might arise from the hydrogen donating capacity of flavonoids to reactive substances (8, 22, 31) Moreover, lipophilic features of HES might contribute to the protection of membrane lipids from oxidation and might cause a decrease in MDA levels, which, in turn, might be beneficial for liver therapy.

Shaban et al. (35) found an increase in the number of bi-nucleated cells in rats treated with DEN and PB. In another study DEN and PB were showed to induce inflammatory infiltrate (33). Consistent with these studies (33, 35), in our study double nucleated hepatocytes, inflammatory cells, and mild congestion were determined

to increase, which might be the result of macrophages activation due to DEN and PB stimulated ROS generation. In a recent study demonstrating the effects of HES on hepatic injury in rats, HES was found to improve hepatocyte architecture (12). Similarly, in our study HES was showed to improve hepatocellular architecture in DEN+PB+HES group. Improvement in hepatocytes structure following HES treatment might probably be related to the antioxidant effects of HES.

SOD and CAT are two important antioxidant enzymes responsible for radical scavenging activity (10). SOD, which stands as the first enzymatic defense line against cellular oxidation, is responsible for the conversion of superoxide radicals to hydrogen peroxide. Subsequently, hydrogen peroxide is decomposed to water and oxygen by CAT activity (37). DEN and PB application have been reported to increase superoxide radical generation and solely DEN application has been shown to decrease liver SOD activity (15). Owumi et al (23) reported that DEN administration to 5 weeks old Wistar rats resulted in decreased SOD activity as well. Oppositely, Hussein et al. (19) indicated that SOD activity did not change significantly after the 24 week of the DEN and PB administration. In accordance with the previous studies (19, 23); DEN administration followed by PB decreased blood SOD activity in the current study, while liver SOD activity remained unaltered as Hussein et al. (19) reported. Decreased blood and unaltered liver SOD levels might indicate the imbalance between oxidants and antioxidants. Moreover, these findings might suggest that oxidation in blood has been initiated before that of the liver. Antioxidant defense system is crucial to prevent the deleterious effects of oxidants. SOD and CAT which are two antioxidant enzymes, protect cell membranes from integrity loss. In a study evaluating the effects of HES on DEN induced hepatocarcinoma, it was found that HES increased SOD levels compared to DEN administered group (17). Besides, HES has been reported to increase SOD expressions in mice treated with DMBA (10). These studies disclosed the positive impact of HES on SOD levels. In line with these studies (10, 17), we found that HES increased both blood and liver SOD activities. The aforementioned connection of two antioxidant enzymes, CAT and SOD make two antioxidant enzymes work together against oxidative damage. In a recent study, DEN administration to 5 weeks old Wistar rats was shown to result in decreased CAT activity (23). On the other hand, it was demonstrated that at early stage of DEN and PB administration CAT activity increased in liver (19). Consistent with these studies (19, 23), in our study, while blood CAT activity decreased in DEN+PB group, liver CAT activity increased, yet this increase was not statistically significant. In our study, blood SOD and CAT activities in DEN+PB group, and unaltered liver SOD and CAT activities were found to be in parallel, suggested that while at that time period blood ROS levels might cause the depletion of antioxidant enzymes, yet liver still might have the capability to manage oxidative stress partially. The activity loss in SOD and CAT may result with the oxidation of macromolecules such as membrane phospholipids, proteins, and DNA (4). In the present study, increased ROS levels in the liver may probably evoke the MDA increase due to the unaltered liver SOD and CAT activity following DEN and PB distribution. Moreover, the reason for increased liver CAT activity in the DEN+PB group compared to the DEN group might be related to the reciprocal decrease in GSH levels in the same group. Although CAT and glutathione peroxidase (GPx) both catalyze H_2O_2, still GPx requires GSH additionally (34). Decreased GSH levels in DEN+PB group may probably be related to activity of GPx instead of CAT. Antioxidants, such as SOD and CAT, stand as the first line of the antioxidant defense against the both erythrocytes and liver derived oxidative stress (10).

A recent study evaluating the effects of HES on DEN induced hepatocarcinoma reported that HES increases CAT levels compared to DEN administered group (17). Moreover, different amounts of HES addition to the rats' diet, found to increase not only CAT activity but also SOD activity (3). In the current study HES administration also increased CAT and SOD activity which makes sense with the previous studies (3, 17). HES has been documented to exhibit anti-apoptotic properties (14). According to the current knowledge, the best explained anti-carcinogenic effect of HES is its strong antioxidant properties. In fact, HES has been stated to exert antioxidant properties and ROS scavenging activity via regulating the ERK/Nrf2 signaling pathway (24). Furthermore, Wan et al. (41) reported that HES shows inhibitory effects on apoptosis, oxidative stress, and inflammatory cell infiltration by increasing hem oxygenase-1 activity in acetaminophen-induced liver injury in mice.

GSH is a tripeptide that contains thiol groups and is one of the non-enzymatic antioxidant responsible for the antioxidant protection of the body (37). GPx catalyzes the conversion of H_2O_2 to water and oxygen in the presence of hydrogen donated by GSH (35). Banakar et al. (6), and Shaban et al. (35), have reported decreased levels of GSH following DEN and PB administration consistently to our study findings. Increased ROS formation as a result of DEN and PB metabolism might probably cause increased demand for GSH in the present study. Similarly to other flavonoids, HES is found in the blood as sulfate conjugate (14). In the current study, increased GSH levels following HES application is presumably related to increased demand for sulfate which might be subsequently utilized for the conjugation of HES. Moreover, lipophilic features of HES provide rapid and simple entry inside the cell. Therefore, HES might have induced the antioxidants in the liver cell (14). The onset of numerous ailments is indicated

to be associated with increased ROS formation (43). It has been reported that H_2O_2 exposure may lead to apoptosis by activating ROS generation (4). Furthermore, DEN derived ROS formation has been revealed giving rise to apoptosis (46). HES, on the other hand, has been shown to have anti-apoptotic properties, thanks to its' radical scavenging effects (46). In addition to these properties, HES has been reported to inhibit bile derived acid stimulated cell death in liver cells (5). On the contrary of its anti-apoptotic effects in normal cells, HES has been known to induce apoptosis in various tumors, due to the disrupted balance between oxidant and antioxidant status (44). The elevated apoptotic index found in this study after DEN and PB administration could be explained with increased ROS formation. Similarly to findings of Bai et al. (5), in our study HES blocked apoptosis after DEN and PB application. Even though in same study it has been indicated that solely administration of HES up to 50 $\mu mol \cdot L^{-1}$ inhibit apoptosis, in our study we found that 50 mg/kg/day solely administration of HES induce not only apoptosis but also increased MDA levels in liver. Despite the fact that 50 mg/kg/day HES administration decreased liver apoptosis together with liver MDA levels in DEN+PB+HES group, which might be regarded as reducing effects of HES on liver injury, in normal cells 50 mg/kg/day HES administration induced liver damage which is a limitation of our study.

In conclusion HES gives prominent inhibitory effects on liver injury due to its' strong antioxidant and anti-apoptotic properties. Moreover, improved hepatocellular architecture following HES administration is another beneficial effect of HES. All these findings suggest that HES could be utilized as an alternative therapeutic due to obtained major benefits. Further studies should be organized to shed light on the protective role and therapeutic activities of HES and similar flavonoids. Due to the limitation of the study, a further dose-dependent study should be organized to evaluate the effects of HES on the liver. Furthermore, disclosing the molecular mechanism of how HES enter cells might be beneficial for establishing the therapeutic potential of HES.

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Ethical Statement

This study was carried out following the approval of the Regional Local Ethics Committee at University of Firat, Faculty of Medicine (approval number: 09032016-45/34).

Conflict of Interest

The authors declare that there is no conflict of interest.

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Effects of calving year, season, and age on some lactation traits of Anatolian buffaloes reared at farmer conditions in Turkey

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Abstract: The aim of this study was to investigate the effects of some environmental factors like calving year, season, buffalo cow age and village on some milk yield traits of Anatolian buffaloes, such as daily milk yield for lactation length (MYLL), total lactation milk yield (TLMY), lactation length (LL), and daily milk yield for calving interval (MYCI). 1838 milk yield records obtained from 851 Anatolian buffaloes reared under different environmental conditions from 2015 through 2019 within the scope of the Bartın Anatolian Buffalo Breeding Project were assessed. The least square means and standard errors for MYLL, TLMY, LL, and MYCI were 4.07 ± 0.02 kg, 1078.6 ± 7.54 kg, 263.83 ± 1.16 days, and 2.75 ± 0.03 kg, respectively. As a result of the study, the effects of village, calving year and age (P<0.001) and calving season (P<0.05) on MYLL; village, calving year and age (P<0.001) on TLMY; village, calving year and age (P<0.001) and calving season (P<0.05) on MYCI; calving age, village (P<0.001) and calving year (P<0.05) on LL were found statistically significant. Moreover, high positive phenotypic correlations between TLMY and MYLL (r = 0.78, P<0.001) and LL and TLMY (r = 0.67, P<0.001) were estimated. The results revealed that milk production traits might be improved by considering calving season and age in breeding studies and stud-selection programs in Anatolian buffaloes. Besides, it will remarkably contribute to the number of limited studies that have focused on this breed.

Keywords: Anatolian buffalo, calving age, calving season, calving year, milk yield.

Türkiye'de yetiştirici koşullarında barındırılan Anadolu mandalarında buzağılama yılı, mevsimi ve yaşının bazı laktasyon özelliklerine etkileri

Özet: Bu çalışmanın amacı, Anadolu mandalarında çevresel faktörlerden buzağılama yılı, mevsimi, yaşı ve köyün bazı süt verimi özelliklerinden Laktasyon Süresinde Günlük Süt Verimi (LSSV), Toplam Laktasyon Süt Verimi (TLSV), Laktasyon Süresi (LS) ve Buzağılama Aralığında Günlük Süt Verimi (BASV) üzerine olan etkilerinin araştırılmasıdır. Çalışmada Bartın ili Anadolu mandası ıslah projesinde 2015-2019 yılları arasında yetiştirilen 851 baş Anadolu Mandasına ait 1838 adet süt verim kaydı kullanılmıştır. LSSV, TLSV, LS ve BASV özelliklerine ilişkin en küçük kareler ortalama ve standart hatalar sırası ile 4,07 ± 0,02 kg, 1078,6 ± 7,54 kg, 263,83 ± 1,16 gün ve 2,75 ± 0,03 kg'dır. Araştırma sonucunda LSSV üzerine köyün, buzağılama yılı ve yaşının (P<0,001), mevsiminin (P<0,05); TLSV üzerine köyün, buzağılama yılı ve yaşının (P<0,001); BASV üzerine köyün, buzağılama yılı, yaşı (P<0,001) ve mevsiminin (P<0,05); LS üzerine buzağılama yaşı, köy (P<0,001) ve buzağılama yılının (P<0,05) etkisi önemli bulunmuştur. Aynı zamanda TLSV ile LSSV (r = 0,78, P<0,001) ve LS ile TLSV (r = 0,67, P<0,001) arasında yüksek pozitif fenotipik korelasyonlar hesaplanmıştır. Sonuçlar, buzağılama mevsimi ve buzağılama yaşının yapılacak olan ıslah çalışmalarında ve damızlık seçim programlarında dikkate alındığında Anadolu mandalarının süt verimi özelliklerinin iyileştirilebileceğini ortaya çıkarmıştır. Ayrıca bu çalışma, bu ırka yönelik sınırlı sayıdaki çalışma sayısının artırılmasına önemli katkılar sağlayacaktır.

Anahtar sözcükler: Anadolu mandası, buzağılama mevsimi, buzağılama yaşı, buzağılama yılı, süt verimi.

Introduction

The buffalo (*Bubalus bubalis*) population was 173 million throughout the world in 2015 and reached 206.6 million by 2018, an increase of 19.4% (4). Buffaloes were originally reared in Asia but are now commonly reared farm animals worldwide for their milk (8). The total buffalo milk yield worldwide in 2018 was 127.3 million tons, which constitutes around 15.6% of total milk production; therefore, these animals are second to only dairy cows in milk production (4). The Anatolian

buffaloes reared in Turkey is originated from the Mediterranean buffaloes, a sub-group of river in buffaloes (11). The population in Turkey in 2010 was 84726 and reached 184192 in 2019 with the aid of the Anatolian Buffalo Breeding Project conducted by the Ministry of Agriculture and Forestry (5). The buffaloes in Turkey are mostly reared in the North, Middle, West, East, and Southeast Anatolia regions. They hold an important place in husbandry due to their resistance to environmental stresses and diseases, great ability to utilize feed, converting even low-quality rough feed into meat and milk, and lower cost of husbandry than cattle (6).

The most important buffalo yield is their milk, which is distinguished from the milk of other animals by its high dry matter and particularly fat content. Buffalo milk is used in the production of yogurt, cream, cheese, and ice cream. The composition of the Anatolian buffalo milk has been reported to be made up of 18.19% dry matter, 7.92% fat, 5.14% lactose, and 4.09% protein (27). In Turkey, 79000 tons of buffalo milk was produced in 2019 (5). The total milk yield from buffaloes depends on both genetic and non-genetic factors such as season, management, and feed amount and quality (1, 22). The milk yield traits are also affected by several environmental factors, such as calving age and season (19). TLMY and LL are important parameters in milk production of these animals (10).

It is aimed this study that is to investigate the opportunities for increasing the milk yield, raising the incomes of farmers, implementing selection studies and stud selection programs through examining the effects of calving year, season, age, and village on some milk yield traits like MYLL, TLMY, LL, and MYCI of Anatolian buffaloes.

Materials and Methods

The research material of the study constitutes of the pedigree records of 851 Anatolian buffalos that were included in the Project conducted in Bartın, its districts, and 48 villages (41° 38′ 29″ N and 32° 19′ 58″ E) with the support of the General Directorate of Agricultural Research and Policies. In that study, 1838 milk yield records obtained on the control day from Anatolian buffaloes that calved during 2015–2019 were used. The data on milk yields were obtained from the 'Manda Yıldızı' data recorder system in which the technical staff working within the body of the Project entered the data (32).

Milk was produced from buffaloes at individual farms that were included in the project. The buffaloes on these farms are milked twice a day, in the morning and evening. The buffalo breeding in the region is conducted extensively and in a manner of management and feeding that is similar among the farms. Within the farms, the buffalo cows are naturally inseminated by the bulls. Buffaloes are manually hand-milked on most of the farms, although a small number of farms use milking machines. The milk controls from the buffaloes were collected monthly using scales with a sensitivity of 10 g/50 kg. The milk yields were recorded in kilograms from the morning and evening milkings. Anatolian buffaloes data, which were recorded at least 5 test days for each lactation, were included in the analyzes (12, 34).

The study comprised milk yield traits including MYLL for TLMY/LL, MYCI for TLMY/CI (16, 33), TLMY, and LL. In the study, the records between 147≤LL≤404 days for LL and 300≤CI≤700 days (20) for CI were evaluated. In this study, Alkoyak and Öz's (2) findings obtained in a study carried out in the same study area were also used. The calving years were grouped into those between 2015 and 2019. The calving seasons were divided into four groups according to the geo-climatic conditions in Turkey as follows: (1) winter (December, January, and February), (2) spring (March, April, and May), (3) summer (June, July, and August), and (4) fall (September, October, and November). The calving ages were divided into the following five groups: (1) 3–4 years, (2) 5–6 years, (3) 7–8 years, (4) 9–10 years, and (5) ≥11 years. The study was conducted in 48 villages (The villages are listed numerically from 1 to 48). The environmental factors that were investigated for their effects were the calving year, season, age and village on MYLL, TLMY, LL, and MYCI and were determined using the least-square method. The phenotypic correlation was calculated using the Pearson correlation coefficient. For the statistical analyses, the general linear model (GLM) using Minitab ver. 18 was used. The differences between the mean values were analyzed using Tukey's multiple comparison test (3). Since the inadequate data at subgroups, two or three-way interactions were not included in analyzes.

The equation and statistical model below were used to examine the effects of the environmental factors on MYLL, TLMY, LL, and MYCI.

$$Y_{ijklm} = \mu + Y_i + S_j + A_k + V_l + e_{ijklm},$$

where, Y_{ijklm} is the quantities of traits obtained from the individual buffaloes (i. year, j. season, k. age, l. village, m. observation value for an investigated trait); μ is the overall (expected) mean value; Y_i is the effect of i^{th} calving year (i = 2015, 2016, 2017, 2018, 2019); S_j is the effect of j^{th} calving season (j = 1,2,3,4); A_k is the effect of k^{th} calving age (k = 1,2,3,4,5); V_l is the effect of l^{th} village (l = 1-48) and e_{ijklm} is the random error, presumed to be normally and independently distributed with a mean value of zero and constant variance (NID, 0, σ^2).

Results

The overall mean values and standard errors for MYLL, TLMY, LL, and MYCI are given in Table 1. The

effects of environmental factors including calving year, season and age on MYLL, TLMY, LL, and MYCI were investigated and the standard mean values, errors, and affective factors are provided in Table 2.

As a result of the study, the effects of village, calving year and age (P<0.001) and calving season (P<0.05) on MYLL; village, calving year and age (P<0.001) on TLMY; village, calving year and age (P<0.001) and calving season (P<0.05) on MYCI; calving age and village (P<0.001) and calving year (P<0.05) on LL were found statistically significant. However, the effect of the calving season on TLMY and LL was determined to be non-significant. Since the number of villages studied (48 villages) is too high, it is not given in the Table 2. Variance analysis results for all environmental factors are given in Table 3. Moreover, high positive phenotypic correlations between TLMY and MYLL (r = 0.78, P<0.001) and LL and TLMY (r = 0.67, P<0.001) were calculated.

Table 1. Descriptive statistics for milk yield traits in Anatolian buffaloes.

Paramaters	**MYLL (kg)**	**TLMY (kg)**	**LL (d)**	**MYCI (kg)**
Number of Animals	851	851	851	606
Number of Records	1838	1838	1838	1188
Mean ($\bar{x}$)	4.07	1078.6	263.83	2.75
Standard Error of Mean (SEM)	0.02	7.54	1.16	0.03
Minimum	1.23	294.9	147	0.73
Maximum	8.45	2114.3	404	6.08

MYLL: Daily milk yield for lactation length; TLMY: Total lactation milk yield; LL: Lactation length;
MYCI: Daily milk yield for the calving interval

Table 2. The least square means (±SE) of some milk yield traits for calving year, season, and age in Anatolian buffaloes.

Environmental factors	**MYLL (kg)**		**TLMY (kg)**		**LL (d)**		**MYCI (kg)**	
	n	**(Mean± SEM)**	**n**	**(Mean± SEM)**	**n**	**(Mean± SEM)**	**n**	**(Mean± SEM)**
Calving year								
2015	231	3.16 ± 0.05^{d}	231	856.5 ± 19.1^{d}	231	269.40 ± 3.62^{ab}	94	2.09 ± 0.09^{c}
2016	302	3.64 ± 0.04^{c}	302	964.7 ± 16.2^{c}	302	263.46 ± 3.06^{b}	190	2.34 ± 0.07^{c}
2017	423	4.28 ± 0.04^{b}	423	1148.8 ± 14.2^{b}	423	268.83 ± 2.70^{ab}	261	2.77 ± 0.06^{b}
2018	429	4.59 ± 0.03^{a}	429	1252.5 ± 13.8^{a}	429	273.47 ± 2.60^{a}	324	3.00 ± 0.05^{a}
2019	453	4.64 ± 0.04^{a}	453	1238.7 ± 14.0^{a}	453	266.51 ± 2.65^{ab}	319	3.00 ± 0.06^{a}
p		**		**		*		**
Calving season								
Winter	252	4.07 ± 0.04^{ab}	252	1089.1±17,4	252	266.67±3.30	163	2.50 ± 0.07^{b}
Spring	445	3.99 ± 0.04^{b}	445	1089.0±14.2	445	271.81±2.69	264	2.64 ± 0.06^{ab}
Summer	654	4.10 ± 0.03^{a}	654	1097.5±12.7	654	267.47±2.41	425	2.68 ± 0.05^{ab}
Autumn	487	4.09 ± 0.03^{ab}	487	1093.4±13.5	487	267.38±2.55	336	2.74 ± 0.05^{a}
p		*		NS		NS		*
Calving age (year)								
3–4	416	3.59 ± 0.03^{d}	416	935.2 ± 13.8^{d}	416	259.97 ± 2.62^{b}	67	2.28 ± 0.10^{b}
5–6	591	3.98 ± 0.03^{c}	591	1055.1 ± 12.3^{c}	591	264.09 ± 2.32^{b}	376	2.53 ± 0.05^{b}
7–8	400	4.14 ± 0.04^{b}	400	1113.5 ± 14.5^{b}	400	268.83 ± 2.74^{ab}	351	2.71 ± 0.05^{a}
9–10	277	4.28 ± 0.04^{a}	277	1183.8 ± 17.0^{a}	277	276.33 ± 3.23^{a}	251	2.89 ± 0.06^{a}
≥11	154	4.32 ± 0.06^{a}	154	1173.6 ± 22.3^{ab}	154	272.45 ± 4.22^{ab}	143	2.79 ± 0.08^{a}
p		**		**		**		**

MYLL: Daily milk yield for lactation length; TLMY: Total lactation milk yield; LL: Lactation length; MYCI: Daily milk yield for the calving interval;
NS: non-significant (P>0.05); *P<0.05; **P<0.001; $^{a, b, c, d}$ The different superscripts within a column in a subgroup symbolize the difference significantly (P<0.05).

Tablo 3. Analysis of variance of factors affecting MYLL, TLMY, LL and MYCI.

Environmental factors	MYLL			TLMY		LL		MYCI		
	D.F	M.S	F-Value	M.S	F	M.S	F	D.F	M.S	F
Village	47	4.6604	13.02**	749046	13.24**	18244	8.99**	47	3.4958	6.22**
Calving year	4	98.6520	275.52**	7446927	131.63**	4866	2.40*	4	22.3327	39.76**
Calving season	3	0.9983	2.79*	7313	0.13^{NS}	2029	1.00^{NS}	3	1.9451	3.46*
Calving age	4	25.1611	70.27**	2884177	50.98**	11383	5.61**	4	6.6675	11.87**
Error	1779	0.3581		56574		2029		1129	0.5616	

M.S: Mean square; D.F: Degrees of freedom; NS: non-significant (P>0.05); *P<0.05; **P<0.001.

Discussion and Conclusion

The MYLL value found in this study (4.07 ± 0.02 kg) is compatible with the value (4.07 ± 1.3 kg) determined in the study on Murrah buffaloes conducted by Jorge et al. (17). The results found in this study are lower than those reported by other studies (16, 23, 33) for buffaloes (5.61 - 6.37 kg). The MYLL amount elevated with increasing calving age. This is attributable to improvement management and experience on farms each year.

The TLMY value found in the present study (1078.6 ± 7.54 kg) is higher than those found in the studies conducted (29, 30) for Anatolian buffaloes in Turkey (657.7-894.3 kg). On the other hand, it is similar to those reported by some other studies (2, 20, 31) on the same breeds (1000.7-1087.49 kg); however, the TLMY value is lower than those found by other researchers (1, 10, 16, 18, 24, 25) for Mediterranean buffaloes in Italy (2286 kg), Murrah buffaloes in Brazil (1594 kg), Nili Ravi buffaloes in Pakistan (1831.6 L), Murrah buffaloes in India (1984-2164.13 kg). These milk yield differences are attributable to differences in breed, feeding and managerial applications and seasonal or periodic changes in environmental factors (9). In the present study, the TLMY increased with the elongation of LL (263.83 ± 1.16 d) (Table 2). There was a strong and significant correlation between the two traits. Similarly, various researchers have reported a strong and significant correlation between LL and TLMY in buffaloes (1).

LL was longer than those reported by some other studies (20, 29, 31) for Anatolian buffaloes (146.6 -245.43 days). This can be a result of the farmers' desire to obtain milk from the buffaloes for as long as possible, thus ignoring the economy of life-long milk production (15). The LL value found in this study is similar to those reported by other researchers (1, 2, 24) for Italian buffaloes (270 d), Anatolian buffaloes (260.2 d), and Nili Ravi buffaloes in Pakistan (273.3 d); however, it is shorter than that reported (10) for Nili Ravi buffaloes (302 d). The differences in the LL values may arise from different management, care, and feeding practices on farms. The number of studies on the MYCI value of Anatolian buffaloes is limited. The MYCI value found in this study (2.67 ± 0.028 kg) is lower than those reported by other studies (16, 33) for Murrah buffaloes in India (4.26 kg), and in Pakistan (3.61 kg). The low MYCI value in the present study may be the result of differences in buffalo breeds and their higher productivity.

In the present study, the effect of calving season on MYLL was significant (P<0.05). Similar to our results, those of Khosroshahi et al. (19) have indicated that calving season has an important effect on MYLL, and Şahin and Ulutaş (29) have reported that buffaloes produce the highest MYLL in autumn and the lowest in summer. Unlike the results of this study, some researchers (14, 21) have found that the effect of calving season on MYLL was non-significant. The highest MYLL was observed in summer and the lowest in spring. The differences between the results are mostly attributable to different management methods on farms, environmental factors, and breed differences (13). In this study, the effects of calving year and age on MYLL were significant (P<0.001). MYLL gradually increased and reached the highest values when the cows were ≤11 years old (Table 2). In accordance with the results of the present study, Eskandar and Karimpour (13) and Şahin and Ulutaş (29) have found that calving age had significant effects on MYLL for both Iran Khuzestan and Anatolian buffaloes, respectively.

In accordance with our study, Kul et al. (21) and Ghaffar et al. (14) have reported that the effect of calving season on milk production was non-significant for Anatolian and Nili-Ravi buffaloes, respectively. In contrast, some studies (7, 10, 22) have reported that the calving season had a considerable effect on TLMY. In the present study, the lowest TLMY was in spring and winter. Unlike this study, Şahin and Ulutaş (29) have found that milk yield from Anatolian buffaloes in summer was lower than in other seasons. The farms in the study area were mostly individual family-owned farms. The milk they produce contributes to the family income when sold as raw milk, buffalo yogurt, and cream. Thus, it is suggested that more attention is paid to feeding their buffaloes during all seasons. The lowest milk yield was observed in 2015,

while the highest was observed in 2018 (Table 2). The differences in milk yield throughout the years stemmed from the level of farm management and environmental factors. In agreement with our study, the effects of calving year and age were significant in several studies (2, 20, 29). The highest TLMY value was observed in buffaloes calving at 9–10 years old, while the 3- to 4-year-old group produced the lowest TLMY. Bashir et al. (7) have emphasized that age could be a more important factor for inclusion in the models of TLMY. In this study, one of the environmental factors of village's effect on TLMY ($P<0.001$) was found significant (Table 3). There are some studies reporting that the study region has a significant effect on TLSV (2, 28).

In the present study, the effect of calving year on LL was found significant ($P<0.05$) (Table 2). The results found in this study were similar to the Charlini and Sinniah (9), Koçak et al. (20) and Alkoyak and Öz (2). The effect of calving season on LL was found non-significant (Table 2). These findings have supported by various studies (1, 10, 14, 18, 21). In contrast, Hussain et al. (15) and Şahin and Ulutaş (29) have reported that calving season had a significant effect on LL for Nili Ravi and Anatolian buffaloes, respectively. The longest LL was from buffaloes calving in spring, while the shortest was from those calving in winter. The village effect on LL ($P<0.001$) was found significant (Table 3). There are some studies reporting that the study region has a significant effect on LL (2, 28, 29).

There are limited numbers of studies on the environmental factors that affect MYCI in Anatolian buffaloes. In their study on Murrah buffaloes in India, Jakhar et al. (16) have found that calving year and season have significant effect on MYCI, similar to present study. Unlike to the results of the present study, Singh et al. (26) and Thiruvenkadan (33) have reported that the calving season did not have a significant effect on MYCI for Nili-Ravi buffaloes in India and Murrah buffaloes, respectively.

Consequently; in Anatolian buffaloes, both MYLL and MYCI were significantly affected by all environmental factors, while TLMY and LL was significantly affected by calving year and age. However, the effect of calving season on TLMY and LL were non-significant. In general, the milk yield traits elevated with increasing age (TLMY especially peaked in the 9–10 years) and those traits from buffaloes calving in summer were better than those calving in other seasons. It is concluded that the improvements in care, feeding, and herd management, considering calving age and season during studies to increase yields can contribute to higher milk production. Besides, taking calving age and season into consideration will help to determine the best studs for breeding programs.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Determination of factors affecting competitiveness through technical and economic analyses of dairy cattle enterprises in Balıkesir province

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Abstract: In this study, technical and economic analysis of dairy cattle enterprises in Balıkesir province were carried out. The factors which affect the competitiveness of enterprises and are important in terms of the continuity of production were determined. The stratified sampling method was used to determine the sample, and the study material was two-year (2017–2018) data obtained from 147 enterprises. In addition to the economic analysis of the data obtained, factors affecting unit profit in enterprises were estimated using the multiple linear regression model. The cost elements in total enterprises in Balıkesir province in 2017–2018 include feed expenses (47.90%-47.29%), livestock depreciation (16.64%-16.13%), labor expenses (13.84%-14.30%), veterinary health expenses (4.03%-4.50%), fuel transportation expenses (3.43%-4.15%), building equipment depreciation (3.37%-3.64%), and other expense items. A distinct difference was observed between scales in terms of profit and loss states of the enterprises in the study, with an increase in the profitability level from small toward large scale enterprise. The small-scale enterprises, in particular, are at a loss. The most important determinant of competitiveness in the study is the region's development level, where the enterprises are established in the borders of Balıkesir province. Additionally, among the technical parameters, the calving interval and the reduction of the disease rate have a positive effect on the competitiveness of the enterprises. Therefore, improving the financial structure of the enterprises and boosting the rate of technology utilization while simultaneously increasing enterprise scales will contribute positively to competitiveness.

Keywords: Balıkesir province, competitiveness, dairy cattle, economic analysis.

Balıkesir ilinde bulunan süt sığırcılığı işletmelerinin teknik ve ekonomik analizi ile rekabet gücünü etkileyen faktörlerin belirlenmesi

Özet: Bu araştırma, Balıkesir ili süt sığırcılık işletmelerinin teknik ve ekonomik analizini gerçekleştirmek ve işletmelerin üretimde devamlılıkları açısından önemli olan rekabet güçlerini etkileyen faktörlerin işletme düzeyinde belirlenmesi amacıyla yapılmıştır. Örneklem tespitinde, tabakalı örnekleme yöntemi kullanılmış olup, çalışma materyalini 147 adet işletmeden elde edilen iki yıllık (2017-2018) veriler oluşturmaktadır. Elde edilen verilerin değerlendirilmesinde yapılan ekonomik analizlerin yanı sıra işletmelerde birim kâra etkili faktörler çoklu doğrusal regresyon modeli ile tahmin edilmiştir. Balıkesir ili genelinde 2017-2018 yıllarında toplam işletmelerde masraf unsurları sırasıyla yem gideri (%47,90-%47,29), canlı demirbaş amortisman (%16,64-%16,13), işçilik giderleri (%13,84-%14,30), veteriner sağlık gideri (%4,03-%4,50), akaryakıt nakliye gideri (%3,43-%4,25), bina ekipman amortismanı (%3,37-%3,64), diğer gider kalemleri yer almıştır. Çalışmadaki işletmelerin kâr zarar durumlarında ise ölçekler arasında belirgin bir farklılık oluşmakta, kârlılık seviyesi küçük ölçekten büyük ölçeğe doğru artış göstermektedir. Özellikle küçük ölçekli işletmelerin zarar ettiği tespit edilmiştir. Çalışmada rekabetin en önemli belirleyicisi işletmeler bakımından Balıkesir ili sınırları içerisinde kurulu olduğu bölgenin gelişmişlik düzeyidir. Buna ek olarak teknik parametrelerden buzağılama aralığı ve hastalık oranın azaltılmasının işletmelerin rekabet gücüne olumlu etki ettiği anlaşılmıştır. Sonuç olarak işletme ölçeklerinin büyütülmesi ile eş zamanlı olarak işletmelerin finansal yapısının iyileştirilebilmesi ve teknoloji kullanım oranının artırılabilmesi rekabet gücüne olumlu yönde katkı sağlayacaktır.

Anahtar sözcükler: Balıkesir ili, ekonomik analiz, rekabet gücü, süt sığırcılığı.

Introduction

Livestock activities fulfill critical economic functions as a source of livelihood and employment of Turkey's rural population. Although animal production activities in Turkey are carried out across the country, it could not provide the expected and desired added value increase to the general population. Besides, positive developments have not been continuous (11). The success not achieved in terms of increasing added value has a negative effect on the competitiveness of exports of animal products (35, 41).

The "Revealed Comparative Advantage" Indices have been calculated as comparative advantage and competitiveness for Turkey and European Union countries with respect to the product group under the title of live animals. According to this index, Turkey's comparative advantage and competitiveness are not at par with the level of FAO's livestock category classification in European Union member country markets (38).

Balıkesir province is a developed region in terms of livestock production (21), and a significant part of the province's economy is based on livestock farming (42); thus, making the economic evaluation of dairy cattle breeding strategic. In Turkey, dairy cattle breeding is one of the most dynamic branches of production among the sub-sectors of the farming industry (2). Considering the dairy cattle breeding sector in Turkey, particularly the dairy cattle enterprises in Balıkesir province, as well as the potential changes in domestic and foreign markets, in addition to the performance of milk producers, the importance of competitiveness among enterprises is also increasing. It is important to examine and reveal the level of competitiveness among dairy cattle enterprises (28). As an outcome of the identification and analysis of the factors affecting competitiveness in dairy cattle enterprises, it is important to adopt the measures necessary to boost the competitiveness and the performance of the enterprises in terms of setting relevant policies.

In summary, dairy cattle enterprises that cannot make a profit arguably do not have competitiveness (20). In other words, under perfectly competitive conditions, a product introduced to the market and having a higher cost than the market price reveals that dairy enterprises do not have competitiveness (19). Therefore, profitability is the basic measure of competitiveness in enterprises (9) and has been considered as a measure of competitiveness in line with the objective of this study.

This study aimed to perform technical and economic analyses of dairy cattle enterprises in Balıkesir province and determine the factors affecting competitiveness at the enterprise level, which is critical in terms of the production continuity of the enterprises.

Materials and Methods

Primary data from Balıkesir Province Cattle Breeders Association-member enterprises located in Balıkesir province was obtained using study materials and the data collection form.

In determining the sample, the average and variance weights of each layer were considered using the stratified sampling method, and a single sample volume of the layers was determined. Accordingly, the study's sample size was 135 Balıkesir Province Cattle enterprises Association member enterprises from the 6066 enterprises registered in the Ministry of Agriculture and Forestry system. Twelve more enterprises were added to the study to create a reserve; finally, 147 enterprises were included in the study (7, 18). Among the enterprises with dairy cattle in Balıkesir province, the enterprises containing 1–10 milked cows were classified as small, those with 11–50 milked cows as medium-scale, and with 51 and above milked cows were considered as a large-scale enterprise.

For this study, a data collection form was used for face-to-face interviews with representatives from dairy cattle enterprises. In the data collection form, questions were designed to evaluate the physical and technical structures of the enterprises and their economic analysis. The data were transferred to the computer, and expense elements comprising the cost determined for milk production, income elements, and the cost of producing one liter of milk (25, 32), enterprises' input, output values, and capital structures were calculated (26, 32).

The Republic of Turkey's central bank values has been calculated based on the recent United States Dollar (USD) rate in 2017 and 2018. As of the last day of December, 1 unit of USD was announced as 3.77 TRY - 5.26 TRY in 2017 and 2018 (12).

Calving interval is measured as the time between two live calves from a cow. The disease rate was calculated as the number of sick animals / total animals (4).

Statistical analysis: The Microsoft Excel (Microsoft Office Professional Plus 2010) and SPPS 25 (23) statistical package programs were used to process the data obtained in 2017–2018 from the dairy cattle enterprises included in the study. As variables, the mean ±standard deviation and the median (Maximum-Minimum) percentage, and frequency values were used. Variables were evaluated after checking the normality and homogeneity pre-conditions of the variances (Shapiro-Wilk and Levene Test). During the data analysis, when the comparison of three or more groups was not provided using One-Way Analysis of Variance and the multiple comparison test Tukey HSD, Kruskal Wallis, and the Bonferroni-Dunn multiple comparison tests was used (3, 6, 27). The values accepted for the significance level of the tests are $P<0.05$ and $P<0.01$.

Multivariate regression analysis was performed for a large number of determined and measured variables. No elimination method (backward, forward) was used. Analyses were evaluated in accordance with the partial regression parameters obtained in the developed model. β The coefficients show how and in which direction a change of 1 unit in the relevant variable will cause a change in Y when other variables are kept fixed (39).

The regression equation divided into developed and undeveloped regions was applied for profit and loss, and for this, all 2017–2018 data were used.

The function to be applied for study data is,

$Y = f(X_1b_1, X_2b_2, X_3b_3, X_4b_4, \ldots X_nb_n)$,

and is formulated as follows:

$$\mathbf{Y = b_0 + b_1 X_1 + b_2X_2 + b_3X_3 + b_4X_{4+} \ldots\ldots\ldots\ldots\ldots b_nX_n + \varepsilon}$$

$$\mathbf{Y_i = b_0 + b_1 X_1 + b_2X_2 + b_3X_3 + b_4X_{4+} b_5X_5 + b_6X_6 + b_7X_7}$$

In the model developed within the scope of the study, the dependent variable was taken as profit/loss (TRY) per L of milk. **(Y)**

ε = Error Term.

X_1 = Milk yield per cow/year (tons);

X_2 = Feed (coarse + concentrate) cost (TRY) per L of milk;

X_3 = Active Capital (TRY) per Lt of milk;

X_4 = Amount of loan (TRY) used per liter of milk;

X_5 = Number of cows per enterprise;

X_6 = Calving interval (days);

X_7 = Rearing disease rate (Number of sick animals/Total number of animals);

X_8 = Developed and underdeveloped regions (Categorical variable encoded as a dummy)

Note: X_8 is the developed and underdeveloped region variable that was evaluated considering the table of socio-economic development level according to the districts of Balıkesir province published by the Ministry of Agriculture and Forestry (40).

Results

In the economic analysis tables prepared for each enterprise in this study, the expense elements comprising the cost and their ratio within the total expense were evaluated in detail according to the scale of enterprises. The expense elements comprising the average cost of the dairy cattle enterprises in Balıkesir province for 2017 and 2018 are presented in Table 1 and Table 2, respectively.

According to Table 1, in 2017, the largest share in total operating expenses in Balıkesir province was that of feed expenditure at 47.90%, followed by an average livestock depreciation at 16.64%, while the average labor expenses were 13.84%, and other expenditure items had a 21.62% share.

Analysis on the basis of scales revealed that while feed cost and the rates of building and equipment depreciation were higher for large-scale enterprises, labor cost and fuel transportation expense rates were higher for small-scale enterprises.

Further analysis in terms of scales revealed that the difference between scales statistically significant in 2017 in terms of veterinary health, labor, electricity, water, insurance, and building equipment depreciation costs ($P<0.05$).

Table 1. Cost elements constituting the 2017 cost of enterprises according to scales ($X \pm S_x$).

2017	0–10 Small n = 58 (%)	11–50 Medium n = 74 (%)	51+ Large n = 15 (%)	Total n = 147 (%)	p
Feed	47.90 ± 14.26	48.44 ± 8.67	51.46 ± 6.59	47.90 ± 11.05	0.56
Veterinary health	4.32 ± 1.66	3.73 ± 1.7	4.58 ± 1.68	4.03 ± 1.70	<0.05
Labor	16.81 ± 5.31a	12.8 ± 3.83a	7.76 ± 2.79b	13.84 ± 5.15	<0.001
Electricity water	1.47 ± 0.89 a	1.09 ± 1.11 a	2.23 ± 0.9 b	1.34 ± 1.06	<0.001
Fuel transportation	3.81 ± 3.09	3.35 ± 1.64	2.42 ± 1.3	3.43 ± 2.30	0.12
Insurance	0.05 ± 0.2a	0.16 ± 0.45a	1.71 ± 1.84b	0.26 ± 0.81	<0.001
Loan interest	0.13 ± 0.58	0.54 ± 1.48	0.35 ± 0.85	0.36 ± 1.16	0.14
Land rent	1.46 ± 2.17	1.87 ± 2.67	1.07 ± 1.98	1.63 ± 2.42	0.42
Inventory value decrease	2.30 ± 5.44	2.69 ± 7.98	0.59 ± 2.06	2.33 ± 6.79	0.56
Milk food	1.90 ± 1.04	2.11 ± 0.78	2.22 ± 1.11	2.04 ± 0.92	0.33
Building equipment depreciation	3.47 ± 2.63a	2.62 ± 1.5a	7.07 ± 4.71b	3.37 ± 2.72	<0.001
Maintenance repair	0.70 ± 0.5	0.97 ± 1.59	0.8 ± 0.79	0.85 ± 1.21	0.45
Livestock depreciation	16.00 ± 5.45	17.43 ± 4.36	15.47 ± 3.37	16.64 ± 4.77	0.14
General administration	2.23 ± 0.3	2.20 ± 0.27	2.27 ± 0.35	2.22 ± 0.23	0.67

* a; b; c; There is a statistically significant difference for variables with different letters in the same row.

According to Table 2, of the total operating expenses in Balıkesir province in 2018, feed expenses had the largest share, with an average of 47.29%, followed by livestock depreciation with an average of 16.13%, labor expenses at 14.30% on an average, and other expenditure items had a 22.28% share. Further analysis revealed that while feed cost, building, and equipment depreciation rates were higher for large-scale enterprises, labor cost and fuel transportation expense rates were higher for small-scale enterprises.

The difference between scales was found to be statistically significant in 2018 in terms of labor, electricity, water, insurance, building equipment depreciation, and living stock depreciation costs ($P<0.05$).

The average cost of milk/L and the amount of profit and loss per enterprise across the dairy cattle enterprises were estimated for the period of 2017–2018 and are presented in Table 3.

Analysis of Table 3 shows a decrease in the average cost of milk/L as the enterprise-scale increases. In small-scale enterprises, the cost of milk, which was 1.63 TRY (0.43 USD) in 2017, increased to 1.70 TRY (0.32 USD) in 2018. In the medium-sized enterprises, the cost of milk, which was 1.36 (0.36 USD) TRY in 2017, increased to 1.39 TRY (0.26 USD) in 2018, and in the large-scale enterprises, the cost of milk increased from 1.13 TRY (0.29 USD) in 2017 to 1.09 TRY (0.20 USD) in 2018.

Investigation of the profit and loss statement of the enterprises shows distinct differences between the enterprises, with the profitability level increasing from small scale toward large scale.

Table 2. Cost elements constituting the 2018 cost of enterprises according to scales ($X \pm S_x$).

2018	0–10 Small n = 58 (%)	11–50 Medium n = 74 (%)	51+ Large n = 15 (%)	Total n = 147 (%)	p
Feed	45.56 ± 14.25	48.4 ± 7.88	49.71 ± 5.68	47.29 ± 11.03	0.23
Veterinary health	4.53 ± 2.17	4.34 ± 1.84	5.18 ± 2.21	4.50 ± 2.02	0.35
Labor	17.45 ± 6.48a	12.77 ± 3.26b	7.79 ± 2.75c	14.30 ± 5.78	<0.001
Electricity water	1.52 ± 1.02a	1.24 ± 1.11a	2.57 ± 1.2b	1.49 ± 1.14	<0.001
Fuel transportation	4.55 ± 3.37	4.04 ± 2.01	2.94 ± 1.39	4.15 ± 2.67	0.1
Insurance	0.17 ± 0.59a	0.40 ± 0.88a	1.27 ± 1.46 b	0.39 ± 0.89	<0.001
Loan interest	0.30 ± 1.54	0.47 ± 1.25	0.65 ± 1.19	0.41 ± 1.37	0.61
Land rent	1.76 ± 2.78	1.57 ± 1.93	1.01 ± 2.45	1.59 ± 2.37	0.54
Inventory value decrease	0.51 ± 2.29	0.47 ± 2.17	0.03 ± 0.12	0.44 ± 2.10	0.73
Milk food	2.02 ± 1.21	2.17 ± 1.3	2.15 ± 0.91	2.10 ± 1.22	0.78
Building equipment depreciation	3.48 ± 2.74a	3.08 ± 2.01a	6.87 ± 4.98b	3.64 ± 2.95	<0.001
Maintenance repair	0.72 ± 0.54	0.94 ± 1.04	0.72 ± 0.57	0.82 ± 0.72	0.24
Livestock depreciation	14.78 ± 5.24a	17.38 ± 4.69b	16.26 ± 2.55a	16.13 ± 4.91	<0.01
General administration	2.65 ± 0.71	2.71 ± 0.41	2.86 ± 0.44	2.69 ± 0.55	0.42

* a; b; c; There is a statistically significant difference for variables with different letters in the same.

Table 3. Financial findings of the milk production according to the enterprise scale ($X \pm S_x$).

Scale		One Lt Milk Cost (TRY)		Profit Loss (TRY)	
		2017	2018	2017	2018
Small	n	58	58	58	58
	$X \pm S_x$	1.63 ± 0.62	1.70 ± 0.58	-4671.53 ± 24758.32	-4915.96 ± 21791.7
Medium	N	74	74	74	74
	$X \pm S_x$	1.36 ± 0.5	1.39 ± 0.5	20552.66 ± 69203.88	21111.07 ± 77537.96
Large	n	15	15	15	15
	$X \pm S_x$	1.13 ± 0.64	1.09 ± 0.41	816264.38 ± 1190627.66	804057.58 ± 1118220.17
Total	n	147	147	147	147
	$X \pm S_x$	1.44 ± 0.58	1.49 ± 0.56	86382.32 ± 430690.23	89672.07 ± 425923.66

Footnote: At the end of December 2017 and 2018, 1 unit of USD was 3.77 TRY -5.26TRY.

Table 4. The results of the regression analysis of the variables affecting profit per liter of milk (2017–2018).

		(2017–2018)				
Parameters		**N**	**β**	**t**	**Sig.**	**VIF**
Fixed			-6.249	-1.917	.056	
Milk yield per cow, tons	X_1	294	.097	.658	.511	1.420
Feed cost per 1 liter of milk	X_2	294	-.025	-.063	.950	1.207
Active capital per 1 liter of milk	X_3	294	.083	6.164	.000	1.425
Loan amount per 1 liter of milk	X_4	294	.368	1.949	.050	1.303
Number of cows milked	X_5	294	.013	3.712	.000	1.224
Calving interval, day	X_6	294	-.021	3.091	.002	1.167
Disease rate%	X_7	294	-.163	-3.219	.001	1.044
Region	X_8	294	1.182	2.832	.005	1.062

Footnote: R^2 : 0.330, F: 17.563, P<0.05

$\mathbf{Y} = 6.249 + 0.097\mathbf{X_1} – 0.025\mathbf{X_2} + 0.083\mathbf{X_3} + 0.368\ \mathbf{X_4} + 0.013\mathbf{X_5} – 0.021\mathbf{X_6} – 0.163\mathbf{X_7} + 1.18\ \mathbf{X_8}$

The regression analysis of the variables affecting profitability per liter of milk from the 147 enterprises in Balıkesir province in 2017–2018 is presented in Table 4.

According to Table 4, the adjusted determination coefficient, which expresses the ratio of the independent variables to explain the dependent variable, was estimated to be R^2:0.330, and the F test, which states the significance of the model, was F: 17.563. Further, the amount of profit/loss per liter of milk, which is a dependent variable in the regression method, is explained by 33.0% of variables, and that the model is valid (P<0.05).

Discussion and Conclusion

In studies on the economic analysis of dairy cattle enterprises conducted in the previous years, feed expenditures had the largest share among the cost elements comprising expenses, followed by labor, depreciation, veterinary health expenses, maintenance and repair costs, and other expenses (5, 13, 15, 22, 24, 31).

There has been a change in the order of the expense of cost elements in dairy cattle enterprises in Balıkesir. This change resulted from the great increase in cattle values and energy expenses.

Despite the dairy cattle enterprises in Balıkesir province failing to cultivate their coarse fodder crop at low costs with their own means, the share of feed expenses in the grand total of costs was lower than the rates found by other researchers (13, 22, 24, 31, 36), as the share of livestock depreciation and energy costs were high.

When labor costs are evaluated in terms of scale size, the ratio of labor expenses among expenses clearly decreases in percentage as the enterprise-scale grows. Compared to results obtained in other studies, labor expense rates were similar for small-scale enterprises and lower for medium and large-scale enterprises (13, 22, 24, 31, 36). The reason for this difference is the development of technology and increased use of machinery and technology in production in recent years; thus, highly boosting the rate of machinery use in medium and large-scale enterprises. Accordingly, an increased rate of machinery use significantly increases the rates of equipment depreciation, especially in large-scale enterprises compared to the other scale enterprises. It, therefore, causes a proportional decrease in labor costs. Additionally, the widespread recruitment of foreign nationals, especially in medium and large-scale enterprises, is another factor that reduces labor costs (1, 14).

The ratio of veterinarian - medicine costs among total expenses seems to differ among certain studies (5, 13, 15, 22, 24, 31). The primary reasons for this are the increase in the rate of dairy culture and crossbreeds in the Balıkesir province in recent years, and inadequate suitable care and hygiene conditions for these breeds in enterprises milk yield. Considering enterprise owners' easy access to information through technology, and the importance of the economic value of veterinary health services in cattle enterprises in recent years, the veterinary health expenses item may increase.

Evaluation of the insurance expense item has shown that the proportional share of animal life insurance expenses in small and medium-scale enterprises is very low and is proportionally higher in large-scale enterprises. The most important feature of animal life insurance is that large-scale enterprises understand its function as a guarantee for the continuity of producers' income from the enterprise (29).

The most important challenge is controlling the costs without negatively affecting production, reproduction, animal welfare, and continuity in personnel employment. Costs can be successfully controlled by considering these factors and regular reviews (37).

In addition to the fluctuations in livestock enterprise production costs and milk prices, problems in production and marketing directly affect the producer (5).

According to the National Milk Council data for enterprises having ten or more cattle, the cost of one liter of milk was calculated to be 1.18 TRY - 1.37 TRY at the end of 2017–2018 (43). This is similar to the results of a study conducted in Konya province in 2017 in terms of scales to the cost of one liter of milk (33). However, the cost of one liter of milk in 2017–2018 announced by the national milk council was lower than the values calculated in this study. In the evaluation made according to enterprise scales, the large-scale enterprises clearly differed significantly from other scales in terms of their understanding of the enterprise and professional management and reduction in the production cost with effective cost control. It has been observed that the required cost control and follow-up on the basis of small and medium-scale enterprises are not up to the desired standard. Thus, production cost drives dairy cattle enterprises to face the complex dilemma of aiming to maximize economic efficiency and minimize costs (30).

For all the dairy cattle enterprises included in this study, the average profit was calculated as 86382.32 TRY (22913.08 USD) in 2017, and 89672.07 TRY (17047.92 USD) in 2018, indicating that the resource use of small-scale enterprises in Balıkesir province is not rational. Small and medium-scale enterprises, in particular, do not consider important cost items such as depreciation and labor in their calculations. This situation shows that enterprise owners are critically mistaken while calculating costs, assuming that their costs are low and that their enterprises are profitable.

The relationship between the enterprise's profit per liter of milk and various variables was analyzed using the regression model and the data of the enterprises included in the study.

According to the regression equation, active capital per liter of milk, the number of cows milked, calving interval, rearing disease rate, and the region variable significantly affect the profit-loss dependent variable per liter of milk (Table 4) ($P<0.05$).

The regression model data obtained from dairy cattle enterprises included in the relevant regression model revealed that the active capital is not being used efficiently. Increased and efficient use of active capital investment in animal husbandry enterprises is directly proportional to the growth of technology investment (8, 10). The importance of adopting new technologies to enterprises and increasing the rate of technical innovation has been understood, especially in small and medium-sized enterprises, for Balıkesir dairy cattle enterprises to switch from loss to profit and ensure the sustainability of the enterprises.

The small scale of enterprises and lack of expertise in animal production prevent output growth and the continuity of increasing returns (16). Evaluation of the statistical significance of the independent variable, the number of cows milked, in terms of the scale's efficiency, based on regression model data, reveals that the productivity of the scale may increase in Balıkesir province. In the regression model established in enterprises with high milk yield due to profitability, a significant relationship between the rate of disease and breeding was observed. The primary reason for this is predicted to be the higher rate of certain breeding diseases in these enterprises with high milk yield. Though the calving interval presents less effect statistically, shortening this interval will reduce economic loss and thus enable further competitiveness of enterprises. In a study, it was found that shortening the calving interval increased the gross margins between 13% and 35% (34).

Greater profitability per liter of milk in developed regions compared to underdeveloped regions is found to be statistically significant. In this case, it is understood that the location of the dairy cattle enterprises and their interactions with the environment significantly affect profitability per liter of milk. Based on the location of the enterprise, supporting economic enviroment and main sectors in the environment can be advantageous in terms of cost-effectiveness, priority, and rapid raw material input supply (17, 20).

From this study, it is clear that the socio-economic data used in the analysis of dairy cattle enterprises are mostly quantitative but do not provide a qualitative result. In case dairy cattle enterprises incur a high cost for one liter of milk and low profitability, it is often recommended to reduce costs and increase milk production. However, the origin of these problems is not specified. If regression models are used to determine the factors affecting enterprises' competitiveness, both quantitative and qualitative results can be obtained.

Feed costs can be reduced by increasing feed conversion rates by supporting more active use of pasture areas and increasing the use of feed mixer machines over a certain scale. Furthermore, different additional practices can be implemented to encourage producers, who possess a certain area per cattle and have knowledge and experience to expand their enterprise scale.

It should be known that developing medium-scale enterprises have different needs than large-scale enterprises to grow and benefit from the scale economy. Insufficient funding and the need for financial management expertise can be cited as appropriate examples.

According to the model developed, it is obvious that the dairy cattle enterprises in developed regions have a greater advantage in competitiveness as they earn higher profits. Reducing the disease rate by implementing effective herd health management will positively contribute to the competitiveness of enterprises.

Additionally, the measures to be taken to improve enterprises' financial structure and increase the rate of technology use with simultaneous upsizing of enterprise scales will increase competitiveness.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Comparison anti-oxidant and neuroprotective effects of extra-virgin olive oil, donepezil and rosmarinic acid on aluminum chloride-induced Alzheimer's in rat models

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Abstract: In this study, it was aimed to investigate the effects of EVOO, rosmarinic acid and donepezil in Alzheimer's model to be created with $AlCl_3$ in rats. For this reason, administration of 100 mg/kg aluminum chloride (AlC_3) for 15 days to Sprague Dawley adult male rats; donepezil, Extra-virgin olive oil (EVOO) and rosmarinic acid were administered to three different groups for 21 days by applying treatment protocols. With this study, we were able to demonstrate that cognitive impairment has been occurred after 15 days of $AlCl_3$ administration by oral gavage and treatment protocols prevented the occurrence of AD pathology histopathologically. We also showed that oxidative damage findings which are positively Congo-red stained cell cytoplasm and impaired cell integrity have been observed in serum and hippocampus. Besides, treatment groups showed better cognitive and motor performance, and there was no damage to the cells in control and treatment groups. In the rotarod motor performance test, a significant increase was observed in the donepezil group compared to the $AlCl_3$ group at speeds of 26 and 30 rpm. In MVM, on the 5th day of the experiment, a significant increase was observed in the donepezil group compared to the $AlCl_3$ group, as they were spending more time in the hidden platform area. These results show that 15 days of aluminum exposure is effective in creating a moderate Alzheimer's pathology, but further chronic research is necessary to explain the efficiencies of rosmarinic acid and EVOO in treatment.
Keywords: Aluminum chloride, Alzheimer, donepezil, EVOO, rosmarinic acid.

Ratlarda alüminyum klorür ile oluşturulmuş Alzheimer modelinde sızma zeytinyağı, donepezil ve rosmarinik asidin antioksidan ve nöroprotektif etkilerinin karşılaştırılması

Özet: Bu çalışmada; sıçanlarda $AlCl_3$ ile oluşturulan Alzheimer modelinde, EVOO, rozmarinik asit ve donepezilin etkilerinin araştırılması amaçlanmıştır. Bu sebeple, Sprague Dawley yetişkin erkek sıçanlara 15 gün boyunca 100 mg/kg alüminyum klorür (AlC_3) uygulanmasının ardından; üç farklı gruba 21 gün süreyle donepezil, erken hasat sızma zeytinyağı (EVOO) ve rosmarinik asit tedavi protokolleri uygulanmıştır. Bu çalışmada, oral gavaj ile $AlCl_3$ uygulamasından 15 gün sonra bilişsel bozulmanın ortaya çıktığı ve tedavi protokollerinin histopatolojik olarak AH patolojisinin ortaya çıkmasını engellediğini görülmüştür. Serum ve hipokampusta da oksidatif hasar bulguları olan Congo-red pozitif boyanmış hücre sitoplazması ve bozulmuş hücre bütünlüğü gösterilmiştir. Ayrıca, tedavi grupları daha iyi bilişsel ve motor performans göstermiş, kontrol ve tedavi gruplarındaki hücrelerde hasara rastlanmamıştır. Rotarod motor performans testinde donepezil grubunda 26 ve 30 rpm hızlarda $AlCl_3$ grubuna göre anlamlı artış gözlenmiştir. MWM testinde deneyin 5. gününde donepezil grubu, platformun bulunduğu alanda $AlCl_3$ grubuna göre anlamlı olarak daha fazla zaman geçirdiği gösterilmiştir. Bu sonuçlar, 15 günlük alüminyum maruziyetinin ılımlı AH patolojisi oluşturmada etkili olduğunu göstermektedir ancak rosmarinik asit ve EVOO'nun tedavideki etkinliklerini açıklamak için daha fazla kronik araştırmaya ihtiyaç duyulmaktadır.
Anahtar sözcükler: Alüminyum klorür, Alzheimer, donepezil, EVOO, rosmarinik asit.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder with a gradual deterioration in cognitive function, leading to reduced quality of life in humans (41). AD is the most common neuropathological form of dementia and characterized by senile plaques and neurofibrillary tangle lesions in the brain, mostly formed by the accumulation of β-amyloid (Aβ) and hyper-phosphorylated tau protein (37). Early identification of risk factors for AD will allow early diagnosis of the disease and therefore, develop successful treatment strategies (13). Although metals such as aluminum, copper, zinc, lead, mercury, and iron cause neurotoxicity, it has been reported that aluminum (Al) metal is the biggest risk factor for the cause and development of AD (12). Aluminum accumulates in the cortex, cerebellum, and hippocampus, which are responsible for memory and cognition (36). It has been shown in many studies that chronic aluminum chloride ($AlCl_3$) application is used as an AD model in rodents (22, 29).

Currently, there is no drug group or treatment method that can fully treat AD; however, to increase the welfare of the individuals who have the disease, dietary adjustments or treatment programs with certain drugs are implemented. Acetylcholinesterase enzyme inhibitor (AChEI) is one of the frequently preferred groups used in drug treatment. AChEI's act by preventing enzymatic degradation and are seen as first-line treatment options for AD. AChEI group drugs act without changing the development of the underlying pathology, relieving the symptoms and increasing the patient's quality of life and are considered "symptomatic drugs" (18, 43). Donepezil, which is still preferred as the first choice for AD treatment, remains popular due to its low toxicity and good tolerability (44). Oxidative stress is considered to be one of the most important causes of pathological phenomena, such as aging and AD in humans (19). Therefore, the interest of researchers working in this field are: directed more towards natural foods containing high levels of antioxidants recently. Among them, extra-virgin olive oil (EVOO) has an important place. EVOO is an essential component of the Mediterranean diet and has been associated with a long healthy life. EVOO is a complex mixture with 98% fatty acids esterified with mono and diglycerols and 2% non-saponified components (14). Despite our knowledge of EVOO phenol's protective role in different pathologies, few studies describe the molecular mechanisms that show how olive oil affects longevity. Studies in rodents have shown that animals fed a diet containing high polyphenols increased cognitive ability and reversed oxidative damage in the brain (31). Another phenol carboxylic acid derivative known to have antioxidant effects is rosmarinic acid (RA); which is in various foods and plants. RA contains antioxidant, anti-inflammatory, anti-apoptotic and neuroprotective phenolic compounds (30).

In this study, we aimed to investigate the effects of EVOO, rosmarinic acid, and donepezil on behavioral paradigms and the therapeutic effects of substances in the prefrontal cortex and hippocampus of rats in the Alzheimer's model to be created with $AlCl_3$ administration.

Materials and Methods

***Subjects*:** Thirty-five adult male Sprague Dawley rats (300-350 gr) were used in this study. All rats were housed in pairs in cages with free access to water and laboratory chow. They were kept in a 12h-light/12h-dark cycle at constant room temperature (22±1°C) and humidity (60%). Dokuz Eylül University Animal Experiments Local Ethics Committee approved all experimental procedures (Ethics committee approval date: 14.12.2017 decision number: 25/2017).

***Experimental Design*:** 35 healthy male Sprague Dawley rats were divided into five groups: control group, $AlCl_3$ group, donepezil group, EVOO group, and rosmarinic acid group (n=7 in each group). Physiological saline (PS) was administered to the control group at a dose of 1 ml orally once a day during the study. $AlCl_3$ was obtained from the chemical named $AlCl_3$ Aluminum chloride Anhydrous (Sigma-Aldrich CAS number: 7446-70-0). $AlCl_3$ was administered orally at a dose of 100 mg/kg for 15 days to the animals once a day to induce cognitive impairment (24). Donepezil was obtained from the drug (Pfizer ARICEPT ® 10 mg donepezil hydrochloride) equivalent to 9.12 mg donepezil as the active ingredient frequently preferred at the onset of AD. Donepezil was administered orally at a dose of 5 mg/kg once a day to the animals (5). EVOO; supplied from oral preparation (Cardiolive, TUAY, TURKEY). 1 ml of EVOO was administered orally to the animals once a day for 1 day (27). Rosmarinic acid was obtained from rosemary juice (Arifoğlu ®). It is obtained from the aboveground parts of the rosemary plant by the method of water vapor distillation. Rosmarinic acid was administered orally at a dose of 1 ml once a day for 21 days to be used in animals (35). At the end of the 5 weeks, learning and memory were assessed by Morris Water Maze, anxiety was assessed by open field and elevated plus maze tests. Motor functions assessed by rotarod motor performance test. Animals were euthanized under carbon dioxide anesthesia, blood samples were obtained drawing all intracardiac blood, and brain tissues were removed rapidly. The right hemisphere of the brain was placed in 10% formol for histological examination, while the other hemisphere was separated from the hippocampus

and prefrontal cortex. Homogenate and supernatant were prepared from these separated pieces for biochemical analysis. The thymus and adrenal gland tissues of the rats were also removed and their weight was recorded. Relative adrenal and thymus weight adrenal weight/body weight ratio; calculated by thymus weight/body weight ratio.

***Morris Water Maze Test (MWM)*:** Learning experiments were conducted using Morris water maze test. By placing signs such as a clock and a painting on the room walls where the pool is located, rats were allowed to determine their direction using these signs. A video camera system was installed to monitor and record rats's behavior at an average height of 2 m from the center of the tank. On each test day, rats were placed in the water facing the pool edge from one of the 3 randomly selected quadrants without the hidden platform and allowed to swim until they found the hidden platform. Maximum swimming time was limited to 60 seconds. If the rat could not find the platform within 60 seconds, the rat placed on the for 15 seconds after the try. Each rat was subjected to five consecutive experiments per day, with intervals of 60 seconds. After completing each experiment, the rats were taken from the platform, dried, and placed in their cages. These procedures were repeated for 4 consecutive days. Thus, a total of 20 experiments was applied to each rat for 4 days. On day 5, a probe trial was applied to each rat. The platform was removed from the pool, and the rat was allowed to swim for 60 seconds. Behavioral data were evaluated using the HVS image video tracking system as swimming distance, time to find the platform, and the time spent in each quadrant (39).

***Open Field Test (OF)*:** This test is commonly used to assess spontaneous locomotor activity and anxiety. The open field consists of a 1x1m area surrounded by a wall of 50 cm in height. A video camera was installed 2.5 m above the apparatus. Each rat was placed in the center of the open field, and locomotor activity was measured for 5 min in a soundproof observation room, illuminated with controlled light (100lx) (7).

***Elevated Plus Maze (EPM)*:** The elevated plus maze is another commonly used experimental rodent model to assess anxiety. The elevated plus maze apparatus consists of a central platform (5cm×5cm) with two open arms (50 cm long, 10 cm wide and 0.5 cm high borders) and two closed arms (50 cm long, 10 cm wide with 40 cm high walls), each elevated 50 cm above the floor. Rats were placed on the platform facing the open arm and were observed for 5 min. The total number of entries into the open and closed arms, as well as the entire time spent on the open and closed arms was measured (15).

***Rotarod Test (RT)*:** The rotarod test setup enables the evaluation of motor performance by measuring balance, coordination, and motor control. The apparatus measures the rodent's ability to stand on a rotating shaft at a certain speed or with increasing speed. In the speed-increased protocol, graded speeds between 4-40 rpm are tried in each trial. The test, which was applied for 300 seconds, it was started with a speed of 16 rpm and measurements were made gradually at 20, 26, and 30 rpm. In the five-lane set-ups, two rats were tested simultaneously. With the help of the mechanism with a timer on the floor, how long the rat can stand on the shaft without falling down at any speed was measured, and the speed it fell for the consequent three times was recorded as the maximum speed it could walk (2).

***Biochemical Analysis*:** Blood and all tissue samples were stored at –85°C. Acetylcholinesterase (AChE), lactate dehydrogenase (LDH) enzyme levels by spectrophotometric method (Fully automated Roche/ Hitachi cobas c501). Malondialdehyde (MDA) levels were analyzed by Bioassay Technology Laboratory Rat MDA ELISA Kit (catalog no: E0156Ra, Shanghai, China-Assay sensitivity 0.01 nmol/ml and detection range is 0.05-10 nmol/ml). Aβ-42 precursor protein accumulation was analyzed by Bioassay Technology Laboratory Rat Soluble Amyloid Precursor Protein Beta ELISA Kit (catalog number: E01010Ra, Shanghai, China-Assay sensitivity 0.053 ng/ml and its detection 01-40 ng /ml). BCA protein Assay kit (Cat No: E- BP-500, Elabscience, Wuhan, China) was used for protein analyses, according to the manufacturers' description. Serum corticosterone (CORT) levels were analyzed Bioassay Technology Laboratory CORT - Rat ELISA kit (catalog no: E0496Ra, Shanghai, China-Assay sensitivity 0.24 ng/ml, detection range 0.5 ng/ml-100 ng/ml). Protein and serum CORT levels were measured in the hippocampus, prefrontal cortex homogenates, and serum.

***Histological Analysis*:** Hematoxylin-Eosin (HE) staining of brain tissue samples taken from all groups was performed following the hematoxylin (HX86017674, Merck Hematoxylin, Darmstadt, Germany) protocol and congo red staining (ChemBio laboratory research) protocol.

***Statistical Evaluation*:** All statistical procedures were performed by SPSS software for Windows, Version 23.0 (SPSS, Chicago, IL). Descriptive statistics for each variable were calculated. Prior to hypothesis testing, data were examined with Shapiro- Wilk test for normality and Levene test for homogeneity of variances as parametric test assumptions. Differences between groups were analyzed using one-way ANOVA for data that provide parametric test assumptions. Bonferroni test was used as post hoc. Kruskal Wallis test was used to examine the differences among the groups for the variables that violates parametric test assumptions. Dunn-Bonferroni

test was used as post hoc analysis. A value of $P<0.05$ was considered to be statistically significant.

Results

Histopathologically, H&E staining showed atrophy, decreased neuronal cell density, and gliosis in the prefrontal cortex in the Alzheimer group compared to the control group. Similarly, in H&E staining in the hippocampus, the neuronal cell density in the Alzheimer group compared to the control group decreased, and gliosis was observed (Figure 1. A). Also, neurofibrillary tangles were found in the cytoplasm of cells in basophilic staining in the Alzheimer group (Figure 1. B). Congo red staining showed positively stained cell cytoplasm and impaired cell integrity in the hippocampus and prefrontal cortex of the Alzheimer's group (Figure 1. C). There was no damage to the cells in the control and treatment groups.

There was no significant difference between the groups in the analyzes of the prefrontal cortex MDA, LDH, cholinesterase, and sAppBeta (Table 1 and Table 2). The hippocampus LDH level was found to be significantly lower in the $AlCl_3$ group compared to the control group (Table 1). There was no significant difference in serum CORT level between the groups, serum MDA and cholinesterase level in the $AlCl_3$ group compared to the control group (Table 1), serum LDH level in the $AlCl_3$ group (Table 1) were significantly higher than all groups.

The relative thymus weight showed a significant decrease in the $AlCl_3$ group compared to the control group, and a significant increase in the treatment groups compared to the control group. There was no significant difference between relative adrenal weights (Table 3).

There was no significant difference between groups in the OF (Table 4) and EPM (Table 5). In the rotarod motor performance test, a significant increase was observed in the donepezil group compared to the $AlCl_3$ group at speeds of 26 and 30 rpm. At speeds of 16 and 20 rpm, all groups showed better walking performance compared to $AlCl_3$ group (Table 6). In MWM, an increase in swimming speed was observed in the $AlCl_3$ group on the 3rd and 4th learning days compared to the control group (Table 7). On the 5th day of the experiment, a significant increase was observed in the donepezil group compared to the $AlCl_3$ group, as they were spending more time in the hidden platform area (Table 8). EVOO and rosmarinic acid groups also spent more time in the hidden platform area compared to the $AlCl_3$ group as shown in Table 7.

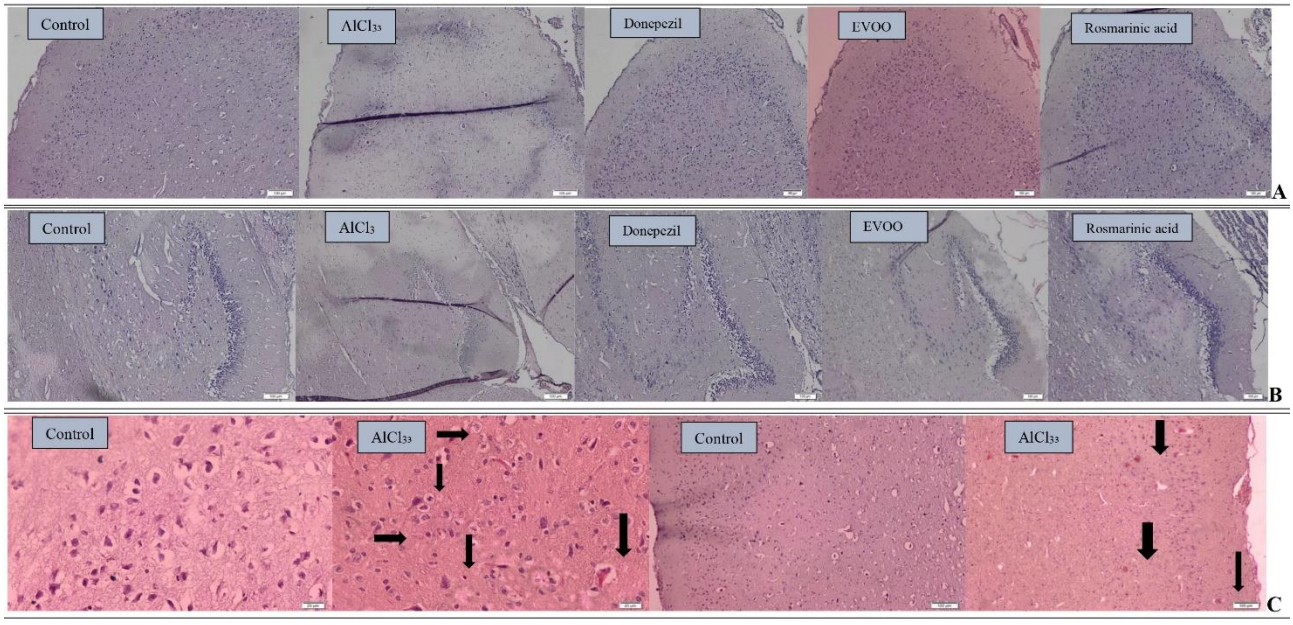

Figure 1. Histopathology results.
A. H&E staining in the prefrontal cortex (x100). **B.** H&E staining in the hippocampus (x100). **C.** Congo red staining in hippocampus (x400) and prefrontal cortex (x100).

Table 1. Analyzes of CORT, MDA, LDH in prefrontal cortex, hippocampus, and serum.

Prefrontal Cortex	**CORT**		**MDA ng/mg protein**		**LDH**	
			Mean ± S.D.	**Median (Min-Max)**	**Mean ± S.D.**	**Median (Min-Max)**
Control	-	-	0.0067±0.00137	0.01 (0.01-0.01)	11148.3333±5924.61073	11737 (1965-17748)
$AlCl_3$	-	-	0.0084±0.00511	0.01 (0-0.02)	11643.3333±2221.60281	12349 (7507-13453)
Donepezil	-	-	0.0045±0.00271	0 (0-0.01)	12704.25±3425.95645	14292.5 (7588-14644)
EVOO	-	-	0.004±0.00122	0 (0-0.01)	15763.2±5902.21795	15522 (9207-25234)
Rosmarinic acid	-	-	0.0072±0.00286	0.01 (0-0.01)	8603.2±3251.96198	8065 (5212-13954)
P Value	-		**0.159**		**0.187**	
Hippocampus	**CORT**		**MDA ng/mg protein**		**LDH**	
			Mean ± S.D.	**Median (Min-Max)**	**Mean ± S.D.**	
Control	-	-	0.028 ± 0.024	0.018 (0.01 – 0.07)	6433 ± 569 [a]	
$AlCl_3$	-	-	0.016 ± 0.008	0.015 (0.007 – 0.028)	5119 ± 664.03 [b]	
Donepezil	-	-	0.029 ± 0.016	0.028 (0.012 – 0.049)	6671 ± 1059 [a]	
EVOO	-	-	0.025 ± 0.005	0.026 (0.017 – 0.032)	6074 ± 588 [ab]	
Rosmarinic acid	-	-	0.034 ± 0.013	0.036 (0.015 – 0.05)	5200 ± 306 [b]	
P Value	-		**0.366**		**<0.05**	
Serum	**CORT**		**MDA ng/mg protein**		**LDH**	
	Mean ± S.D.	**Median (Min-Max)**	**Mean ± S.D.**	**Median (Min-Max)**	**Mean ± S.D.**	**Median (Min-Max)**
Control	4.61±0.48 [a]	4.46 (4.17-5.37)	0.4737 ± 0.0101 [b]	0.4754 (0.4619 – 0.4845)	222 ± 44 [b]	227 (173 - 260)
$AlCl_3$	2.31±0.67 [b]	2.03 (1.85-3.68)	1.1001 ± 0.1184 [a]	1.1149 (0.9651 – 1.2053)	484 ± 121 [a]	433 (408 - 663)
Donepezil	3.24±0.64 [b]	3.35 (2.48-4.13)	1.6355 ± 0.5817 [a]	1.5641 (1.0053 – 2.4084)	258 ± 58 [b]	249 (190 - 324)
EVOO	2.73±0.65 [b]	2.83 (1.65-3.66)	1.0793 ± 0.271 [ab]	1.1238 (0.7947 – 1.4636)	276 ± 91 [b]	289 (157 - 400)
Rosmarinic acid	4.61±0.48 [a]	4.46 (4.17-5.37)	1.0517 ± 0.285 [ab]	1.0471 (0.748 – 1.5056)	205 ± 45 [b]	204 (157 - 267)
P Value	**<0.001**		**0.001**		**<0.001**	

[a],[b],[c]: Different letters on the same line indicate a statistically significant difference. Values are mean ± standard deviation and Median (Min-Max)

Table 2. Analyzes of cholinesterase, and sAppBeta in prefrontal cortex, hippocampus, and serum.

Prefrontal Cortex	**sAppbeta ng/mg protein**		**Cholinesterase**	
	Mean ± S.D.	**Median (Min-Max)**	**Mean ± S.D.**	**Median (Min-Max)**
Control	0.1477±0.03227	0.15 (0.11-0.19)	94±52.78257	110 (28-150)
$AlCl_3$	0.1754±0.0531	0.16 (0.12-0.25)	106.1667±24.9593	104.5 (80-145)
Donepezil	0.1675±0.10917	0.14 (0.06-0.32)	101±38.02631	102.5 (53-146)
EVOO	0.0933±0.03645	0.1 (0.04-0.13)	312.4±471.62623	103 (92-1156)
Rosmarinic acid	0.1597±0.10184	0.14 (0.07-0.33)	55.6±29.2113	45 (36-107)
P Value		**0.376**		**0.356**
Hippocampus	**sAppbeta ng/mg protein**		**Cholinesterase**	
	Mean ± S.D.	**Median (Min-Max)**	**Mean ± S.D.**	**Median (Min-Max)**
Control	0.2821 ± 0.1222	0.2566 (0.1452 – 0.4455)	41 ± 27	48 (1 - 73)
$AlCl_3$	0.2821 ± 0.1222	0.2566 (0.1452 – 0.4455)	41 ± 27	48 (1 - 73)
Donepezil	0.3873 ± 0.2	0.406 (0.1478 – 0.5893)	34 ± 24	31 (9 - 67)
EVOO	0.3104 ± 0.0977	0.3132 (0.2077 – 0.4291)	36 ± 23	35 (9 - 69)
Rosmarinic acid	0.4916 ± 0.3466	0.4559 (0.1733 – 1.0575)	13 ± 8	11 (3 - 22)
P Value		**0.406**		**0.296**
Serum	**sAppbeta ng/mg protein**		**Cholinesterase**	
	Mean ± S.D.	**Median (Min-Max)**	**Mean ± S.D.**	**Median (Min-Max)**
Control	1.1896 ± 0.188	1.2999 (0.935 – 1.3471)	151 ± 36	136 (114 - 197)
$AlCl_3$	0.9961 ± 0.2956	0.8932 (0.6542 – 1.3939)	202 ± 64	201 (137 - 292)
Donepezil	1.1294 ± 0.2122	1.2205 (0.7918 – 1.3392)	140 ± 32	128 (110 - 190)
EVOO	0.9765 ± 0.2926	1.0506 (0.5227 – 1.2285)	137 ± 34	136 (88 - 180)
Rosmarinic acid	1.079 ± 0.2706	1.1644 (0.7747 – 1.3392)	130 ± 18	121 (113 - 155)
P Value		**0.658**		**0.061**

[a,b]: Different letters on the same line indicate a statistically significant difference. Values are mean ± standard deviation and Median (Min-Max).

Table 3. The relative adrenal and thymus weights in the all groups.

Groups	**Relative Adrenal Weight**		**Relative Thymus Weight**	
	Mean ± S.D.	**Median (Min-Max)**	**Mean ± S.D.**	**Median (Min-Max)**
Control	0.0236±0.0064	0.0202 (0.0189 – 0.0355)	0.0564 ± 0.0073 [b]	0.0567 (0.047 – 0.0665)
$AlCl_3$	0.0161±0.003	0.0166 (0.0113 – 0.0198)	0.0363 ± 0.008 [c]	0.036 (0.0249 – 0.0473)
Donepezil	0.0202±0.0074	0.0184 (0.0118 – 0.0333)	0.0833 ± 0.0133 [a]	0.0763 (0.075 – 0.1064)
EVOO	0.0192±0.0067	0.0176 (0.0118 – 0.03)	0.086 ± 0.0076 [a]	0.085 (0.0772 – 0.1)
Rosmarinic Acid	0.0213±0.0022	0.0208 (0.018 – 0.0242)	0.0711 ± 0.0065 [ab]	0.0711 (0.0625 – 0.0783)
P		0.166		<0.001

[a,b,c]: Different letters on the same line indicate a statistically significant difference. Values are mean ± standard deviation and Median (Min-Max).

Table 4. In open field test in the total distance moved, time spent in the middle-edge areas and average speeds.

Groups	Distance moved (cm)		Middle area-time (s)		Thigmotaxis (s)		Speed (cm/s)	
	Mean±SD	Median (Min-Max)	Mean±SD	Median (Min-Max)	Mean±SD	Median (Min-Max)	Mean±SD	Median (Min-Max)
Control	1824.52±621.82	1920.67 (981.88-2578.14)	4.87±6.67	1.04 (0-17.68)	244.72±58.92	267.36 (114.8-281.76)	6.08±2.06	6.43 (3.26-8.57)
$AlCl_3$	1556.66±468.85	1390.64 (1077.1-2510.89)	57.02±108.19	20.8 (0-301.04)	197.97±93.52	213.84 (0-276.72)	5.19±1.58	4.62 (3.58-8.41)
Donepezil	1541.01±401.96	1403.4 (934.36-2079.81)	7.28±5.64	5.84 (0-16.32)	254.54±45.22	266.96 (164.96-298)	5.19±1.39	4.66 (3.1-6.99)
EVOO	1706.83±608.49	1847.25 (572.59-2407.01)	3.52±7.17	0 (0-17.92)	276.95±15.22	277.4 (254.8-300.24)	5.81±2.1	6.2 (1.9-8.08)
Rosmarinic acid	1885.32±561.63	1930.2 (1316.43-2811.62)	3.23±3.75	2.16 (0-8.56)	268.64±27.24	266.72 (221.6-295.84)	6.28±1.86	6.49 (4.37-9.34)
P Value		**0.75**		**0.083**		**0.105**		**0.728**

There was no significant difference between the groups. Values are mean ± standard deviation and Median (Min-Max).

Table 5. In elevated plus maze test in the all groups in total distance moved, open-closed arms, time spent in the middle area and average speeds.

Groups	Distance moved (cm)		Open arms (time) (s)		Closed arms (time) (s)		Middle area (time) (s)		Speed (cm/s)	
	Mean±S.D.	Median (Min-Max)	Mean±S.D.	Median (Min-Max)	Mean±S.D.	Median (Min-Max)	Mean±S.D.	Median (Min-Max)	Mean±S.D.	Median (Min-Max)
Control	813.42±262.71	754.87 (477.49-1260.43)	4.13±5.06	0.8 (0-11.52)	284.74 ± 14.59	278.56 (265.12-301.04)	11.62 ± 12.2	12.56 (0 – 35.12)	2.72 ± 0.89	2.51 (1,62 – 4.25)
$AlCl_3$	804.68±492.87	513.3 (322.11-1616.7)	6.21±8.98	1.6 (0-20.56)	286.58 ± 21.84	298.88 (253.04-301.04)	8.25 ± 12.88	0.56 (0 – 27.44)	2.67 ± 1.64	1.71 (1,07 – 5.37)
Donepezil	942.47±333.59	815.81 (654.58-1528.01)	8.55±14.74	0.24 (0-36.8)	293.77 ± 10.55	298.64 (227.6-301.04)	8.14 ± 13.7	2.16 (0 – 36.64)	3.13 ± 1.11	2.71 (2,18 – 5.08)
EVOO	1062.43±250.96	1005.61 (785-1450.68)	2.8±3.95	0.76 (0-9.52)	293.72 ± 5.1	292.28 (287.12-300.24)	4.51 ± 5.38	2.04 (0.08 - 13.04)	3.53 ± 0.84	3.34 (2,61 – 4.84)
Rosmarinic acid	1141.79±420.57	1079.06 (602.49-1738.09)	21.22±28.24	11.28 (0-80)	275.57 ± 25.55	278.72 (119.6-295.28)	14.04 ± 14.35	9.36 (3.44 - 42.64)	3.8 ± 1.4	3.59 (2 – 5.78)
P Value		**0.241**		**0.392**		**0.358**		**0.414**		**0.241**

There was no significant difference between the groups. Values are mean ± standard deviation and Median (Min-Max).

Table 6. Rotarod motor performance test results.

Groups	16 rpm		20 rpm		26 rpm		30 rpm	
	Mean ± SD	Median (Min-Max)	Mean ± SD	Median (Min-Max)	Mean ± SD	Median (Min-Max)	Mean ± SD	Median (Min-Max)
Control	0,429 ± 0,534	0,001 (0,001 - 1) ^{ab}	0,286 ± 0,487	0,001 (0,001 - 1) ^{b}	0,144 ± 0,378	0,001 (0,001 - 1) ^{b}	0,001 ± 0	0,001 (0,001 - 0,001) ^{b}
$AlCl_3$	0,286 ± 0,487	0,001 (0,001 - 1) ^{b}	0,144 ± 0,378	0,001 (0,001 - 1) ^{b}	0,144 ± 0,378	0,001 (0,001 - 1) ^{b}	0,144 ± 0,378	0,001 (0,001 - 1) ^{b}
Donepezil	1 ± 0	1 (1 - 1) ^{a}	1 ± 0	1 (1 - 1) ^{a}	1 ± 0	1 (1 - 1) ^{a}	0,715 ± 0,487	1 (0,001 - 1) ^{a}
EVOO	0,834 ± 0,408	1 (0,001 - 1) ^{ab}	0,501 ± 0,547	0,501 (0,001 - 1) ^{ab}	0,001 ± 0	0,001 (0,001 - 0,001) ^{b}	0,001 ± 0	0,001 (0,001 - 0,001) ^{b}
Rosmarinic acid	0,857 ± 0,378	1 (0,001 - 1) ^{ab}	0,429 ± 0,534	0,001 (0,001 - 1) ^{ab}	0,429 ± 0,534	0,001 (0,001 - 1) ^{b}	0,001 ± 0	0,001 (0,001 - 0,001) ^{b}
P Value		**0.021**		**0.021**		**0.001**		**0.001**

^{a,b}: Different letters on the same line indicate a statistically significant difference. Values are mean ± standard deviation and Median (Min-Max).

Table 7. In MWM, an increase in swimming speed observed in the all group on the 3rd and 4th learning days.

Groups	1.day	2.day	3.day	4.day
Control	21.07±1.47	18.67±2.95	16.44±3.07[a]	19.56±1.89[a]
$AlCl_3$	21.92±2.23	20.47±4.20	34.93±10.64[b]	27.55±5.62[b]
Donepezil	21.69±2.46	20.79±5.19	20.12±3.29[a,b]	21.58±6.71[a,b]
EVOO	21.19±3.05	21.49±3.66	27.45±9.12[a,b]	16.62±4.09[a,b]
Rosmarinic acid	20.18±4.245	23.01±7.09	30.16±5.64[a,b]	18.37±2.88[a,b]

[a,b]:Different letters on the same line indicate a statistically significant difference (P<0.05). Values are mean± standard deviation.

Table 8. On the 5th day of the experiment spending time in the hidden platform area in the all group.

Groups	Hidden platform area (time) (s)	Other platforms (time) (s)	Distance swim (cm)	Swim speed (cm/s)	Thigmotaxis (s)
Control	23.89±4.52[a,b]	11.31±1.73	1604.82±128.51	27.10±2.10	21.15±6.28
$AlCl_3$	19.80±3.37[a]	13.59±1.09	1227.42±148.53	20.27±2.63	31.08±10.40
Donepezil	31.89±9.051[b]	10.19±3.31	1207.3±263.33	19.77±4.31	24.11±6.73
EVOO	27.52±3.32[a,b]	11.17±1.10	1335.89±246.51	20.51±4.03	21.98±12.28
Rosmarinic acid	28.49±6.62[a,b]	10.06±1.84	1376.38±230.69	23.57±4.53	22.29±2.25

[a,b]**:** Different letters on the same line indicate a statistically significant difference (P<0.05). Values are mean ± standard deviation.

Discussion and Conclusion

In this study, we were able to demonstrate that cognitive impairment has been occurred after 15 days of $AlCl_3$ administration by oral gavage and treatment protocols prevented the occurrence of AD pathology histopathologically. We also showed that oxidative damage findings have been observed in serum and hippocampus. Besides, treatment groups showed better cognitive and motor performance, and there was no damage to the cells in the control and treatment groups.

Neuropathologically, extracellular β-amyloid plaque deposition, intracellular neurofibrillary tangle deposition, neuron and synapse loss, basal forebrain, hippocampus, and gliosis in learning-related regions are the histopathological findings for AD (9). In a study in which 100 mg/kg $AlCl_3$ was administered orally to rats, pathological changes were not observed in the control group, while vacuolated cytoplasm, neurodegeneration, and inflammation were shown in the $AlCl_3$ group (22). Consistent with the studies in the literature, in this study, hematoxylin&eosin staining showed atrophy, decreased neuronal cell density, and gliosis in the prefrontal cortex and hippocampus of rats in the $AlCl_3$ group to compare control group. In the hippocampus, neurofibrillary tangles in the form of basophilic staining were also detected in the $AlCl_3$ group, unlike the control group. In congo red staining, positively stained cell cytoplasms and degenerations were found in the prefrontal cortex and hippocampus in the $AlCl_3$ group, unlike the control group.

Oxidative stress is defined as the imbalance between reactive oxygen/nitrogen species (ROS/RNS) and the capacity of anti-oxidative protection systems of cells to neutralize these reagents (32). Recent evidence indicates that some oxidation products act as biomarkers in some neurodegenerative diseases, and the lipid peroxidation product malondialdehyde (MDA) is one of them (3). Increased MDA levels are observed in the cortex and hippocampus in Alzheimer's patients (3), but no significant difference between groups was observed in the tissue level in this study. This may be because the $AlCl_3$ application was performed for 15 days, and this period did not cause oxidative stress at the tissue level. However, serum MDA level was significantly higher in the $AlCl_3$ group compared to the control group, which can be interpreted as the onset of oxidative stress changes in the $AlCl_3$ group.

Lactate dehydrogenase (LDH) is an enzyme found in almost every cell and tissue in the body. The high total activity in the LDH enzyme indicates tissue damage or cell destruction in the body (23). In a study that using the model induced by $AlCl_3$, it was found that the serum LDH level of the $AlCl_3$ group was significantly higher than the control and treatment groups (19). Interestingly, some studies using an Alzheimer's mouse model suggest that the loss of aerobic glycolysis in the brain is associated with AD. In a study conducted with APP/PS1 mice, it was shown that there was a decrease in the expression of the aerobic glycolysis kinase isoenzyme 1 (PDK1) and LDH enzyme in the frontal cortex compared to the same age group control mice at the age of 12 months (28). Besides, brain imaging studies in humans have shown that the brain regions most sensitive to amyloid toxicity are in the regions where aerobic glycolysis is at the highest level (42). This situation is defined as a protective mechanism

against Aβ accumulation, and it is accepted that the loss of this mechanism triggers AD (40). In this study, consistent with the results in the literature, it was observed that the serum LDH level of the $AlCl_3$ group was significantly higher than the control and treatment groups, and the LDH activity in the hippocampus was significantly lower in the $AlCl_3$ group than in the other groups. This result can be considered an indicator that a moderate level of Alzheimer's pathology has begun to occur.

In recent studies, the ratio of organ weight to body weight is considered the most widely used index to show stress-induced organ weight changes. Accordingly, absolute thymus weight can be used as biological indicators generated in response to stress (16). In this study, no statistically significant difference was found in the relative adrenal weights of the control, Alzheimer's, and treatment groups. However, a significant decrease was observed in the relative thymus weights in the $AlCl_3$ group compared to the control group, and a statistically significant increase was observed in the donepezil and EVOO treatment groups compared to the control group. In human, animal, and in vitro studies, it has been shown that phenolic compounds in EVOO are antioxidant molecules that can scavenge the toxic effects of oxygen metabolism, such as free radical formation, and thus protect cells against oxidative damage (25). Donepezil also shows significant effectiveness in reducing the severity of neuropsychiatric symptoms in mild to moderate AD (11). According to the results of this study, results are consistent with the conclusion in the literature that the use of EVOO and donepezil reduces the negative effects of stress.

Cognitive impairment is the main clinical symptom of AD; however, non-cognitive problems such as motor dysfunctions are also associated with the disease (6, 33). The decline in motor skills in mild to moderate Alzheimer's patients is substantial than in patients with moderate and severe periods (45). Recent studies suggest that AChEI's may improve some of these changes (4). In another study, it was reported that donepezil in Alzheimer's patients did not have any positive effect on motor functions (17). Also, in another study, enriching the diet with herbal antioxidants can improve brain damage and cognitive functions (30). The anti-amnesic activation effect of rosmarinic acid against neurotoxicity and neurodegeneration induced by Aβ in mice was also shown (20, 26). But in this study, in the rotarod motor performance test, there was a statistically significant difference between donepezil and the other groups at 26 and 30 rpm speeds and showed better walking performance. Studies on the effect of donepezil on motor performance in the literature are limited, and more research is needed on this subject.

MWM test is one of the most important methods that can be used to evaluate learning and memory in experimental animals with an Alzheimer's model (38). In a study, administration D-galactose and $AlCl_3$ in rats reported that the donepezil treated group found the hidden platform, and the swimming distance was significantly shorter than the $AlCl_3$ group (8). In this study, consistent with the results in the literature, it was observed that the time spent on the platform hidden area on the 5th day of the experiment was significantly higher in the donepezil group than the $AlCl_3$ group. Increased swimming speed in the MWM is considered one of the indicators of increased anxiety. In a study shown that the platform finding time of mice with Alzheimer's pathology took longer than the control group, and at the same time, the swimming speed increased compared to the control group (21). Again, in a study in which 3xTg-AD mice were used, control mice were more comfortable swimming; it has been observed that mice with Alzheimer's are more stressed and faster (10). As shown in the literature, MWM measures not only spatial learning but also anxiety and sensorimotor skills (1). In this study, it was observed that the swimming speed of the $AlCl_3$ group increased significantly on the 3^{rd} and 4^{th} days of the learning experiment compared to the control group. This situation is consistent with the results of increased anxiety and prolonged platform finding in animals with mild Alzheimer's pathology, as stated in the literature.

In OF, avoiding the middle area is considered as an indicator of anxiety. Thigmotaxis is defined as the desire to spend time near the wall, and it is accepted that animals displaying anxiety-like behavior show this behavior more frequently (34). In this study, there was no difference between the groups in OF and EPM. This result can be interpreted as that the disease-inducing and treatment protocols applied to all groups for 5 weeks may cause similar anxiety.

In conclusion, these studies results show that 15 days of aluminum exposure is effective in creating a moderate Alzheimer's pathology, but further chronic researches are necessary to explain efficiencies of rosmarinic acid and EVOO in treatment.

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Ethical Statement

This study was approved by the Animal Experiments Local Ethics Committee of Dokuz Eylül University (Ethics committee approval date: 14.12.2017 Decision number: 25/2017).

Conflict of Interest

The authors have no conflict of interests to disclose.

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The effects of adding waste sesame seeds to diets on performance, carcass characteristics, and meat fatty acid composition of Karayaka lambs

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Abstract: This study aims to determine the effects of the inclusion of waste sesame seed (WSS) to concentrate feed on performance, carcass characteristics, and meat fatty acid profile of Karayaka lambs. A total of twenty-three lambs were randomly allocated to a control group (11 lambs, with an average live weight of 23.39±0.58 kg) and an experimental group (12 lambs, with an average live weight 23.55±0.41 kg). Also, each of the group was divided into two subgroups according to their gender. The lambs were fed with isonitrogenous (17% CP) and isocaloric (2650 kcal/kg ME) concentrate feeds containing either 0% WSS (control) or 10% WSS (experimental) for 60 days. Final live weight, average daily weight gain (ADG), feed conversion ratio (FCR), and carcass traits were not affected by diet, although they were higher in males than females (P<0.01). In addition, males presented a higher percentage of C17:1, C18:2 n6, C20:3 n6 (P<0.01), and polyunsaturated fatty acid (P<0.05) than females. In conclusion, the addition of WSS to the lamb diet did not increase the concentration of unsaturated fatty acids in meat. However, WSS can be included up to 10% in concentrate feed of lambs during fattening period without negative effects on performance and carcass yield.
Keywords: Fatty acid profile, Karayaka, lamb meat, waste sesame seed.

Atık susam tohumlarının rasyonlara ilavesinin Karayaka kuzularının performans, karkas özellikleri ve et yağ asidi bileşimine etkileri

Özet: Bu çalışmada, atık susam tohumlarının (WSS) konsantre yemlere ilavesinin Karayaka kuzularının performans, karkas özellikleri ve et yağ asidi profili üzerine etkilerinin belirlenmesi amaçlandı. Toplam yirmi üç kuzu, rastgele bir kontrol (11 kuzu ve ortalama canlı ağırlığı 23,39 ± 0,58 kg) ve bir deneme grubuna (12 kuzu ve ortalama canlı ağırlığı 23,55 kg ± 0,41 kg) ayrıldı. Ayrıca, her iki grup da cinsiyetlerine göre iki alt gruba bölündü. Kuzular, WSS içermeyen (kontrol) ve %10 WSS ilave edilen (deneme grubu) izonitrojenik (%17 HP) ve izokalorik (2650 kcal/kg ME) konsantre yemlerle 60 gün süresince beslendi. Kuzuların deneme sonu canlı ağırlıkları, günlük canlı ağırlık artışları ve yemden yararlanma oranlarının rasyondan etkilenmediği, ancak bu değerlerin erkek hayvanlarda dişilere göre daha yüksek olduğu saptandı (P<0,01). Ayrıca, et yağ asidi kompozisyonu bakımından erkek kuzuların dişilere kıyasla daha yüksek oranda C17:1, C18:2 n6, C20:3 n6 (P<0,01) ve çoklu doymamış yağ asidi içerdiği (P<0,05) tespit edildi. Sonuç olarak, kuzu rasyonlarına WSS ilavesinin, etteki doymamış yağ asitleri konsantrasyonunu artırmadığı tespit edilmiştir. Bununla birlikte besi dönemindeki kuzuların konsantre yemlerine performans ve karkas verimi üzerinde negatif bir etkiye yol açmaksızın %10'a kadar WSS dahil edilebileceği kanısına varılmıştır.
Anahtar sözcükler: Karayaka, kuzu eti, susam tohumu atığı, yağ asidi profili.

Introduction

The agriculture sector producing feedstuffs for domestic livestock is confronted with problems such as the consistent supply of raw materials and price fluctuations (25). Researchers usually recommend the use of local agro-industrial by-products to decrease feeding costs. Therefore, the most appropriate local agro-industrial by-products should be utilized in livestock

feeds, providing they do not adversely affect animal health and are cost-effective in terms of productivity.

Sesame (*Sesamum indicum* L.) is an oilseed plant that is cultivated primarily for the production of sesame oil, tahini (tehineh). It is used as an ingredient in foods such as halva, sesame bread, java beans, and salads (13). In addition, many by-products are obtained during the processing of sesame seeds and these are used in animal nutrition. Some studies have been conducted with refused and waste sesame seeds (1), sesame oil (18), sesame meal (22), and sesame hull (8) included. Waste sesame seed (WSS) is a by-product obtained during the sorting and sieving processes of sesame seed. It consists mainly of low-quality sesame seeds (e.g., broken or small-sized seeds) and some harvest residues. It is rich in nutritional values and could be used as an alternative feed ingredient for ruminants (1).

Meat is one of the crucial protein sources for human nutrition. Red meat is the most preferred because it best provides the nutrients, such as essential fatty acids, amino acids, minerals, and vitamins, needed for bodily functions and growth (16). However, the fatty acid profile of meat, especially its saturated fatty acid (SFA) composition, is criticized for contributing to cardiovascular diseases, obesity, and diabetes. On the other hand, the unsaturated fatty acids (UFA) in meat are considered important due to their nutritional properties and beneficial effects on human health (11). Therefore, safe, healthy and high-quality red meat production is needed to ensure the health of consumers.

Sesame by-products are used in some countries to enhance the growth performance and carcass characteristics of lambs (22). However, there is scarce information about the effects of waste sesame seed on fatty acid profile of ruminant meat, particularly in lambs (8). Therefore, we aimed to investigate the effects of WSS inclusion in diets on performance, carcass characteristics and the fatty acid profile of lamb meat.

Materials and Methods

Animals, diets, and experimental design: This study was approved by the Local Ethics Committee of Ondokuz Mayis University for Experimental Animals (Approval number: HADYEK/2018/18). A total of twenty-three lambs with aged 2.5 to 3 months, were randomly divided into a control group (6 males and 5 females) and an experimental group (6 males and 6 females). Each group was divided into two subgroups according to their gender (2x2 factorial design). The mean live weight of the control group was 23.39 kg (±0.58 kg) and the experimental group was 23.55 kg (±0.41 kg). Before starting the trial, the lambs were treated against internal and external parasites, vaccinated against enterotoxaemia and put through two weeks of dietary adaptation. The lambs were fed as group *ad libitum* with an isonitrogenic (17% CP) and isocaloric (2650 kcal/kg ME) ration consisting of concentrate (85%) and alfalfa hay (15%) throughout the study. The diets included: control diet without waste sesame seed (WSS) and experimental diet (10% WSS). The WSS was provided by a sesame seed processing factory that produced tehineh. The ingredients and the chemical composition of experimental diets, forage and WSS are presented in Table 1. During the 60-day intensive fattening period, the amount of diet offered to animals daily was 10% more than the previous day's consumption.

The chemical composition (dry matter, ash, crude protein, ether extract, and crude cellulose) of the diets were analyzed according to the methodology of the AOAC (6).

Dry matter intake and performance: Dry matter intake (DMI) was determined by weighing the amounts of feeds offered and refusals daily. The animals were weighed individually at the start of the experiment (day 0) and every 30 days during the experimental period. Average daily gain, average feed intake and feed conversion ratio were calculated.

***Slaughter and carcass traits*:** The final weight of the lambs was determined at the end of the fattening period. Animals were slaughtered after being deprived of feed for 16 h with having free access to water (28). Hot and cold dressing percentages were calculated according to the slaughter weight of the lambs. The carcasses were kept at 4 °C for 24 h, and then the cold carcass weights were recorded. Following that, the cold carcass percentages and chilling losses were calculated.

Fatty acid composition of WSS and meat samples: 10 g of WSS and 10 g of meat samples from each animal were added to a chloroform/methanol solution (2:1 v/v) and the samples were homogenized. Ten milliliters of 10 mM $CaCI_2$ was added to the homogenate, and the mixture was stirred vigorously for 30 seconds. The mixture was centrifuged at 2000xg for 15 minutes. The chloroform phase was transferred to a flask and evoparated by a vacuum evaporator. The fat mass was then used for the determination of total fatty acids composition (10). The content of major fatty acids in the lipid mixture was determined after methylation (5) by gas chromatography-mass spectrophotometry (GC-MS) (Shimadzu model of QP2010 Plus, Shimadzu Corporation, Kyoto, Japan), using an Agilent HP-88 column (60 m x 0.25 mm I.D., 0.20 µm) (Agilent, USA). The temperatures of the injector port and detector were maintained at 250 °C. The injected volume was 1.0 µL and the carrier gas was helium at a pressure of 200 kPa (split ratio 1:100). The temperature of the column was maintained at 90 °C for 7 min, then raised to 240 °C at 5 °C/min and finally held at 240 °C for 10 min. The main fatty acids were identified by comparing retention times with the original standards (Supelco 37

Components FAME Mixture, Cat. No. 18919-1AMP, Bellefonte PA, USA) and are reported as a proportion (%) of the total fatty acids recorded (5).

Statistical analyses: The data were analyzed with SPSS (IBM Inc., USA, version 21). The Kolmogorov-Smirnov Test was used to check for normal distribution. The homogeneity of variances were evaluated with the Levene Test. The performance parameters, carcass traits and fatty acid profile of the diet (control and treatment) and gender (male and female) groups and their interactions were compared with General Linear Model (GLM). A level of $P<0.05$ was accepted as statistically significant.

Results

Feed ingredients and the chemical composition of concentrates, forage and WSS are shown in Table 1. In addition, the fatty acid profile of the WSS used in the experimental concentrate was determined to be C14:0 (0.118%), C16:0 (12.77%), C17:0 (0.049%), 17:1 (0.077%), C18:0 (22.22%), C18:1 (24.97%), C18:2 (38.21%), C18:3 (1.07%), C20:0 (0.096%), C20:1 (0.026%), 20:2 (0.096%) and 20:3 (0.072%).

Data on average daily weight gain (ADG) and final weight are shown in Table 2. Dry matter intake (DMI) and feed conversion ratio (FCR) are demonstrated in Table 3. DMI and FCR were not available for individual animals due to group feeding. However, calculated values showed that feed intakes of lambs in all groups were similar and ranged from 1253 to 1283 g. Final live weights, ADG and FCR were unaffected ($P>0.05$) by diet but were higher ($P<0.01$) for males than females.

Carcass characteristics for all groups are presented in Table 2. Mean values for the carcass characteristics were similar for the control and experimental. In terms of gender groups, the values for the carcass characteristics of male lambs, except for cold dressing percentage, were tend to be higher than those of female lambs. The mean final pH (24 h) in the musculus longissimus dorsi was 5.77 and 5.8 for the control and treatment groups respectively, and 5.83 and 5.74 for males and females respectively.

The fatty acid profiles for the groups are presented in Table 4. No significant differences were generally observed for the fatty acid profile of meat from the control and experimental groups ($P>0.05$). In both diet and gender groups, the percentages for the fatty acids C16:0 (21.48-22.22%), C18:0 (15.78-17.25%), and C18:1 (40.44-41.77%) were by far the highest. The mean fatty acid percentages in the male lamb meats were generally higher than those of female lamb meats. The males presented a higher percentage of C17:1 (heptadecanoic), C18:2 n6 (linoleic), 20:3 n6 (eicosadienoic) ($P<0.01$), and total polyunsaturated fatty acid ($P<0.05$) than females. In addition, significant differences were found between the genders with regard to some saturated fatty acid (C10:0, C15:0, C17:0) ($P<0.05$) and monounsaturated fatty acid (MUFA) concentrations (C17:1) ($P<0.01$). However, the overall SFA and MUFA percentages were unaffected by diet or gender.

Table 1. Feed ingredients and the chemical composition of concentrates, forage, and WSS.

Ingredients, %	Control	Experimental	Alfalfa	WSS
Barley	35.00	40.00		
Corn	25.50	8.50		
Sunflower meal	29.00	21.00		
Wheat Bran	1.70	11.70		
Waste sesame seed	-	10.00		
Molasses (sugar beet)	6.00	6.00		
Salt	0.70	0.70		
Limestone	1.50	1.50		
MCP[1]	0.50	0.50		
Premix[2]	0.10	0.10		
Chemical composition, % DM				
Dry matter	89.63	89.8	90.12	94.47
Organic matter	81.32	81.6	80.73	79.80
Crude Protein	17.03	17.64	17.40	15.55
Ether extract	3.04	5.12	1.34	33.37
Crude Cellulose	8.46	7.99	29.50	14.6
ME[3], kcal/kg	2657	2658	2258	3450

[1]: Monocalcium phosphate, [2]: Composition (per 1 kg of the mixture): Vit A 8000000 IU, Vit D_3 2000000, Vit E 20000 mg, Mn 50000 mg, Zn 50000 mg, Cu 10000 mg, Co 150 mg, I 800 mg, Se 150 mg, $CaCO_3$ 310000 mg, Cibus 32 Vanilla aroma 200000 mg. (Icon Agro Novimix Ruminant VM01+Aroma), [3]ME: Metabolizable energy; calculated (20, 27).

Table 2. Effects of diet and gender of lambs on performance and some carcass traits.

Item	Diet		Gender		SEM	P value		
	Control	Treatment	Male	Female		D	G	D*G
Initial weight	23.47	23.38	22.91	23.93	0.36	NS	NS	NS
Final weight	32.32	32.59	34.66	30.25	0.57	NS	**	NS
ADG (kg)	0.139	0.147	0.190	0.102	0.008	NS	***	NS
Slaughter weight (kg)	31.80	32.19	34.30	29.68	0.57	NS	***	NS
HCW (kg)	15.40	15.30	15.97	14.74	0.36	NS	NS	NS
HDP (%)	48.22	48.33	47.20	49.36	0.46	NS	*	NS
CCW (kg)	14.99	14.85	15.51	14.33	0.36	NS	NS	NS
CDP (%)	45.65	46.32	46.04	45.92	0.90	NS	NS	NS
Chilling loss (%)	2.68	3.02	2.91	2.78	0.18	NS	NS	NS
pH0	6.22	6.23	6.32	6.14	0.07	NS	NS	NS
pH24	5.77	5.8	5.83	5.74	0.02	NS	NS	NS

D: Diet, G: Gender, ADG: Avarage daily gain, HCW: Hot carcass weight, HDP: Hot dressing percentage, CCW: Cold carcass weight, CDP: Cold dressing percentage, *: P<0.05, **: P<0.01, ***: P<0.001, NS: non-significant.

Table 3. The mean feed consumption and feed conversion ratio of lambs (Dry matter basis).

Item	Control		Treatment	
	Male	Female	Male	Female
Concentrate intake, g /day	1069	993	1033	978
Forage intake, g /day	214	270	238	275
Total dry matter intake, g /day	1283	1263	1272	1253
Feed convertion ratio	6.66	12.31	6.61	10.81

Table 4. Effects of diet and gender of lambs on meat fatty acid profile (%).

	Diet		Gender		SEM	P value		
Item	Control	Treatment	Male	Female		D	G	D*G
C10:0	0.17	0.15	0.17	0.15	0.01	NS	*	NS
C12:0	0.21	0.16	0.20	0.17	0.01	NS	NS	*
C14:0	2.86	2.50	2.72	2.64	0.11	NS	NS	*
C14:1	0.25	0.21	0.23	0.23	0.01	*	NS	*
C15:0	0.46	0.42	0.47	0.41	0.01	NS	*	**
C15:1	0.14	0.12	0.14	0.13	0.01	*	NS	*
C16:0	22.22	21.48	21.89	21.81	0.18	NS	NS	NS
C16:1	1.39	1.10	1.28	1.21	0.04	**	NS	NS
C17:0	1.27	1.13	1.30	1.09	0.04	NS	*	NS
C17:1	0.77	0.61	0.77	0.62	0.02	**	**	NS
C18:0	15.78	17.56	16.09	17.25	0.43	NS	NS	NS
C18:1	41.77	40.44	40.76	41.45	0.42	NS	NS	*
C18:2 n6	4.78	4.54	5.17	4.01	0.16	NS	**	NS
C18:3 n3	1.17	1.38	1.32	1.23	0.08	NS	NS	NS
C20:0	0.94	0.72	0.75	0.98	0.04	*	NS	NS
C20:3 n6	0.74	0.77	0.86	0.65	0.03	NS	**	*
C20:3 n3	0.26	0.40	0.36	0.31	0.04	NS	NS	NS
C20:4 n6	0.20	0.38	0.33	0.25	0.04	NS	NS	NS
C22:3	0.24	0.26	0.29	0.22	0.02	NS	NS	NS
C24:0	0.11	0.19	0.16	0.14	0.01	**	NS	NS
C24:1	0.25	0.27	0.24	0.28	0.02	NS	NS	NS
SFA	44.05	44.35	43.79	44.6	0.49	NS	NS	NS
MUFA	44.84	43.06	43.74	44.17	0.44	NS	NS	NS
PUFA	7.17	7.48	8.05	6.60	0.24	NS	*	NS

D: Diet, G: Gender, SFA: Saturated fatty acid, MUFA: Monounsaturated fatty acid, PUFA: Polyunsaturated fatty acid, *: P<0.05, **: P<0.01, ***: P<0.001, NS: non-significant.

Discussion and Conclusion

The effects of adding fat to the ruminant diets depend on the amount and structural characteristics of the lipid source (12). Oils with high UFA content added to ruminant diets may cause adverse effects on dry matter intake due to their toxic effects on cellulolytic bacteria (21). In our experiment, the addition of WSS with a high UFA content to the diet did not affect the total dry matter intake. These results can be attributed to two factors: the dietary fat content (51.3 g EE/kg DM) and the form of WSS (oilseed). The DMI reduction by lipid supplementation is observed generally in animals fed diets with a higher lipid content (>60 g EE/kg DM) than used in our study (19). In rumen, oilseeds have less direct contact with rumen microorganisms and tend to release its fatty acid content slower than free oils. Therefore, fat supplementation in the form of oilseed has less detrimental effects on DM intake than free oils (12).

Final live weights, average daily weight gain, and feed conversion ratio were not affected by diet. Similar findings have also been reported for fattening lamb fed with sesame oil (18) or other lipid sources (23). The fact that the diets were isoenergetic and isonitrogenous may explain the absence of significant differences in performance. However, growth performance was affected by gender. Although dry matter consumption was similar in all groups, the lower fattening performance was observed in female groups regardless of diet. These results are consistent with other studies in which female lambs showed lower growth performance than males (14). This may related to the physiological and hormonal difference between male and female lambs (14). On the other hand, the ADG for males and females of Karayaka lambs in this experiment was lower compared to other studies (24, 29).

The lambs in all groups were fed with isonitrogenous and isocaloric diets. However, the ether extract content of the experimental diet was 2.08% higher than the control diet. Haddad and Younis (19) reported that adding fats to the diet of fattening lambs did not modify growth performance or carcass characteristics. Another study also reported no differences in growth performance and carcass characteristics when lambs received up to 6% of supplementary fat (9). Therefore, the absence in the present study of significant differences concerning carcass characteristics may be related to the same energy:protein ratio of the diets.

The slaughter weight of male lambs was significantly higher than the females (12.72%; $P<0.001$). This difference between genders can be explained by the presence of the muscle-building, anabolic hormone testosterone in males (14). The hot and cold carcass weights were unaffected by diet and gender ($P>0.05$). However, overall, the hot and cold carcass weight of male lambs was higher than that of female lambs but not significantly ($P>0.05$). Contrary to this situation, the hot carcass percentage of male lambs was lower than that of female lambs. The higher carcass percentage of female lambs can be explained by the lower percentage of the non-carcass component of female lambs. These results are in line with hot carcass characteristics; the chilling loss was 2.78% and 2.91% for the male and female lambs, respectively. However, these chilling losses were higher than the reported optimum range of 1% to 2% (2).

A carcass pH value of greater than 5.8 at 24 h postmortem is regarded as undesirable (26). In the present study, the mean pH values at 24 h postmortem were 5.73 and 5.87 for the diet and gender groups, respectively. The pH values obtained from the current study were within the acceptable range.

Sheep meat has been reported to contain mainly C14:0 (myristic acid), C16:0 (palmitic acid), C18:0 (stearic acid), and C18:1 (oleic acid) (17). However, the composition of the diet was reported to affect on body fat stores and the fatty acid profile of sheep meat (23). A study that investigated the effect of different diets on the fatty acid profile reported that oleic acid (C18:1), palmitic acid (C16:0) and stearic acid (C18:0) predominated in the longissimus thoracis muscle (51). The fatty acid profile of the meat obtained at the end of the experiment, namely C14:0 (2.50-2.86%), C16:0 (21.8-22.2%), C18:0 (15.7-17.2%), C18:1 (40.4-41.7%) and C18:2 (4.0-4.7%), was comparable with the fatty acid profile generally seen in meat of lambs (18). Additionally, SFA and MUFA levels predominated and polyunsaturated fatty acid (PUFA) levels were low. Therefore, it appears that PUFA in waste sesame seeds oil is dehydrogenated and broken down to short-chain fatty acids by microbial lipases which are absorbed, transferred in the blood, and then reassembled in the tissues as long-chain fatty and that only a small proportion is transferred to the muscle.

In the current study, the fatty acid profiles of male lambs from the different feeding groups were similar, whereas the means for C17:1, C18:2 n6 and C20:3 n6 and MUFA were all significantly lower ($P<0.05$) than for the same characteristics of the females. It has been reported that a fatty carcass is obtained from female lambs that have been fattened (3). The Karayaka sheep breed is lean and long-tailed, and the accumulation of intramuscular fat contributes to fatness in the carcass. Moreover, the PUFA proportion decreases with increasing intramuscular fat accumulation in comparison to the SFA ratio (15). The high proportion of fat in the ration when the WSS was added may have increased the carcass fatness in the female group. Therefore, the low amount of PUFA in the muscle tissue of female lambs can be explained by the increase in carcass fatness of female lambs.

In one study from Turkey, the meat quality characteristics of Karayaka lambs were determined at

different slaughter weights after they were fed with lentil straw with high fiber content in addition to concentrated feed. SFAs of 48.94% and 48.58% respectively, MUFAs of 42.66% and 44.41% respectively, and PUFAs of 7.77% and 6.98% respectively, were determined for the fatty acid profile of meat of lambs of 30 kg and 35 kg slaughter weight, respectively (4). In the present study, alfalfa hay was used as forage, and it was determined that the ranges of SFA, MUFA, and PUFA were 43.7% to 44.6%, 43% to 44.84%, and 6.6% to 8.05%, respectively, in the fatty acid profile of the lambs. It has been reported that rumen pH may increase when rations have high fiber content and that this situation can change the fatty acid profile (7). Therefore, the difference between the SFA profiles of lambs of the same breed and same body weight in different studies may be related to the use of diets with different fiber content level.

The proportion of PUFA in waste sesame seeds was approximately 40%. Despite that, the saturated fat percentage was also high. Also, the MUFA level in the fatty acid profile of the obtained lamb meats was high but the level of PUFA level was low. These results are comparable with the general fatty acid profile seen in lamb meat. Also, the mean slaughter weight of the experimental group was higher than that of both genders in the control group, and for the control and experimental groups, the carcass percentage of female lambs was higher than that of male lambs.

Overall, even though the waste sesame seed had a high percentage of UFA, it did not significantly change the fatty acid profile of the lamb meats. However, the use of waste sesame seed in combination with an additive that prevents the rumen biohydrogenation of unsaturated fatty acids would be a worthwhile follow-up study.

In conclusion, WSS may not always be effective as a nutritional strategy for increasing UFA percentage in meat. However, it can be included up to 10% in the concentrate feed of Karayaka lambs during fattening periods with no negative effects on performance and carcass yield.

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Ethical Statement

The procedures and protocols used in this experiment were approved by the Local Ethics Committee of Ondokuz Mayis University for Experimental Animals (Approval number: HADYEK/2018/18).

Conflict of Interest

The authors declared that there is no conflict of interest.

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Explant culture and multilineage differentiation of amniotic membrane derived stem cells

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Abstract: Amniotic membrane derived stem cells (AMSCs) are reported to have a comparatively higher potency than multipotent stem cells. These cells are shown to have low immunogenicity and no teratogenicity. Among various conventional methods of isolation using enzymes, explant culture method is believed to be an easy and cost-effective way to harvest stem cells. The purpose of this study was to isolate AMSCs from amniotic membrane of rats and to characterize them for multilineage differentiation, including generation of neurospheres to use them later in *in-vivo* experiments. Amniotic membranes were collected from Wistar rats on 17th day of pregnancy. After processing of the tissues, AMSCs were isolated by the explant culture method and continued to grow until 10th passage. The doubling time was estimated and the cells were analyzed for growth curve parameters at passages 5 and 9. The osteogenic and adipogenic differentiation studies were carried out from the same cells after 3rd passage. Neurospheres generation from AMSCs was performed using neurogenic induction media. The cells were further assessed for their mesenchymal, haemopoietic, and neurogenic marker expressions by immunofluorescence staining and PCR analysis The study suggests that AMSCs isolated through explant culture are reliable stem cells which could generate neurospheres under proper induction conditions and could be a potential candidate to be used on *in-vivo* neural degeneration models.

Keywords: Amniotic membrane, differentiation, explant culture, neurosphere.

Eksplant kültür ve amniyotik membran kaynaklı kök hücrelerin çok yönlü farklılaşması

Özet: Amniyotik membran kaynaklı kök hücrelerin (AMKH) multipotent kök hücrelere göre daha yüksek potansiyele sahip hücreler olduğu bildirilmiştir. Bu hücreler düşük immunojeniteye sahiptir ve teratojenik etkileri yoktur. Enzimatik yöntemlere dayanan çeşitli konvansiyonel metodların yanında eksplant kültür metodu, kök hücrelerin elde edilmesinde kolay ve ekonomik bir yöntem olarak görülmektedir. Çalışmada sıçan amniyotik membranından AMKH'lerin izole edilmesi ve daha sonra *in vivo* çalışmalarda kullanmak adına yapılan nörosfer farklılaştırması da dahil olmak üzere çoklu farklılaştırma çalışmaları ile hücrelerin karakterizasyonu amaçlandı. Amniyotik membranlar Wistar ırkı sıçanlardan gebeliklerinin 17. gününde toplandı. Bir takım işlemler sonrasında amniyotik membranlardan eksplant kültür metodu ile AMKH'ler izole edildi ve 10. pasaja kadar çoğaltıldı. Hücrelerin iki katına çıkma süreleri hesaplandı ve 5 ile 9. pasajlarda hücrelerin büyüme eğrisi parametreleri analiz edildi. Üçüncü pasajdan sonraki hücrelerde osteojenik ve adipojenik farklılaştırma çalışmaları yapıldı. AMKH'ler nörojenik indüksiyon medyumu ile nörosferlere farklılaştırıldı. Daha sonrasında hücreler mezenkimal, hemapoietik ve nörojenik belirteçlerin ifadesi bakımından immunositokimyasal boyama ve PCR analizi ile incelendi. Çalışma eksplant kültür ile izole edilen AMKH'lerin uygun koşullar altında nörosfer oluşturma kapasitesine sahip, güvenilir bir kök hücre kaynağı olduğunu ve *in-vivo* nöral dejenerasyon modellerinde kullanılabilecek yeni bir potansiyel aday olduğunu önermektedir.

Anahtar sözcükler: Amniyotik membran, eksplant kültür, farklılaştırma, nörosfer.

Introduction

Amniotic membrane derived stem cells (AMSC) stands between embryonic stem cells and adult stem cells since they have a better potential to differentiate into many different types of cells compared to adult stem cells. Moreover, there are no ethical problems in isolation of AMSCs as these are easy to obtain non-invasively at parturition and do not induce immunogenic rejection. These features of AMSCs make them a preferable source for stem cell therapy (12, 24, 26, 29, 37).

The two methods, enzymatic dissociation and explant culture are used to obtain stem cells from amniotic membrane. There are certain advantages of explant culture over enzymatic dissociation method. In explant culture method, it is possible to obtain higher numbers of cells (15) which are also more homogenous in context of their morphologies (35). The proliferation rates (35) and the viability of the obtained cells (43) are both reported to be higher for explants culture when compared to the enzymatic dissociation method. It is thought that these disadvantages in enzymatic applications may be caused by a decrease in adhesion properties due to enzyme application and loss of enzymes in washing and filtration stages (15). Further to that, the explant culture is more economical, and the processing of the tissues takes place in a much shorter time (15).

Stem cells are known to have potential to differentiate into neural progenitor cell clusters called as neurospheres (25) when stimulated by high concentrations of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) in culture conditions (6). A variety of stem cell types such as adipose tissue derived stem cells (9, 28), bone marrow derived stem cells (9), olfactory mucosa derived stem cells (1), as well as amniotic membrane derived stem cells (42) are reported to have the capability to generate neurospheres. The generated neurosphere precursor cells can differentiate into astrocytes, neurons and oligodendrocytes by culturing in a proper culture medium containing different growth factors depending upon the desired differentiation, (31). Neurosphere generating precursor cells might also be used to treat neurodegenerative diseases in animals and humans without differentiating them (14). The subset of rat AMSCs, also have been reported to express neuroectodermal (neurofilament-M), mesodermal (fibronectin), and endodermal (a-1 antitrypsin) genes (25). There are few studies on neurosphere generation from human AMSCs (3, 42, 44), however, authors failed to find any report on neurosphere generation from rat AMSCs. *In-vivo* experimental studies about neurological disorders are being performed on experimental animals especially on rat (40), mouse (38) and rabbit (21). Therefore, it is important to obtain AMSCs derived neurospheres from experimental animals to learn the role of AMSCs derived neurospheres on neurological disorders and to use them in experimental models. In this study, we aimed to isolate the stem cells from the rat amniotic membrane by using explant culture method to show their proliferation potential and generate neurospheres from AMSCs.

Materials and Methods

Isolation of Amniotic Membrane Derived Stem Cells: All procedures were approved by the Ethical Committee of Afyon Kocatepe University, Turkey (AKÜHADYEK-29-16; 17.03.2016). The Wistar rats were obtained from the Experimental Animal Application and Research Center of Afyon Kocatepe University, Afyonkarahisar. The Wistar rats on the 17^{th} day of the pregnancy were anesthetized by intraperitoneal 21.1 mg/kg ketamine and 4.2 mg/kg xylazine injections, and later euthanized with cervical dislocation to isolate amniotic membrane. Amniotic membrane pieces were seeded into 4 well plates. In each well, 500 µl of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) was added. The content of the culture medium was adapted from Nawaz et al. (2020) (27). The tissue explants were allowed to proliferate and generate cells for a week with renewal of culture media in every 48 hours (Figure 1 A, B). After one week of culture, the adhered cells were detached from the surface by using 0.25% trypsin-EDTA (Gibco, USA) and seeded into T-25 cell culture flasks.

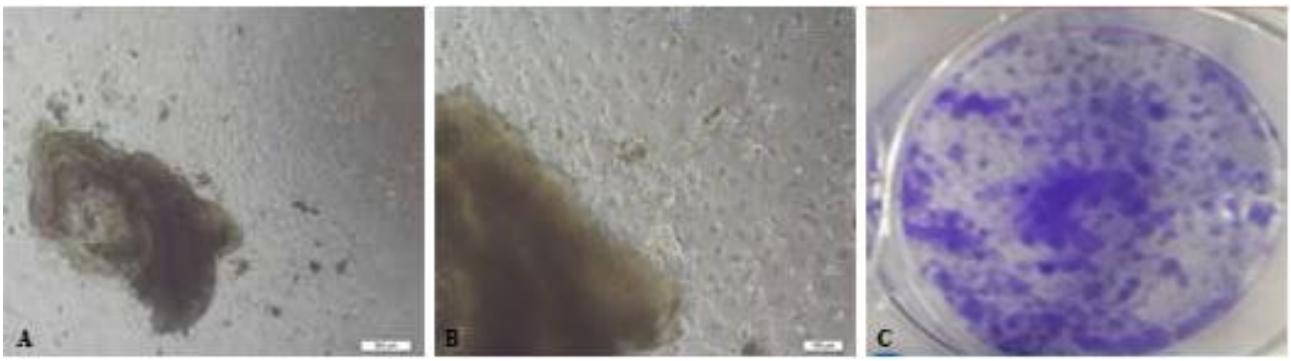

Figure 1. A, B: The cells were derived from the surroundings of the amniotic membrane tissues. C: Formed colonies of amniotic membrane derived stem cells. Crystal violet staining. Bar = A:500µm, B:100µm.

Colony Forming Unit: To observe the colony-forming capabilities, the cells from passage 1 were seeded in a 4-well plate at 1900 cells per well density. After 15 days, colony formation was confirmed by crystal violet (Premed, Turkey) staining (Figure 1).

Population Doubling Time and Growth Curve: The cells were cultured until 10th passage for doubling time studies. The seeded and harvested cell numbers were recorded alongside culture durations for each passage. Population doubling time was calculated through the following formula (33).

$$\text{PDT(h)} = \frac{\text{Duration of culture(h)} * \log(2)}{\log(\text{Final concentration (M)of cell}) - \log(\text{Initial concentration (M)of cells})}$$

Growth curve analysis was performed at P5 and P9. Briefly, cells were seeded in 4-well plates at 8×10^3cells/cm^2 concentration. Culture media was changed every 3rd day. Cells were trypsinized and counted at 2 days intervals.

PCR Analysis: Stem cell samples at P3 were collected in 1.5 ml centrifuge tubes and immediately frozen in liquid nitrogen. The protocols specified by Nawaz et al. (2020) were used in the PCR analysis (27). PCR primers were designed by using FastPCR 6.0 (16) software. All primer base pairs, Tm values, and cDNA sizes of used genes are given in Table 1. Specific expressions of mesenchymal [CD44, CD105, ITGB1 (CD29), ALCAM (CD166)], neurogenic [Tubb3 (beta tubulin), Nestin, NCAM], and hematopoietic (CD34) genes were investigated by PCR analysis on undifferentiated cells.

Differentiation Studies: For differentiation studies and related staining, the protocols specified by the researchers (27, 28) were modified and applied. Cells in the third passage ($2.9\text{x}10^4$/well) were used for adipogenic and osteogenic differentiation. One 4-well plate was used for each study. A well was spared as negative control for each type of differentiation which was cultured only with normal culture media and the remaining wells were used for differentiation experiments.

After 21 days, osteogenic differentiation was confirmed by alizarin red S staining and adipogenic differentiation was demonstrated by oil red O staining (27).

Neurosphere Generation and Immunofluorescence Staining: A previously utilized protocol was used for the neurospheres generation and immunofluorescence (IF) staining (27, 28) with a minor modification on doses of bFGF (R&D Systems, USA) and EGF (R&D Systems, USA) for neurospheres differentiation medium as 50 ng/ml ratio was used for both growth factors. After 48 hours of incubation, sizes of the generated neurospheres were measured using Image J software (1.48 latest version:1.49e, pp-188, imagej.nih.gov/ij/). In order to confirm the differentiation, generated neurospheres were subjected to IF staining against Nestin (1:500, Mouse Monoclonal; Merck- MAB5326, Germany), ß-III Tubulin (1:200, Mouse monoclonal; Abcam- ab78078, UK), and Sox2 (1:50, Rabbit clonal; Merck- AB5603, Germany). For IF studies, cells were seeded into 4-well Millicell EZslide glasses (Merck, Ireland) at a concentration of 16×10^4 cells per well and cultured with serum-free neurosphere induction media for 48 hours with same conditions as described earlier. Neurospheres were photographed at 24th and 48th hours of induction. Cells were incubated with primary antibody solutions at 4°C overnight in a humidity chamber. Anti-Nestin and anti-ß-III Tubulin primary antibodies were subjected to goat anti-mouse Alexa Fluor 488 conjugated (1:250, Abcam-ab150117, UK), while anti-Sox2 primary antibody was subjected to goat anti-rabbit Alexa Fluor 488 conjugated (1:250, Abcam- ab150077, UK) after 1 hour of incubation with secondary antibodies, antibody solutions were rinsed by 1% Tween-20 supplemented phosphate buffered saline (PBST) and one drop of DAPI Fluoroshield Mounting Medium (Abcam-ab104139, UK) was applied into wells for 1 minute. Expressions were checked under a fluorescence microscope (Zeiss Axio Observer Z.1) immediately after the mounting.

Table 1. Oligonucleotide sequences used for PCR analysis.

Gene	Forwards (5'→3')	Reverse (5'→3')	cDNA Bp	Tm ºC	NCBI No.
TUBB3	GATAGGGGCCAAGTTCTGGGA	CTCTGGGCACATACTTGTGAGAGGA	143	57	XM_032887673.1
CD34	GCCATCTCAGAGACCACGGTC	GGTGGAGTGTTCCACTTCTGGA	156	57	XM_032915361.1
CD44	GCATCCAACACCTCCCACTATGAC	CTGGTCCATCGAAGGAATTGGGTA	106	57	XM_032903796.1
CD105	GCGTCACACTTGAATGGCAACC	GGATGAGAACGGCATCCCCA	180	58	XM_032901802.1
ITGB1	TCCAGAAGGTGGCTTTGATGCA	CGTGGAAAACACCAGCAGTCG	94	57	XM_032888182.1
NESTIN	AGGTGGCTACATACAGGACTCTGC	CCCAAGGAAATTCGGCTTCAGC	119	56	XM_032898121.1
ALCAM	TGAGGCACCTACCCTGGTC	TCTCCATCAACAGGCTGTAGCAC	183	58	XM_032900289.1
NCAM	CACAAAGGCCGAGATGTCATCC	ACCTGAATGTCCTTGAAGTTGATCTC	161	57	XM_039080805.1
GAPDH	GGCAAGTTCAACGGCACAGTC	GACGCCAGTAGACTCCACGAC	144	58	NM_017008.4

cDNA: Complementary deoxyribonucleic acid, Bp: Base pairs, Tm: Melting temperature.

Results

Isolation and Culture of Amniotic Membrane Derived Stem Cells: The cells started to proliferate on the plastic surface around 10 days after seeding the tissue samples (Figure 1A, B). The isolated cells at P0 were observed to possess epithelial and fibroblast-like morphologies (Figure 2 A). The epithelial morphology, however, started to diminish by passaging (Figure 2 B) and only the cells with fibroblast-like morphology were visible at further passages starting from P3 (Figure 2 C, D, E, F).

Cell Proliferation and Colony Forming Ability: The crystal violet staining on 15th day showed that the cells at P0 were able to form colonies when they were seeded at 1900 cells/well density in 4-well plates (Figure 1 C). To determine the doubling time, the cells were cultured up to the 10th passage. In the context of doubling time, better results were obtained beginning from passage 5 and onward. The lowest doubling time was acquired from the 9th passage which means that the maximum proliferation rate was shown in that passage within the shortest duration (Figure 3 A). The viability of cells was higher than 96% after the 7th passage till the 10th passage (Figure 3 B). In growth curve studies, graphics indicated that cells rapidly entered the logarithmic phase at passage 9 after 48 hours of lag phase. The lag phase continued until 10th day whereas the cells showed an exponential growth after 6th day. But the proliferation rates of P5 cells were found to be lower in growth curve studies (Figure 3 C).

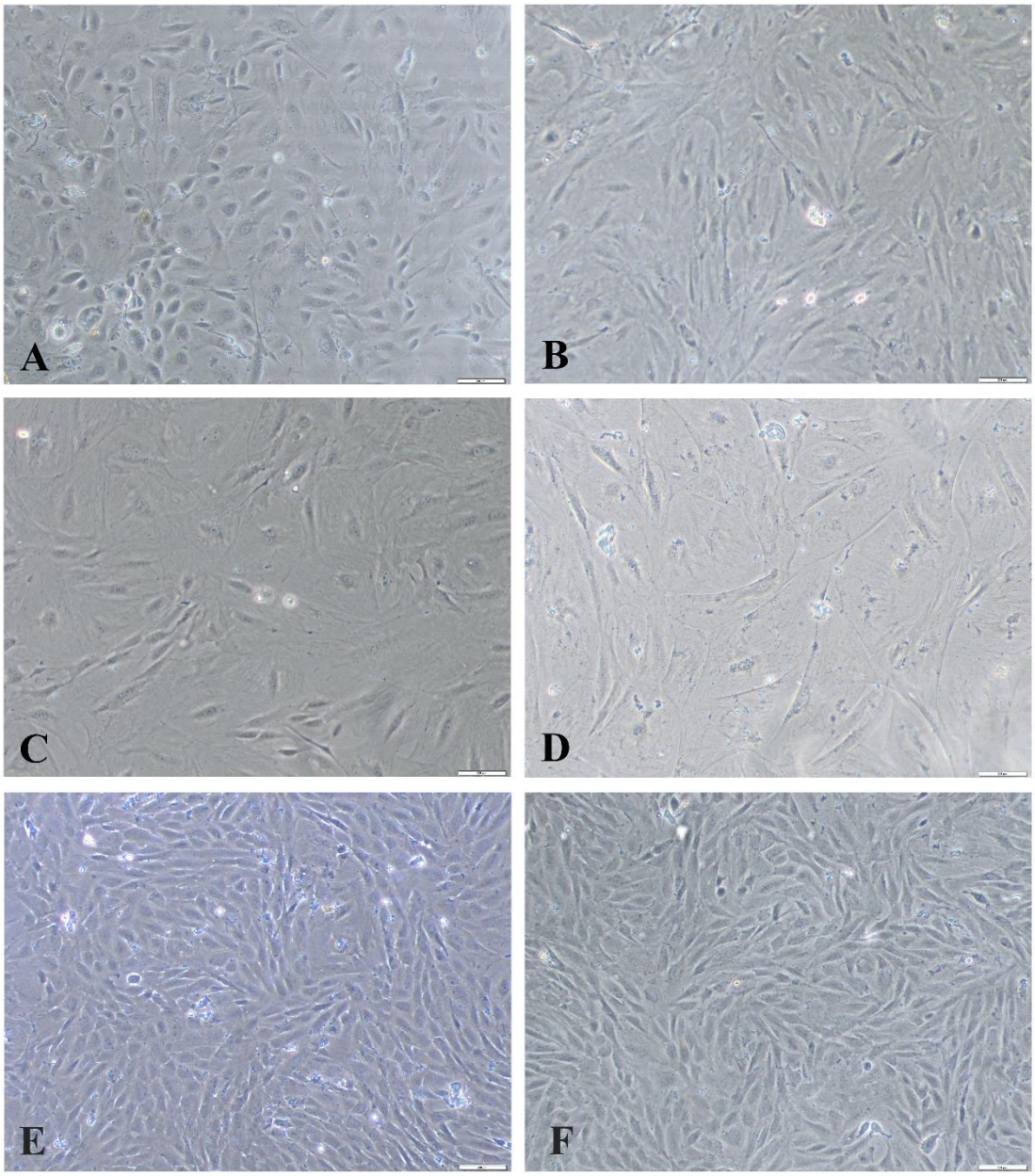

Figure 2. Fibroblast-like morphology of the cells started to be more intense after passage 1. (A: P0, B: P1, C: P3, D: P5, E: P7, F: P9). Bar = 200 μm.

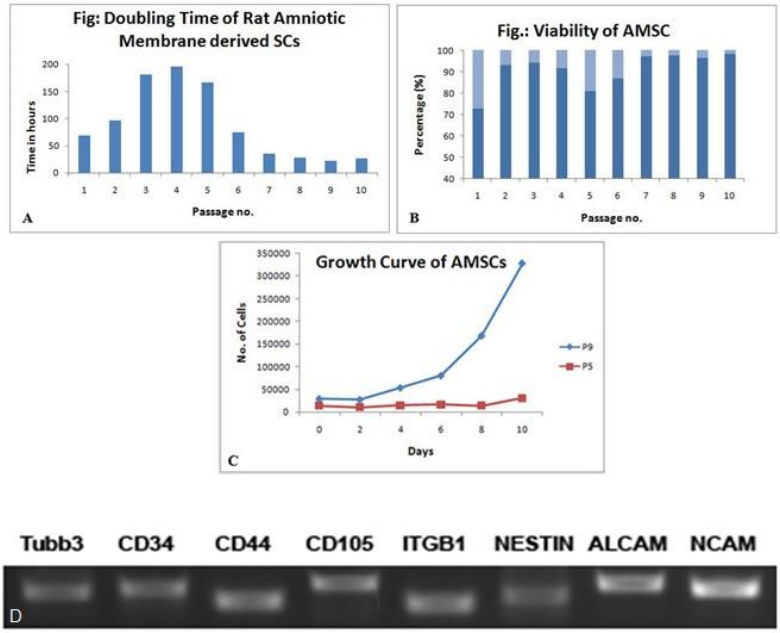

Figure 3. A: Doubling time graph for AMSCs. B: Cell viability graph for AMSCs.C: The growth curve analysis for P5 and P9 cells. D: PCR analysis of mesenchymal, neurogenic and, hematopoietic gene expressions on AMSCs.

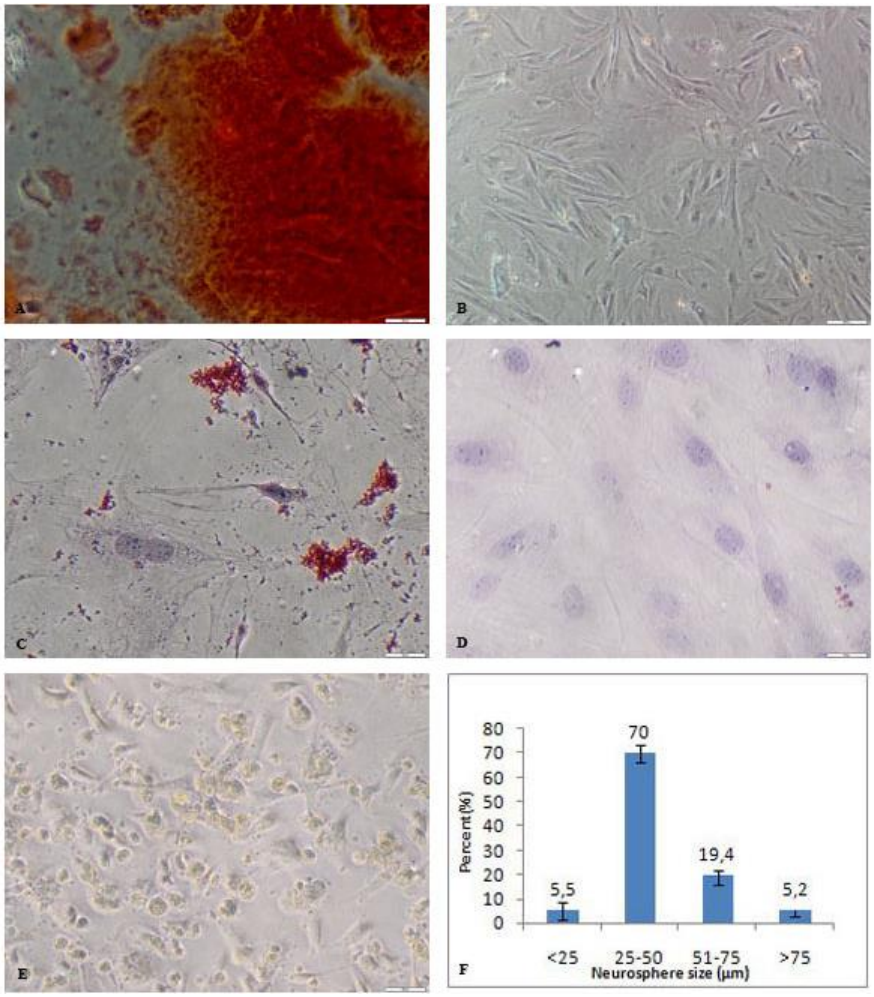

Figure 4. A, B: Alizarin red S staining. A: Osteogenic differentiation in AMSC's. Red color indicates positive staining for the calcium deposition of differentiated cells. Bar =100 µm. B: No staining was visible in control group. Bar =200 µm. C, D: Oil Red-O staining; counter stained with hematoxylin. C: Adipogenic differentiation in AMSC's. Red color indicates positive staining for the lipid vacuoles. Bar =50 µm. D: No staining was visible in control group. Bar =50 µm. E: Neurospheres at 48th hour. Bar =100 µm. F: Percentage distribution of the sizes of neurospheres.

Gene Expression Patterns of AMSCs: A PCR analysis was performed to investigate various genes including mesenchymal stem cell markers [CD44, CD105, CD29 (ITGBI), CD166 (ALCAM)] alongside neurogenic cell markers [Nestin, NCAM, TUBB3 (β-III tubulin)] and hematopoietic marker (CD34) on AMSCs (Figure 3 D). All the aforementioned genes were given positive results for our study.

Differentiation Studies: Amniotic stem cells were differentiated into osteogenic, adipogenic, and neurogenic lineages. Osteogenic differentiation was demonstrated with alizarin red S staining. In differentiation groups, red-orange stained extracellular calcium deposits were shown as there was no staining in the negative control group and the morphology of those cells remained in an undifferentiated state (Figure 4 A, B). Adipogenic differentiation was confirmed by oil red O staining. Bright red stained cytoplasmic lipid vacuoles were demonstrated in the differentiation groups (Figure 4 C). While the morphology of differentiated cells was seen under microscopic evaluations, there were no morphological changes visible in the negative control and also there were no stained vacuoles visible in negative control wells (Figure 4 D). The cells were also subjected to neurogenic differentiation by neurosphere generation studies. For this purpose, cells were cultured with neurosphere induction medium for 48 hours. Neurospheres were started to be formed even at 24th hour. After 48 hours of induction, free floating and round shaped neurospheres were found to be generated (Figure 4 E). At this point, sizes of the neurospheres were measured by using Image J software. It is found that 70% of the generated neurospheres had a radius of 25-50 microns whilst 19,4% of neurospheres were at a size of 51-75 microns (Figure 4 F). There were also much smaller and bigger neurospheres, but the amount of those neurospheres were lesser. In order to confirm neural properties of the generated neurospheres, immunofluorescence staining was also performed at 48th hour. Immunofluorescence staining against Nestin, ß-III Tubulin and Sox2 were all given positive results while there was no reaction at negative control wells as the staining demonstrated that the cells indeed possess neurogenic properties even after a short period of differentiation (Figure 5).

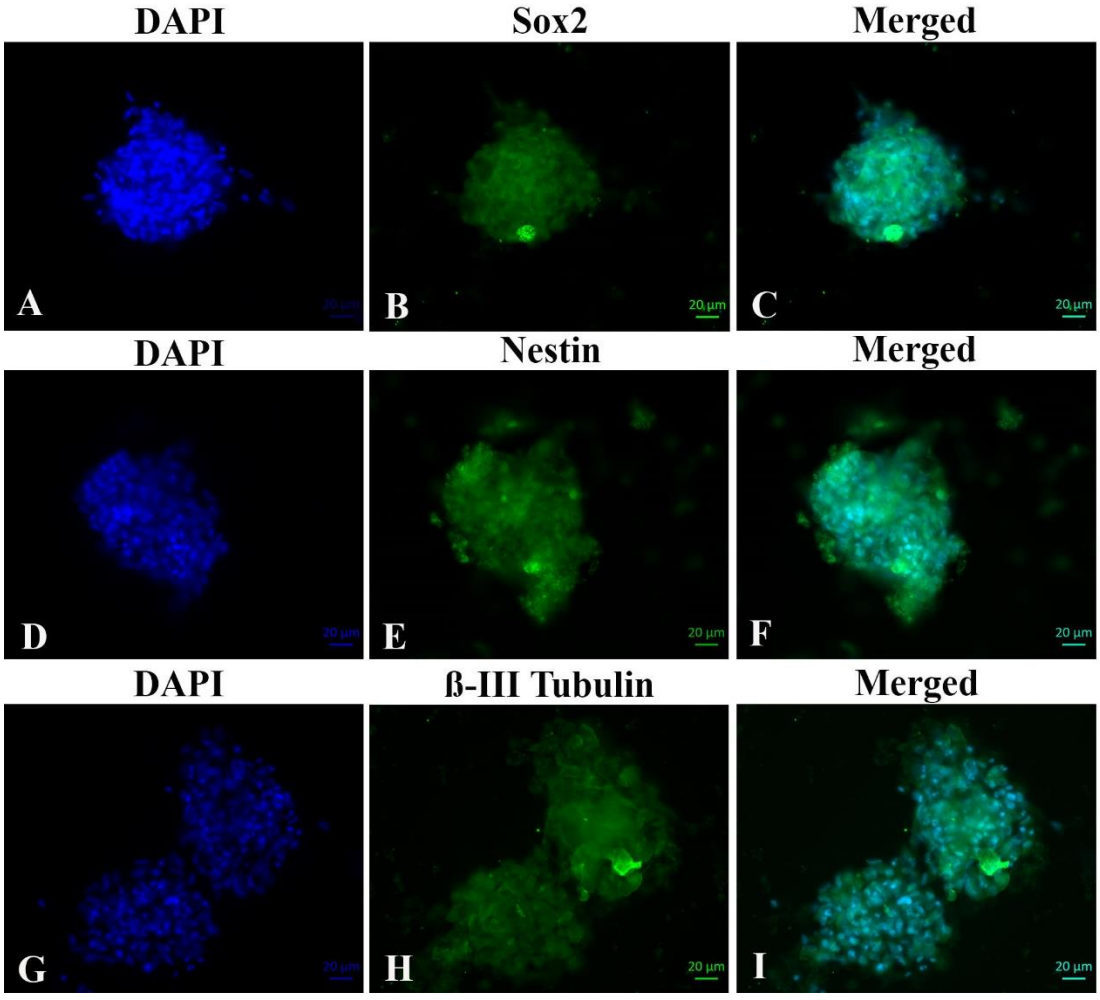

Figure 5. Immunofluorescence staining against Sox2, Nestin and ß-III tubulin in neurospheres at 48 th hour. Nuclei stained with DAPI (A, D, G). The results are pointing that neuropsheres are positive against Sox2 (B, C) and they have strong expression of neural progenitor marker Nestin (E, F) alongside with neurogenic marker β-III tubulin (H, I). Bar: 20 µm.

Discussion and Conclusion

AMSCs were isolated in line with previous studies (25). Isolated AMSCs possess fibroblast-like morphology (Figure 2) with good adhesion potential similar to other studies (10, 11, 19, 25). In primarily cultured P0 cells, the cells usually have a heterogeneous population, consisting of fibroblast-like and/or round shaped morphologies (34). After the first passage, cells with fibroblast-like morphology began to increase (17). Similarly, cells of different morphologies were observed at P0 while fibroblast-like cells were observed to intensify in the following passages (Figure 2) in this study. In stem cell research, cells from passage 3 or further are usually preferable for differentiation analysis since both the uniform morphology generally commences and the cells express necessary stem cell markers from P3 and onwards (18, 20). Stem cells are supposed to be capable of forming colonies. In this study, it was observed that amniotic cells indeed formed colonies proving that they have the potential to colonize in the primary culture at P0. This situation may indicate a paracrine signalization among the cells as it has been reported in umbilical cord perivascular cells (36).

AMSCs were announced to have the capacity to differentiate into osteocytes and adipocytes (25, 27). Our findings from osteogenic and adipogenic differentiation studies match with previously reported researches.

The doubling time graph shows that after the 5th passage, the proliferation rate increased and the doubling time remained very short till the 10th passage (Figure 3 A). Proliferation assays indicated that AMSCs were seen to reach the highest proliferation rate in the 9th passage with the least doubling time (Figure 3 A). Also, it has been shown that AMSCs had a viability ratio higher than 96% starting from passage 7 (Figure 3 B). However, the previous reports indicate that doubling time begins to decrease with P3 (18, 19) and offer using stem cells from passage 3 for therapeutical approaches, as we observed that doubling time of our cells started reducing from passage 5 onward. Growth curve analysis was performed in passages 5 and 9 since the doubling time began to decrease by P5 and the shortest doubling time was measured in P9. In the context of growth curve, the analysis showed similarities with doubling time results. Cells represented a 48 hours of lag phase at passage 9. The cells showed a log phase continued until 10th day afterwards. While the cells represented a long log phase with exponential growth at passage 9; passage 5 cells were found to be less proliferative (Figure 3 C). Those doubling time and growth curve analysis results pointed out that using AMSCs starting from passage 5 and following passages could be more proper for therapeutic approaches.

In this study, the expression of both mesenchymal (4, 18) and neurogenic (41) markers as well as hematopoietic (8, 13) marker CD34 were observed by PCR analysis. Although a negative expression was expected for CD34 (2, 30), we detected mild expression for this gene which was similar to the findings of some previous studies (8, 13).

AMSCs are suitable for neurosphere formation because they express neuronal cell markers with mesenchymal cell markers. In this study, neurospheres were successfully generated from rat AMSCs as well as the other researches did in different species and/or different sources (3, 14, 22, 32). Neurospheres formed *in vitro* are a mixture of stem cells, progenitor cells, and further differentiated cells such as neuronal and glial cells (7, 23, 39). A separation was also made depending on the sizes of the neurospheres. It was observed that a great portion of the neurospheres were small to medium in size with approximately 25-50 microns of diameters. Large neurospheres are the representatives of neuronal stem cells and they can be a good source of neurons. Furthermore, neurospheres with smaller size represent progenitor cells that can enhance and support the neurons as well as replenish nervous tissue by promoting glial cells (5). Neurospheres chosen by the means of those properties could be applied to an area of defect in neurogenic disorders (14). Neurospheres are anticipated to differentiate into neural cells since they are neural progenitor stem cells. Yet, a molecular neuroprogenitor characterization has to be done by checking related genes and proteins in order to prove neurogenic differentiation potentials of the neurospheres generated from AMSCs. To show the neuroprogenitor character of the generated neurospheres, we checked fluorescence expressions of some neural markers and demonstrated positive expressions for Nestin, Sox2 and, ß-III Tubulin after 48 hours of induction in the neurospheres generated from AMSCs. The results are consistent with previous neurospheres studies in that context (22, 28, 32). Although there are many previous authors on the cells from different sources, neurosphere studies with amniotic membrane remain rare. Yan et al. (42), presented that neurospheres generated from human AMSCs express both Nestin and Sox2 similar to our study. They also conducted a comparative study between non-differentiated stem cells and neurospheres and stated that both relative expressions and positive cell percentages for Nestin and Sox2 were significantly greater in neurosphere (42). Zhou et al. (44), also reported positive expression of Nestin in neurospheres at 3rd day in their research on human AMSCs. They further differentiated neurospheres into neuron-like cells (NLC) and reported positive expressions for ß-III tubulin, GFAP, S100 and MBP in those differentiated NLCs (44). In the study, although we did not perform NLC differentiation, we obtained positive results for ß-III tubulin in neurospheres in a short notice like 48

hours. Yet, most of the previous studies on neurospheres which were generated from AMSCs were conducted on humans and stem cells were isolated with enzymatic dissociation method (42, 44). We were unable to find any similar previous reports on neurospheres for both rat AMSCs generated neurospheres and amniotic membrane derived stem cells of any species which the isolation was made by explant culture method. These results may suggest that rat amniotic membrane derived stem cells are a promising source for neurosphere studies and explant culture method is a reliable method for neurosphere generation coupled with other previously reported advantages.

AMSCs isolated by explant culture proved to be a reliable stem cell type and to have high cell viability as well as their multilineage differentiation potential. Besides, neurospheres generated from AMSCs could be a potential candidate to be used on *in vivo* neural degeneration models.

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Ethical Statement

This study was approved by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (AKÜHADYEK-29-16; 17.03.2016).

Conflict of Interest

The authors declared that there is no conflict of interest.

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Investigation of the effects of fetal rat kidney-derived mesenchymal stem cells implementation on doxorubicin-induced nephropathy in male Sprague–Dawley rats

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Abstract: The potential protective effects of mesenchymal stem cells (MSCs) on some kidney diseases have been reported. However, the effect of the fetal kidney–derived (FKD)MSCs on doxorubicin-induced nephropathy has not been studied yet. This study aimed to treat rats with doxorubicin-induced kidney injuries by transplantation of –FKD-MSCs. Twenty-four Sprague-Dawley rats were divided into three groups as control, doxorubicin nephropathy (Sham), and doxorubicin + MSC treated group. Serum biochemistry analysis was performed at the beginning and the end of the study. Functional changes in kidneys were evaluated by scintigraphy. In the doxorubicin nephropathy group, histopathological findings such as mesangial cell proliferation, tubular cast, and glomerular hypertrophy were observed, whereas in the MSC group these findings were significantly reduced. CD133 and CD24 positive immunoreactions were the most severe and frequently observed in the MSC group. While positive staining was detected in the tubular epithelium, there was no immunostaining observed in the glomerulus. The results showed that both functional and histological improvements were achieved in the MSC group compared to the Sham group. In conclusion, transplantation of fetal kidney - derived MSCs into patients with renal damage is thought to contribute to the healing of the renal tissue.
Keywords: Doxorubicin, mesenchymal stem cell, nephrotoxicity, rat.

Erkek Sprague-Dawley ratlarda doksorubisin nefropatisinde fetal rat böbreği kökenli mezenkimal kök hücre uygulamasının etkilerinin araştırılması

Özet: Mezenkimal kök hücrelerin (MKH) bazı böbrek hastalıklarındaki potansiyel koruyucu etkileri bildirilmiştir. Bununla birlikte, fetal böbrek kaynaklı (FBK) MKH'ların doksorubisin ile indüklenmiş nefropati üzerindeki etkisi henüz araştırılmamıştır. Bu çalışmanın amacı, doksorubisin kaynaklı böbrek hasarı olan ratlara FBK-MKH'ların transplantasyonu yapılarak hasarın tedavi edilmesidir. Çalışmada yirmi dört adet Sprague–Dawley ırkı rat üç gruba ayrılmıştır. Bunlar: kontrol grubu, doksorubisin nefropatisi (Sham) grubu ve doksorubisin + MKH ile tedavi edilen gruptur. Çalışmanın başında ve sonunda serum biyokimya analizleri gerçekleştirilmiştir. Böbreklerdeki fonksiyonel değişiklikler sintigrafi ile değerlendirilmiştir. Doksorubisin nefropatisi grubunda mezanjiyal hücre proliferasyonu, tübül içi kast birikimi ve glomerüler hipertrofi gibi histopatolojik bulgular gözlenirken, MKH grubunda bu bulgular anlamlı olarak azalmıştır. CD133 ve CD24 pozitif immünreaksiyonlar, en şiddetli ve en sık olarak MKH grubunda gözlenmiştir. Tübüler epitelde pozitif boyanma tespit edilirken glomerulusta immün boyanma gözlenmemiştir. Sonuçlar, Sham grubuna kıyasla MKH grubunda hem fonksiyonel hem de histolojik iyileşmelerin sağlandığını göstermiştir. Sonuç olarak, böbrek hasarı olan hastalara fetal böbrek kaynaklı MKH transplantasyonunun böbrek dokusunun iyileşmesine katkıda bulunduğu düşünülmektedir.
Anahtar sözcükler: Doksorubisin, mezenkimal kök hücre, nefrotoksisite, rat.

Introduction

Chronic renal failure (CRF) can be defined as a chronic and progressive deterioration of metabolic-endocrine functions and adjustment of the fluid-solute balance of the kidney as a result of a decrease in glomerular filtration value. CRF is characterized by the development of tubulointerstitial inflammation and fibrosis in glomerulosclerosis (21).

Doxorubicin (Adriamycin) is an anthracycline and an anti-tumor class drug that has been used for the treatment of several types of cancer (uterine, ovarian, breast, lung) in humans (1, 9, 24) and in animals (4, 12, 16). It induces nephrotoxicity in the cancer patients, thus its clinical applications are limited (24). Doxorubicin is a well-known inducer of kidney injury in rodents and mimics CRF in humans with primary focal segmental glomerulosclerosis. It is mainly metabolized in the liver. It accumulates mainly in the kidney but is also found in the liver, heart, and small intestine (10).

The kidney is defined as an organ with minimal cellular recovery and low regeneration capacity (21). Cellular or regenerative therapies targeting progenitors in the damaged kidney have emerged as an innovative strategy. In stem cell-based therapies, renal tropism, and regenerative capacity can be increased by using the unique characteristics of stem cells and contributing to kidney healing (1, 7, 23).

This study aimed to treat doxorubicin-induced nephropathy in rats by intraperitoneal administration of fetal kidney - derived MSCs (FKD–MSCs). It has been reported in many studies that transplantation of MSCs has the potential to treat many diseases. However, the effect of FKD–MSCs in doxorubicin-induced nephropathy in rats has not been studied previously. It was hypothesized that repeated intraperitoneal administration of FKD–MSCs would result in both structural and functional renal improvement as evaluated by serum biochemistry analysis, dynamic renal scintigraphy, histological, and immunohistochemical examination.

Materials and Methods

Ethical approval: The experimental protocol was approved by the Local Animal Ethics Committee of Dışkapı Yıldırım Beyazıt Training and Research Hospital.

Experimental rats: Twenty - four male, 10- 12 weeks old, conventional Sprague - Dawley rats (200 ± 20 g) were purchased from Dışkapı Training and Research Hospital Laboratory Animal Facility. All rats were kept under standard laboratory conditions (21 ± 2°C, 65% humidity, and 12 h light / 12 h dark). The animals were fed ad libitum with standard rat chow and allowed access to water continuously.

Preparation of the FKD - MSCs: A hysterectomy was performed on 5 pregnant rats (on the 19th day of pregnancy) by median line laparotomy under sterile conditions, and 23 fetuses were taken. The fetuses were anesthetized and euthanized by ether. Forty-six kidney tissues were removed from the fetuses and stored in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Belgium).

Fetal kidney tissues were mechanically dissected into small pieces using a sterile scalpel. Then, the tissues obtained by the explant culture method were placed in T25 flasks and incubated at 37°C in a 5% CO_2 humidified atmosphere. 77% DMEM (Lonza, Belgium), 20% fetal bovine serum - FBS (Lonza, Belgium), 2% L - Glutamine (Lonza, Belgium), 1% Penicillin, Streptomycin, Amphotericin (Biological Industries, Israel) were added to the medium. The medium was changed once every 2-3 days to remove non-adherent cells. When about 70% adhesion was present, the adherent MSCs were passaged and aliquoted 1:2 with 0.25% Trypsin in PBS. The cells were grown to the 3rd passage. 10 µl of MSCs prepared for transplantation were taken and mixed by pipetting with 10 µl Trypan Blue. The stained cells were counted using the countess automated cell counting device (Invitrogen, Carlsbad, USA) to determine viability.

Differentiation of the FKD - MSCs: The FKD-MSCs were seeded at a density of $5x10^4$ cells/cm^2 in a six-well culture plate at P3 and differentiated into adipogenic, chondrogenic or osteogenic differentiation medium for 21 days. Von Kossa staining, Oil red staining and Alcian blue staining were used to reveal osteogenic, adipogenic and chondrogenic differentiation, respectively.

Sample size determination and experimental protocol: With a plan to have a continuous response variable from three independent groups and two measurement times, using type-1 (alpha) error rate = 0.05, power (1-beta) = 0.80, effect size: 0.25, the minimum required sample size was determined as 24 animals. Animals were randomly divided into Control, Sham, and MSC groups. To establish nephropathy, the rats in Sham and MSC groups received a single dose tail vein injection of 6 mg/kg / BW doxorubicin (Adriamycin, Adriablastina, Deva Ilaç, Turkey) dissolved in 0.9% NaCl. 7 days after these injections, animals were kept in a metabolic cage for a day to collect 24 h urine. Glucose, bilirubin, urobilinogen, ketone, density, pH, erythrocyte, protein, nitrite, ascorbic acid, leukocyte levels in urine were evaluated by urinalysis device (LabU Reader Plus, Hungary) using a strip (Lab Strip U11 Plus, Germany). The protein level above 25 mg in the 24 h urine of the rats in the Sham and MSC groups showed that the animal model was established. BUN, creatinine, albumin, total protein, triglycerides, Na, Cl, K, cholesterol levels were measured using AU5800 chemical autoanalyzer (Beckman Coulter, CA, USA) from the blood collected at the beginning and end of the study.

The following treatments were applied to the groups:

Group 1 (n = 8): Control group. No medication was applied to these animals.

Group 2 (n = 8): Sham group. 0.9% NaCl (1 ml) were administered intraperitoneally 3 times in one-week intervals.

Group 3 (n = 8): MSC group. 2 x 10^6 FKD - MSCs (1 ml) were administered intraperitoneally 3 times in one-week intervals. All rats were monitored for 5 weeks.

***Dynamic renal scintigraphy*:** After 5 weeks of observation, 10 mg/kg xylazine (Xylazinbio 2%, Bioveta, Ivanovicena Hane, Czech Republic) and 75 mg/kg ketamine (Ketasol 10%, Richter Pharma AG, Wels, Austria) were administered intraperitoneally to provide general anesthesia. Renal scintigraphy was performed to observe functional recovery. For this purpose, commercially available MAG3 (TechneScan, Nepha, Ankara, Turkey) labeled with 99 mTc, was administered to rats from the tail vein in a volume of 0.2 ml at a dose of 2 mCi. Conventional double-headed wide-angle gamma camera (ECAM, Siemens, Illinois, USA) and parallel collimator were used for imaging. Animals were placed in the prone position, 7-8 cm away from the collimator. Dynamic images were obtained simultaneously with the drug application. In the perfusion phase, the data were collected at the end of 60 seconds in the form of 1-second windows and the function phase as 10-second windows for 20 minutes. All images were obtained in a 256 x 256 matrix and 3.20 magnification.

Image and data analysis: Renogram curves were achieved from all groups. Quantitative parameters obtained from renogram curves were selected based on statistical significance according to the previous renal animal study findings (5). These are peak counting (C_{max}), normalized residual activity (NORA), renal retention (RR), and split renal function (SRF). RR is the ratio of minimal transit time to the mean transit time. This parameter, as an index to calculate renal retention function, should be set between 0.1 and 1.0. On the other hand, NORA is the ratio of a 1– minute renal activity at 20 minutes of the renogram to the renal activity at the first 1 to 2– minutes interval of the renogram.

Euthanasia of animals: Forty-eight hours after scintigraphy, rats were euthanized by decapitation under the xylazine/ketamine general anesthesia. Blood samples and kidneys were obtained, and histopathologic examination and immunohistochemistry were performed.

Histological and immunohistochemical study: The tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. The tissues were cut into 4 μm thick sections and stained with hematoxylin and eosin (H&E). The samples were examined under a light microscope to evaluate the general histomorphological structure of the kidneys. A blinded and semi-quantitative analysis was used to quantify the level of kidney injury. Three randomly selected areas were evaluated in each kidney section. The cases were classified as mild, moderate, and severe based on the assessment of levels of glomerular hypertrophy, congestion, mesangial cell proliferation, dilatation, and hyaline cylinder deposits in tubules.

CD24 and CD133 positive cells were investigated by using specific antibodies in renal tissue samples of rats. Immunohistochemical staining results were evaluated semi-quantitatively together with the intensity and percentage of staining of the positive cells. Positive staining changed from light yellow to brown. The staining severity score was scored as no color 0, light yellow 1, yellow 2, and brown 3. The percentage of positive cells was scored as 0 for 0 - 5%, 1 for 6 - 25%, 2 for 26 - 50%, 3 for 51 - 75%, and 4 for > 75%. Both data were considered during the evaluation. Five randomly selected sites (x 400) were evaluated in each preparation.

Statistical analysis: Descriptive statistics of the variables included in the study were shown as "Arithmetic Mean ± Standard Error" or "Median (Min-Max)" considering the distribution of data. Kruskal-Wallis test was used to examine the difference between pathological scores and urinalysis results between the groups. In cases where the differences between the groups were found to be significant, the Dunn-Bonferroni test was used as the post-hoc test. Blood analysis and renogram data were subjected to two-way mixed ANOVA using the general linear modeling procedure for repeated measures to analyze the differences between groups and time or laterality, where necessary. The model included the main effects of Group *(Control, Sham, MSC)*, Time *(t^1, t^2)* (or side *(right, left)*) and Time*Group *(or Side*Group)* interaction term. In cases where interaction terms were not significant, the Tukey test was used as post-hoc procedure to analyze main effects. The simple effects analysis was used by applying Bonferroni correction in cases where interaction terms were significant. Data were analyzed using SPSS 14.01 (SPSS Inc, USA). The $P<0.05$ criteria were used for all statistical evaluations.

Results

Biochemical study: It was observed that blood urea levels were increased in the Sham and MSC groups compared to the Control group. However, there was no significant difference between urea levels between MSC and Sham groups. Albumin levels were decreased in Sham and MCS groups when compared to the Control group. Cholesterol levels were increased in Sham and MSC groups when compared to the Control group. Creatin levels were increased in the Sham group, but there was no significant difference between MSC and Control groups.

Triglyceride values were significantly increased in the Sham group when compared to the Control and MSC group. Sodium levels were increased in Sham and MCS groups when compared to the Control group. Chloride levels were increased in the MCS group, but there was no significant difference between the Sham and Control groups. When total protein and potassium levels were evaluated, no significant difference was found between the groups. Biochemical findings are presented collectively in Table 1.

Urine analysis: When the 24 h urine samples were examined, bilirubin, urobilinogen, ketone, ascorbic acid, leukocyte levels were not different between the groups, but glucose and nitrite were positive and protein levels were above 500 mg/dl in the Sham and MSC group compared to the Control group. The pH of the urine was significantly different between the groups (P<0.05). The pH levels were elevated the most in the Control group, then the Sham, and MSC group, respectively. Urine density was found to be significantly different between the groups (P<0.05). As the highest in the MSC group (1.025), then the Sham group (1.020), then the Control group (1.010).

Table 1. Biochemical findings of the rats in all study groups.

Biochemical assays	Rat Groups	t^1	t^2	P		
		Mean ± SEM	Mean ± SEM	**Time**	**Group**	**Time*Group**
ALB	Control	3.09 ± 0.04 A,a	2.72 ± 0.06 B,a			
	Sham	2.99 ± 0.03 A,a	1.59 ± 0.14 B,b	<0.001	<0.001	<0.001
	MSC	2.85 ± 0.03 A,b	1.59 ± 0.16 B,b			
TP	Control	5.86 ± 0.1 A,a	5.44 ± 0.09 B,a			
	Sham	5.44 ± 0.07 A,a	5.16 ± 0.37 B,a	0.008	<0.001	0.492
	MSC	5.06 ± 0.05 A,b	4.31 ± 0.22 B,b			
UREA	Control	47.25 ± 2.14 A,ab	48.25 ± 1.26 A,a			
	Sham	50.75 ± 1.42 B,a	107.88 ± 23.23 A,b	<0.001	0.036	0.027
	MSC	42.25 ± 0.9 B,b	10.13 ± 17.59 A,b			
K	Control	4.71 ± 0.15 B	5.72 ± 0.42 A			
	Sham	5.71 ± 0.09 B	6.06 ± 0.53 A	0.018	0.184	0.572
	MSC	5.26 ± 0.23 B	5.84 ± 0.32 A			
CREA	Control	0.42 ± 0.01 A,a	0.46 ± 0.02 A,b			
	Sham	0.39 ± 0.01 B,b	0.58 ± 0.04 A,a	<0.001	0.001	0.003
	MSC	0.34 ± 0.01 B,c	0.45 ± 0.02 A,b			
CHOL	Control	67.75 ± 2.99 A,a	68.25 ± 3.48 A,c			
	Sham	75 ± 3.58 B,a	541.13 ± 49.26 A,a	<0.001	<0.001	<0.001
	MSC	76.38 ± 1.21 B,a	237.25 ± 35.74 A,b			
TRIG	Control	55.13 ± 3.18 A,a	66.63 ± 5.47 A,b			
	Sham	50.75 ± 4.62 B,a	258.25 ± 55.94 A,a	0.001	0.003	0.001
	MSC	58.13 ± 7.06 A,a	66.88 ± 28.59 A,b			
Na	Control	139.75 ± 0.37 A,a	136.63 ± 1.12 A,b			
	Sham	135.63 ± 0.38 B,b	141 ± 0.6 A,a	0.006	0.923	<0.001
	MSC	136 ± 0.65 B,b	141 ± 1.52 A,a			
Cl	Control	101.38 ± 0.53 A,a	101 ± 0.27 A,b			
	Sham	97.38 ± 0.56 B,b	101.38 ± 1.49 A,b	<0.001	<0.001	0.003
	MSC	99.63 ± 0.73 B,ab	107.25 ± 0.98 A,a			

ALB: Albumin, TP: Total Protein, UREA: Urea, K: Potassium, CREA: Creatinine, CHOL: Cholesterol, TRIG: Trigylceride, Na: Sodium, Cl: Chlorine.
t^1: Beginning of the study, t^2: End of the study.
A,B: Values in the same row with different superscripts show the statistical difference (P<0.05).
a,b,c: Values in the same column with different superscripts show the statistical difference for each parameter (P<0.05).

Scintigraphic evaluation: As a result of the scintigraphic evaluation, peak count (C_{max}) values were significantly low whereas renal retention (RR) was increased in the Sham group. Normalized residual activity (NORA) was increased in the MSC and the Sham groups compared to the Control group (P<0.05). There was no significant difference between the groups in terms of separated renal function (SRF) (Table 2).

Histopathology results: Renal pathology was evaluated (Figure 1) as normal in the Control group (Figure 1A - 1D). In the Sham group, glomerular hypertrophy, congestion of glomerular capillaries, mild mesangial proliferation, intratubular cast, and tubular vacuolization were observed (Figure 1B - 1E). In the MSC group, all these changes were significantly alleviated/regressed (Figure 1C - 1F).

Immunohistochemical results: The staining intensity and percentage of CD24 and CD133 were significantly higher in the MSC group (P<0.05) (Table 3) (Figure 2). The staining intensity and percentage of CD24 and the staining intensity of CD133 were not significantly different in the Control and the Sham groups (Figure 2C - 2F). The staining percentage of CD133 was found to be higher in the MSC than the Control (Figure 2A - 2D) and the Sham groups, respectively (Figure 2B - 2E). Positive staining was mostly observed in tubules epithelia. No staining was observed in the glomerulus and intertubular areas.

Table 2. Distribution of the peak counting, normalized residual activity, renal retention, split renal function and time to peak counting values according to the rat groups.

Renogram Parameters	Group	Right	Left	P		
		Mean ± SEM	Mean ± SEM	Side	Group	Side*Group
C_{max}	Control	554.81 ± 6.8 a	535.14 ± 8.06 a	0.191	<0.001	0.433
	Sham	289.27 ± 12.32 c	291.27 ± 10.52 c			
	MSC	437.2 ± 17.55 b	424.6 ± 36.28 b			
NORA	Control	0.4 ± 0.01 c	0.41 ± 0.01 c	0.319	<0.001	0.962
	Sham	0.87 ± 0.01 a	0.88 ± 0.01 a			
	MSC	0.63 ± 0.02 b	0.64 ± 0.02 b			
RR	Control	0.33 ± 0.01 c	0.32 ± 0.01 c	0.367	<0.001	0.375
	Sham	0.63 ± 0.01 a	0.64 ± 0.01 a			
	MSC	0.39 ± 0.001 b	0.4 ± 0.001 b			
SFR	Control	51.21 ± 0.52	48.79 ± 0.52	0.117	0.445	0.205
	Sham	49.71 ± 0.52	50.04 ± 0.49			
	MSC	50.59 ± 0.73	49.43 ± 0.73			

C_{max}: peak count, NORA: normalized residual activity, RR: renal retention, SRF: separated renal function
a,b,c: Values in the same column with different superscripts show the statistical difference for each parameter (P<0.05).

Table 3. The staining intensity and percentage of CD24 and CD133 in the rat groups.

	Groups	n	Mean ± SEM	Median (Min-Max)	P
CD24 staining intensity	Control	8	1.75 ± 0.16	2 (1 - 2) b	<0.001
	Sham	8	1.88 ± 0.12	2 (1 - 2) b	
	MSC	8	2.87 ± 0.12	3 (2 - 3) a	
CD24 staining percentage	Control	8	1.88 ± 0.12	2 (1 - 2) b	<0.001
	Sham	8	2.25 ± 0.16	2 (2 - 3) b	
	MSC	8	3.75 ± 0.16	4 (3 - 4) a	
CD133 staining intensity	Control	8	1.88 ± 0.12	2 (1 - 2) b	<0.001
	Sham	8	1.75 ± 0.16	2 (1 - 2) b	
	MSC	8	3 ± 0	3 (3 - 3) a	
CD133 staining percentage	Control	8	2.38 ± 0.18	2 (2 - 3) b	<0.001
	Sham	8	1.38 ± 0.18	1 (1 - 2) c	
	MSC	8	3.5 ± 0.19	3,5 (3 - 4) a	

a,b: Values in the same column with different superscripts show the statistical difference for each parameter (P<0.05).

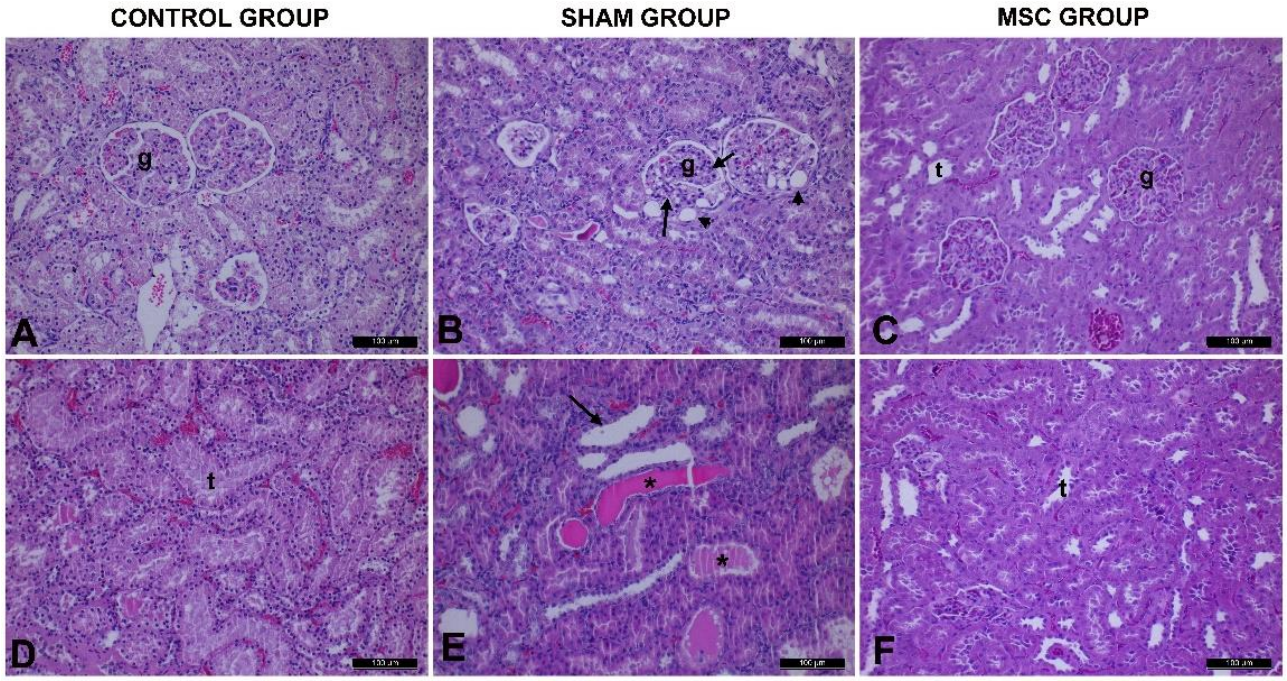

Figure 1. Histopathological appearances of the groups.
A. Control Group. Normal histological view of the glomerulus (g). HE. Bar: 100µm. **B.** Sham Group. Proliferations of mesangial cells (arrows) and enlargement of glomerular capillaries (arrowheads) in the glomerulus (g). HE. Bar: 100µm. **C.** MSC Group. Slightly hyperemic in glomerular capillaries (g) and tubular epithelia in normal view (t). HE. Bar: 100µm. **D.** Control Group. Normal histological view of the tubules (t). HE. Bar: 100µm. **E.** Sham Group. Dilated tubule (arrow) and hyaline cylinder accumulations in the lumen of the tubules (asterisks). HE. Bar: 100µm. **F.** MSC Group. Lesions in the tubules (t) are regressed. HE. Bar: 100µm.

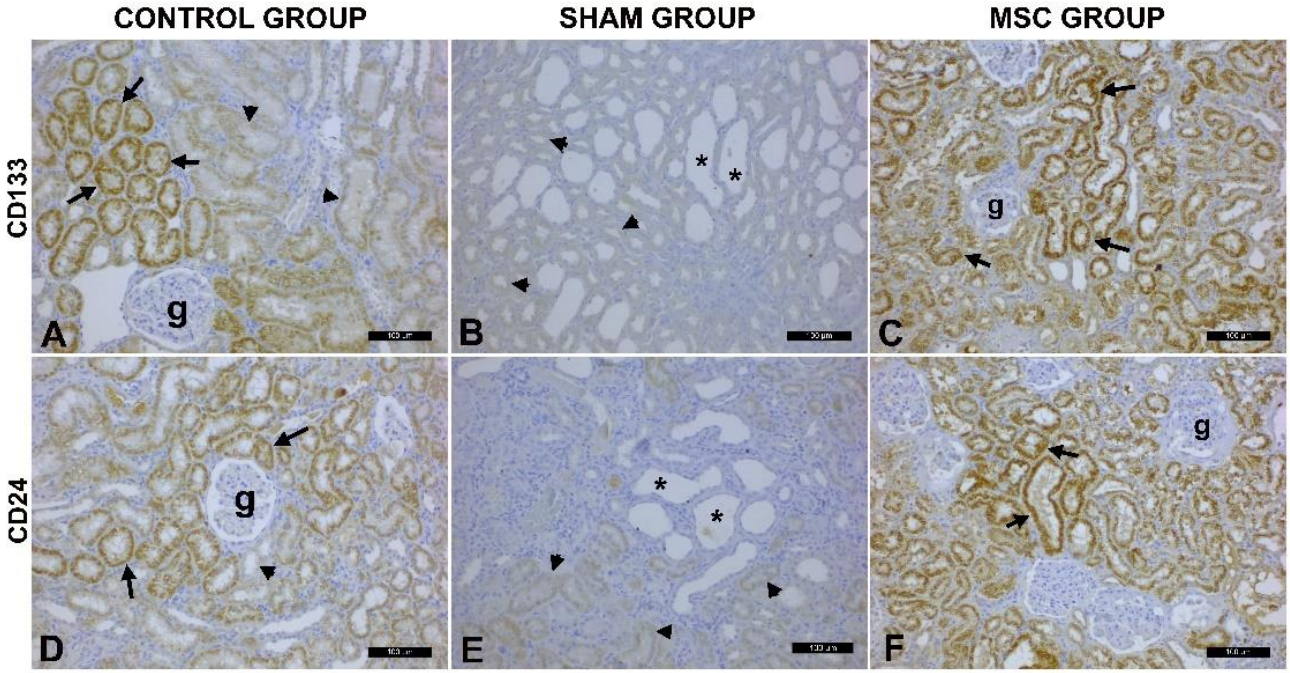

Figure 2. Immunohistochemical staining views of CD133 (A-C) and CD24 (D-F) in the groups.
A. Control Group. Light yellow (+1) (arrowheads) and brown (+3) (arrows) CD133 positive immunoreactions in the tubular epithelia. DAB. Bar: 100µm. **B.** Sham Group. Light yellow (+1) (arrows) CD133 positive staining in tubules epithelia and no positive immunoreactions in the dilated tubules (asterisks). DAB. Bar: 100µm. **C.** MSC Group. Brown (+3) CD133 positive immunostaining in the epithelia of the tubules (arrows). DAB. Bar: 100µm. **D.** Control Group. Light yellow (+1) (arrowheads) and yellow (+2) (arrows) CD24 positive immunostaining in the tubules. DAB. Bar: 100µm. **E.** Sham Group. A small part of light yellow (+1) CD24 positive staining areas (arrowheads) and immunonegative reactions in the dilated tubules (asterisks). DAB. Bar: 100µm. **F.** MSC Group. Brown (+3) (arrows) CD24 positive staining in most tubules in the microscope field (arrows). Glomeruli (g) are negative for CD133 and CD24 antibodies. DAB. Bar: 100µm.

Discussion and Conclusion

Many researchers have studied the benefit of stem cells in the treatment of kidney diseases (1, 3, 11, 15, 21, 25). Human umbilical cord-derived mesenchymal stem cells were transplanted to the rats with doxorubicin-induced nephropathy from the tail vein. Serum interleukin-6, tumor necrosis factor-α, and prostaglandin E_2 levels of the treatment group were decreased for 4 weeks, and interleukin-10 levels were increased. According to these results, it was concluded that MSCs treated doxorubicin-induced kidney damage and inflammation and may play a potential role in the clinical treatment of renal diseases (11).

It has been reported that MSCs are administered to mice with acute renal injury to assist both structural and functional renal repair, which is achieved by trans differentiation of MSCs to the tubular epithelium. However, only 2 to 2.5% of these injected MSCs have been engrafted (18). In another study, intraarterial administration of MSCs reduced necrosis, improved renal function, and increased α -smooth muscle actin (α-SMA) expression, however, no MSC was engrafted in the renal tissue (23). In a clinical study of the CRF model, significant differences in serum creatinine and creatinine clearance levels were detected before and after treatment when 1 x 10^6 MSCs / kg were administered twice intravenously (3). In a study published in 2014, bone marrow-derived MSCs were administered intravenously at a dose of 2 x 10^6 in doxorubicin -induced nephropathy model, and urinary protein, blood creatinine, and triglyceride levels were improved in the treatment group (6).

According to the results of a meta-analysis study, the administration of cellular therapy directly via the renal artery or via tail vein injection (intravenously), resulted in functional and histological improvement in experimental CKD animal models (17). The same study concluded that intraperitoneal administration of cell-based therapy has not showed any significantly improved outcome. However, this conclusion was based on a very limited number of studies.

Studies have shown that intravenously infused human cord-derived MSCs were homed to the renal tubular and renal interstitial area, but intraperitoneally transplanted human cord-derived MSCs were not found in the kidneys of the rats. However no significant differences were found between the groups in terms of renal morphology (11). The intravenous transplantation requires general anesthesia. The injection on the renal artery not only requires general anesthesia but is an invasive method. After evaluating the results of the previous studies, we preferred transplanting the FKD-MSCs intraperitoneally, a less invasive and non-anesthesia requiring method.

According to results of a study conducted in 2019, it was concluded that human umbilical cord blood-derived MSCs attenuated cisplatin-induced nephrotoxicity in mice (20). No significant differences regarding different delivery routes (intravenous and intraperitoneal) were determined when structural injuries and renal function was evaluated . Based on the results of this previous study, we decided to administer FKD- MSCs intraperitoneally at a dose of 2 x 10^6.

The sources from which the stem cells are derived are known to have different effects in treatment (15). For this reason, many researchers are trying to find the best treatment option using different cell sources. In a study conducted by Morigi and Benigni in 2013, the therapeutic properties of stem cells obtained from different sources in acute renal failure (ARF) were compared. Accordingly, bone marrow-derived, cord blood-derived and amniotic fluid-derived stem cells were transplanted into mice, and the results showed that renal histology was maintained in all groups and a decrease in BUN was observed. Survival time was evaluated by looking at the sources from which the stem cells were obtained, and the survival rate was increased in the cord blood derived MSC transplanted group (13). Another study reported that adipose stem cells contribute to treatment in an animal model of CRF (22). In a study conducted in eight cats with CKD, repeated intravenous transplantation of allogeneic adipose derived-MSCs was performed. As a result, no statistically significant difference was present between MSC and placebo groups (19). However, this conclusion was based on a very limited of patients. In our study repeated intraperitoneal transplantation of FKD–MSCs resulted in renal improvement in the MSC group when compared to the sham group.

The most important biochemical finding in CRF is increased BUN levels. Increased proteinuria, serum creatinine, and BUN have been reported in rats treated with doxorubicin intravenously to form a CRF model (11). In our study, it was observed that BUN levels were increased in the Sham and the MSC group compared to the Control group. The application of MSCs did not affect BUN levels but caused a decrease in cholesterol and triglyceride levels. A decrease in BUN levels after MSC transplantation was observed in the ARF model (13). In our study, the CRF model was used, and as irreversible damage occurs in CRF, it is thought that the MSC application did not affect BUN levels. It is known that MSCs that are transplanted systemically without specific targeting migrate through cytokines to other places where inflammation of the organism occurs and ameliorates the damage (13, 14). In our study, the decrease in cholesterol and triglyceride levels in the MSC group, suggests that the stem cells were also located in other organs. Our study focuses on how the kidneys are affected by MSCs.

Therefore, the effects of MSCs on the other organs are beyond the scope of this paper.

Urine analysis plays an important role in renal diseases. Protein levels above 500 mg/dl in doxorubicin induced CRF model, indicates kidney damage (11). In the present study, it was found that protein value was high in the Sham and the MSC groups due to doxorubicin injection. Since kidney damage has become chronic, MSC transplantation has not prevented protein excretion in the urine.

Dynamic renal imaging using MAG3 labeled with 99mTc shows tubular uptake and release at a given time interval. 99mTc-MAG3 can be evaluated using activity curves over some time and quantitative results are obtained from the renogram. In human studies, NORA and transit time have been used to evaluate extraction/excretory function in the kidneys. Besides, when large databases are studied, NORA is an important criterion for the assessment of renal drainage (5). According to the results of this study, NORA and RR values are important in assessing renal dysfunction. In our study, the NORA values of the Sham and MSC groups were the same, but the RR value was higher in the Sham group than the MSC group. When these two parameters are interpreted together, it has been shown that MSCs have a positive effect on healing by decreasing retention, proving that MSC transplantation affects functional recovery.

In CRF, the number of nephrons gradually decreases as fibrous tissue replaces the kidney tissue. After some time, varying according to the rate of progression of the underlying disease, the kidneys no longer meet the needs of the body and uremic syndrome occurs (2). After the kidneys have been damaged to a certain extent and a critical amount of parenchyma has been lost in CRF, even if the primary disease is completely cured, progression to end-stage renal failure cannot be prevented (25). That means terminal renal failure is inevitable after irreversibly decreasing renal function to a critical level. Histopathological examination of kidneys in this period has many common findings: Glomeruli become sclerotic, fibrous tissue develops in renal interstitium, and chronic inflammation of lymphocytes and macrophages occurs (2, 9). Most of the tubules become atrophic and dilated (22). In our study, the renal injury was manifested by glomerular hypertrophy, mesangial proliferation, and tubular vacuolization in the Sham group, whereas these changes were significantly alleviated in the MSC group. These histopathologic results showed that MSCs are effective in kidney healing.

CD133 + and CD24 + cells are renal stem / progenitor cells. Renal CD133 + and CD24 + cells are the cells that determine the potential for self–regeneration, and differentiation of both podocytes and tubular cells. Increased expression of Osr1, Nanog, HGF, BMP - 7, WT - 1, and Pax2 in rat kidneys with CRF is correlated with CD133 + and CD24 + renal stem/progenitor cells. However, this increase reduces TGF-β1 expression and prevents interstitial fibrosis (8). When the data obtained in our study were evaluated, while the number of positive cells was reduced in the Control and the Sham groups; it was found to be higher in the MSC group. CD24 and CD133 staining intensity and percentage were higher in the MSC group compared to the other groups. According to these results, intraperitoneally administered FKD-MSCs reached the damaged kidney tissues and proliferated to treat the tissue. When the findings were evaluated, it was observed that both functional and histological improvements were achieved in the MSC group compared to the Sham group.

In conclusion, transplantation of FKD-MSCs into patients with renal damage is thought to contribute to the healing of the renal tissue. To investigate this functional and histological result in detail, the effective pathways of transplanted MSCs should be investigated in detail with other studies.

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Ethical Statement

This study was approved by the Local Animal Ethics Committee of Dışkapı Yıldırım Beyazıt Training and Research Hospital (Approval number: 2014 / 55).

Conflict of Interest

The authors declared that there is no conflict of interest.

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Fecal microbiota transplantation capsule therapy via oral route for combatting atopic dermatitis in dogs

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Abstract: Given the role of the interaction between gut microbiome with dermatological diseases, namely "gut-skin axis", the present author proved that gut restoration should alleviate canine atopic dermatitis (CAD), which was the purpose of the study. A 4-week, open-label, non-controlled case series involved 8 -owned dogs with CAD which had received no previous treatment. Evaluations included Canine Atopic Dermatitis Extent and Severity Index version 4 (CADESI-04 scores), Visual Analog Scale (VAS) pruritus scores and Polycheck in vitro allergen specific tests. Faecal samples were analysed by dual indexing one-step PCR and 16S rRNA targeted metagenomics for detecting gut microbiota alterations before and after fecal microbiota transplantation (FMT) capsule treatment twice daily for 4 weeks. All cases were presenting pruritus and all of those dogs showed elevated IgE levels. CADESI scores decreased on days 28 (4-21) compared to day 0 initial values (50-128). Similarly, decreased VAS scores were detected on days 28 (0-2) in contrast to prior values (6-10). Regarding epidermal barrier functioning epidermal hydration (55-100 vs. 4-24) and pH (6.-7.8 vs. 4.2-5.7) values were elevated after FMT treatment in contrast to prior ranges, respectively. Alpha diversity revaled both richness and diversity of gut microbiota were improved for all cases on day 28. Furthermore at the end of trial Firmicutes: Bacteroidetes ratio was <8, the benchmark detected for healthy dogs. The present study supports a potential benefit of FMT capsule treatment against CAD. This safe and tolerant treatment modality directed against CAD shifted the gut microbiome composition towards a healthy state for all 8 dogs enrolled.

Keywords: Atopic dermatitis, dog, fecal microbiota transplantation**.**

Köpeklerde atopik dermatitisle mücadelede oral yolla fekal mikrobiyota transplantasyon kapsül sağaltımı

Özet: Bağırsak mikrobiyomu ile dermatolojik bozukluklar arasındaki ilişki göz önünde bulundurulduğunda, sinonim "bağırsak-deri ekseni", bu makalenin yazarı çalışmanın amacı doğrultusunda bağırsak restorasyonu sayesinde kanin atopik dermatitis (KAD) hastalığının hafifletileceğini öne sürmektedir. Dört haftalık, açık uçlu, kontrol grubu bulunmayan çalışmaya daha önceden sağaltım görmemiş KAD'li ve sahipli 8 köpek dahil edildi. Değerlendirmede Köpeklerde Atopik Dermatitis Yaygınlığı ve Şiddetine ait İndeks 4. Versiyonu (CADESI-04) skorları, gözlemsel analog skalası (GAS) ve Polycheck in vitro alerjen spesifik testler kullanıldı. Dışkı örnekleri, 4 hafta boyunca günde 2'şer kez uygulanan fekal mikrobiyota transplantasyon (FMT) kapsül sağaltımı öncesi ve sonrası bağırsak mikrobiyotasında meydana gelen değişimleri değerlendirmek üzere çift indeksli tek-aşamalı PZR ve 16S rRNA hedefli metagenomik analizlerine dayandırıldı. Her olguda kaşıntı saptanırken, bu köpeklerin tamamında artan IgE seviyeleri mevcuttu. CADESI skorlarının 28. günde (4-21), 0. gündeki (50-128) çıkış değerlerine oranla azaldığı tespit edildi. Benzer olarak 28. günde belirlenen GAS skorlarının (0-2), önceki değerlere (6-10) göre azaldığı belirlendi. Epidermal bariyer fonksiyonlarına ilişkin olarak epidermal hidrasyon (55-100'e karşılık 4-24) ve pH (6-7,8'e karşılık 4,2-5,7) değerleri sırası ile FMT sağaltımı sonrası, öncesine oranla artmıştı. Alfa dağılımı mikrobiyotada hem zenginliğin hem de çeşitliliğin tüm olgularda 28. günde geliştiğini gösterdi. Bunun da ötesinde sağlıklı köpeklerde referans noktası kabul edilen Firmicutes: Bacteroidetes <8 oranları tespit edildi. Bu çalışma KAD sağaltımında FMT kapsüllerinin potansiyel yararını destekleyici özelliktedir. Bu KAD'e yönelik güvenilir ve tolere edilebilen sağaltım modeli, bağırsak mikrobiyomunun kompozisyonunu, dahil edilen 8 köpekte de sağlık durumuna göre kaydırarak değiştirmiştir.

Anahtar sözcükler: Atopik dermatit, fekal mikrobiyota transplantasyonu, köpek.

Introduction

The gastrointestinal system of dogs has long been known to be colonized by complex members of microorganisms, namely microbiome (26). Gut microbiota is beneficial for the host, pivotal as a defensive barrier against enteropathogens, modulating the immune system along with other relevant advantages (15, 27), in which alterations in its composition have been associated with acute/ and chronic gastrointestinal disorders in dogs (14, 20). As atopic dermatitis (AD) has been linked with gut microbiota; in which alteration in AD microbiota composition with the absence/depletion of selected bacterial species (24), or diminished biodiversity of the gut microbiome in one-month-old infants caused AD (1). Antibacterial therapy has been linked to elevated risk of AD in humans (28), probably associated with intestinal microbiota alterations. Given those data, briefly reflect participation of the gut microbiome within the pathogenesis of AD, through stimulation and drilling of immune cells (11).

Given changes regarding gut microbiota linked to several diorders, manoeuvre for restoration or optimization of the microbiota might involve fecal microbiota transplantation (FMT), briefly denoted fecal material obtained from a healthy donor (age matched, preferably) to those of administered to diseased case. The latter FMT procedure paid attention in selected study in dogs (5, 7). Satisfactory results were deemed available for gastrointestinal diseases among dogs after FMT treatment (21, 32).

The microbiome, with a pivotal role, is deemed one of the major actor for immune system homeostasis. Dysbiosis, denoted as altered composition and functions in gut microbiome, existing in gut and/or skin, posessess intimate association among “gut-skin axis” could act as a factor for development of AD. Therefore it should not be unwise to claim the association of AD with gut microbiota (GM) composition. Given the limited treatment options reported previously for CAD, it should not be unwise to state for the known facts, long-term risks of immunosuppressive treatment widely preferred elsewhere. There is an unmet need for a safe and efficacious long term and probably non-immunosuppressive treatment option for canine atopic dermatitis (CAD). The aim of this open-label, non-controlled study was to elucidate the probable efficacy of a commercially available FMT capsule containing gut restoration system (obtained from healthy subjects with a known microbiota composition) in CAD. The present author’s interest was aroused for FMT capsules solely might effectively mitigate inflammation in dogs suffering from CAD. It was further suggested that the FMT mediated anti-infectious, anti-inflammatory, anti-allergic effects might account for the observed pruritus alleviation and relevant clinical signs to those of affected dogs of various ages.

Materials and Methods

Prior to conduction of the study, the design was clearly detected and detailed, permitting future (warranted) studies. As AD is a compeller (coercive) disease, owners deny/refuse control cases, which resulted in baseline (prior to treatment; like controls)-therapeutical applications (after FMT treatment) matching. This could allow the present author to compare initial values, thereafter post-treatment data, which could be dedicated as self-control cases. The open-label, non-controlled study - enrolled with written owner consent according to standard clinical practice and compared to mean quantified biomarkers with values on two subsequent re-evaluations at day 0 and at the end of week 4, after therapeutical intervention.

Research period / Inclusion criteria: The present study was conducted at the Aydin Adnan Menderes University, Faculty of Veterinary Medicine, Department of Internal Medicine. A total of eight dogs with a history of frequent itching sensation were initially diagnosed with a presumed CAD based on history, clinical findings, cytology, and necessary dermatological examinations. At the beginning of the present research, all dogs were subjected to a food trial with hydrolyzed proteins for at least 2 months. No prior drug administration was evident, which was one of the inclusion criteria. Dogs were excluded if bacterial or fungal infections (based on laboratory diagnostic); ectoparasites (based on, for example; a flea comb, cytology, dermatoscopy, or skin scraping); flea allergy, metabolic or other nonallergic disorders were diagnosed. Complete blood count, routine serum biochemistry panels and endocrine panels were available on days 0 and 28 for all dogs.

Entire physical analysis were performed on days 0, and 28. The Canine Atopic Dermatitis Extent and Severity Index version 4 (CADESI-04) was used to score the clinical findings [for acute (i.e. erythema) and chronic (i.e. lichenification) and other relevant findings such as alopecia, excoriations, and pruritus]. The animal owners were asked to make interpretation for pruritus via a 10 cm Visual Analog Scale (VAS) involving descriptors similar to another study (9, 13). History involved small samples of quesiton aroused for prior interventions.

In vitro allergen detection: Polycheck Canine Allergy Test (Polycheck, Allergy test, Gmbh, Münster, Germany), an *vitro* test which detects allergen-specific IgE in dog serum) was used (30) to detect underlying etiology as previously described (3).

Treatment protocol: Commercially available (Animal Biome Gut Restoration System FMT capsules,

Animal Biome, Oakland, California, USA) oral FMT capsules were prescribed orally every 12 hours for 1 month.

Microbiome testing: This procedure briefly included i) sample collection (stool sample was collected and stored at 4-8© at initial referral prior to treatment application into 70% ethanol tubes achieved within the test kits provided by the company (Animal Biome Gut Restoration System FMT capsules, Animal Biome, Oakland, California, USA), ii) fecal material isolation, DNA extraction and concentration measurement, iii) 16S rRNA gene (505F/816 R) generation using one step PCR (8, 22) , iv) alpha and beta diversity analysis (6) involving 100% OTUs classified against SILVA reference database (23). All of the procedures were completed at Animal Biome Laboratory at Oakland, USA by experted and specialized researchers.

Corneometric analysis of skin barrier function: Callegari Soft Plus corneometric device (Castello Dei Diritti, Parma, Italy) was used for skin pH (by use of double cell electrode able to determine 2-12 pH ranges) and epidermal hydration (measurement principle: capacitive method by use o special probes detecting 0-100 c.u.) status before and after the treatment. Operating conditions involved room temperature in which dogs were kept there 30 minutes prior to examination.

Statistical analysis: Obtained (interpretation) data were deemed available for descriptive statistics, there afterwards median, standart deviation, minimum-maximum values were forwarded to tables. Wilcoxon test was chosen for interpretation of statistical alterations among enrolled parameters and $P<0.05$ was set as significant. Entire analysis were performed by use of SPSS 21.0 (IBM, Chicago, USA) programme.

Results

Demographic data: Eight dogs at the age of 24 to 119 months, were involved. All dogs were participated, completed the study without any observable side effects related to therapy. History deemed no prior drug application. Endocrine profile were within reference ranges (total t4 values ranged between 1.1–3.7 µg/dL, plasma cortisol levels 2.2–5.4 µg/dL).

CADESI-04 and VAS pruritus scores: Both CADESI-04 and VAS pruritus scores were declined in all 8 dogs enrolled. The day 0 scores (50-128) belonging to 7 severe and 1 moderate CAD cases, were significantly higher than scores on day 28 (4-21) switching the severity of the disease to mild status. Day 0 VAS pruritus scores were ranging between 5 to 10, whereas owner VAS pruritus score had decreased, which corresponds to a reduction from “severe itching” to “absent (0) or normal (2)” on day 28 (after completion of treatment) (Figure 1). Six out of 8 FMT capsule treated dogs were not presenting pruritus at second referall, as detected by the present author. No adverse events attributable to the FMT capsule treatment were reported in any of the dogs. All cases were monitored for 18 months after completion of treatment in which no recurrence was observed. Clinical photographs of three selected cases were shown in Figures 2-4, describing before and after treatment records along with gut microbiota changes. CADESI-04 scores to those of classification of atopic dermatitis along with allergen specific Ig E analysis were shown in Table 1. Six out of 8 dogs with AD presented strong IgE response against *Dermatophagoides farinae*. Statistical analysis deemed available in Table 2, as shown, presented that there were significant alterations (P=0.012) for CADESI-04 and VAS scores along with corneometric analysis before and after treatment.

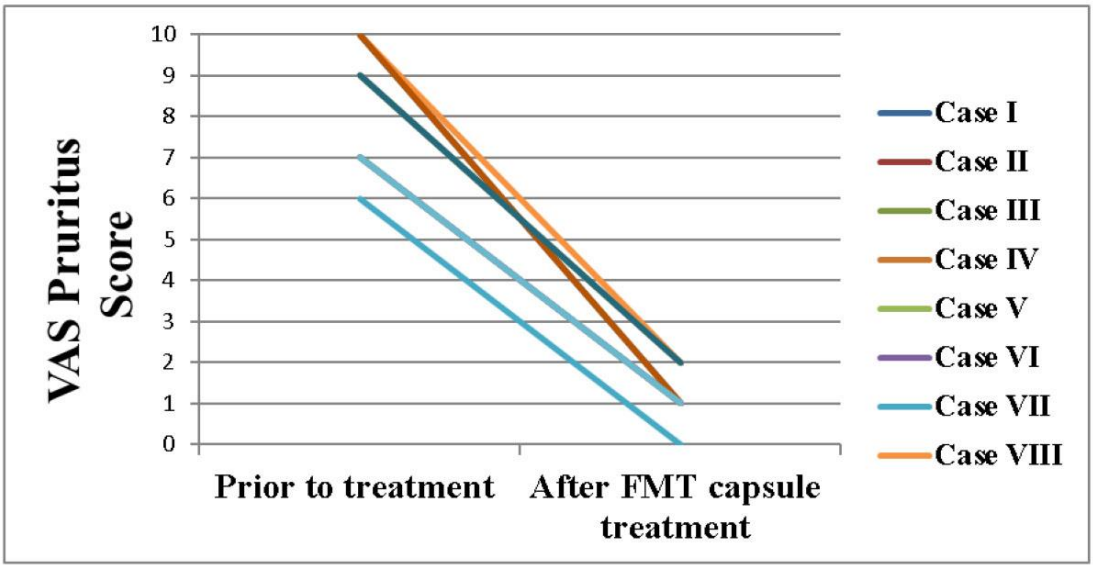

Figure 1. Linear graphic regarding VAS Pruritus Scores for 8 dogs with CAD enrolled in this study (prior to treatment day 0 and after FMT capsule treatment on day 28).

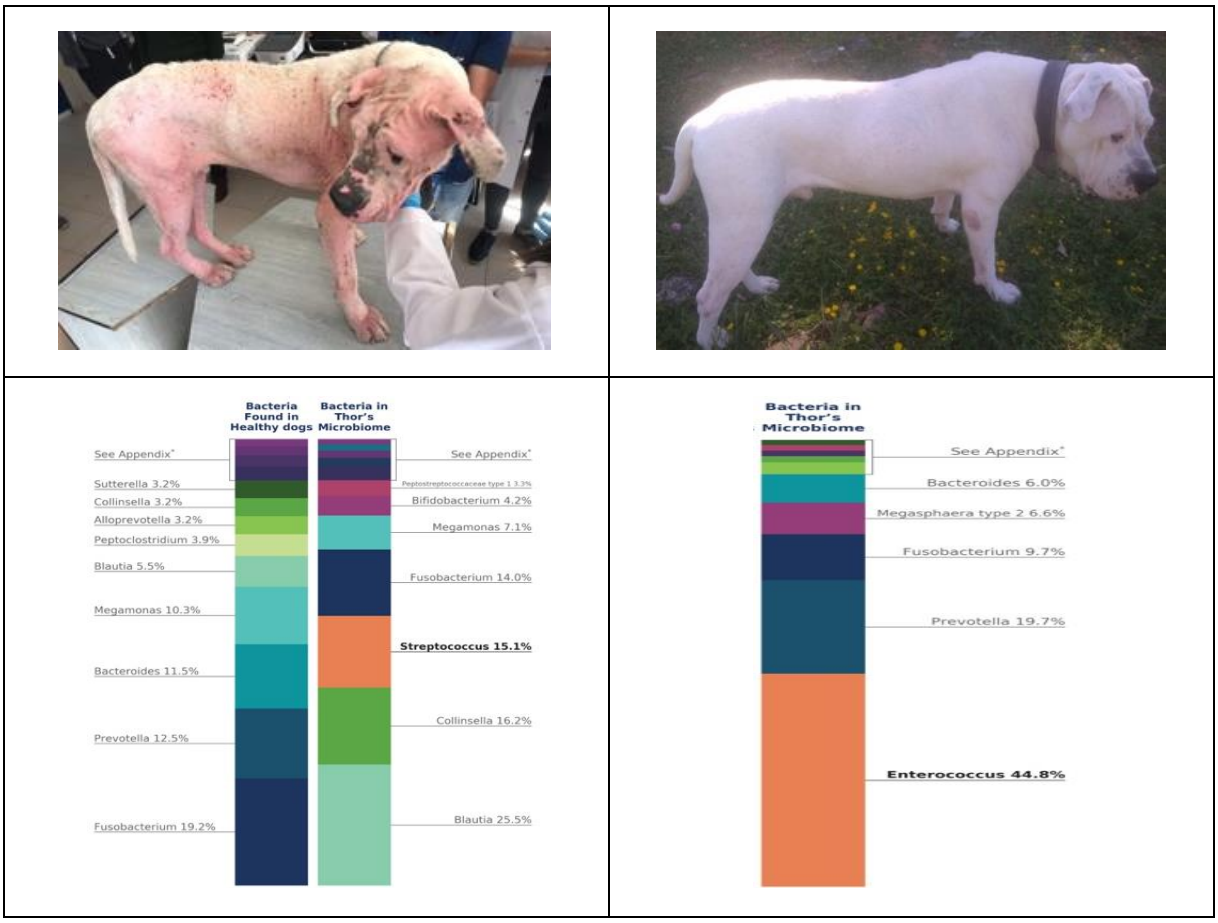

Figure 2. Relative abundaces of gut microbiota belonging to case I, a) before, and b) after completion of FMT capsule treatment. Case 1 before and after treatment showing clinical picture and lesional view along with microbiota analysis. Bar graphic on the left side comperatively showed bacteria found in healthy dogs, to those of on the middle and rigth, before and after treatment, respectively.

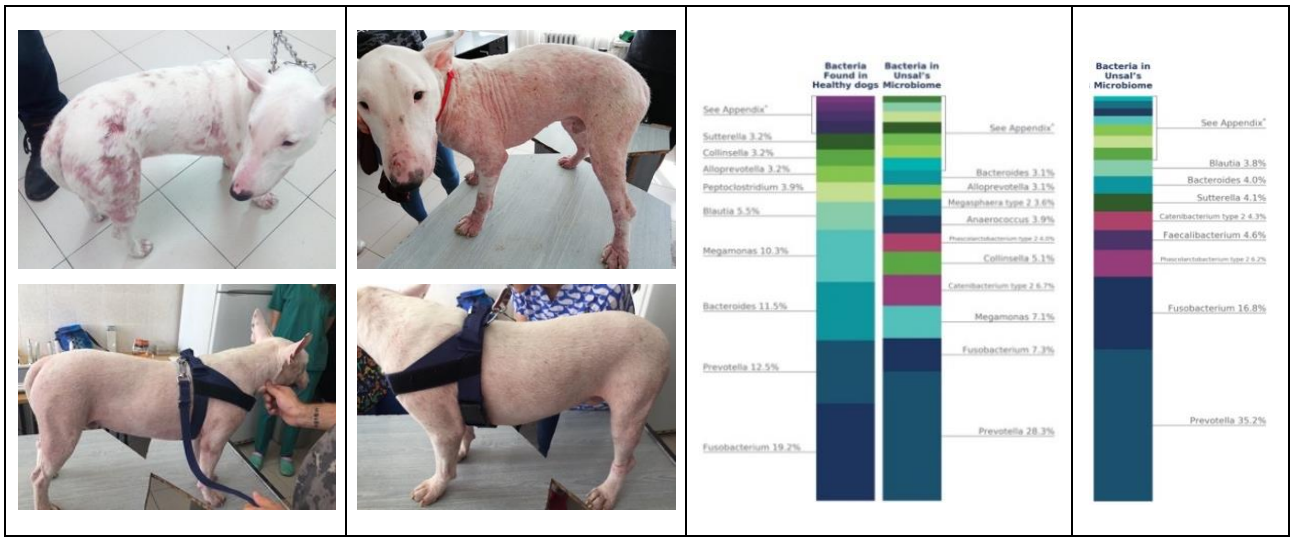

Figure 3. (Case 2) Before and after treatment along with bar graphics showing gut microbiota alterations comperatively (first bar graphic comperatively presents healthy dog microbiota).

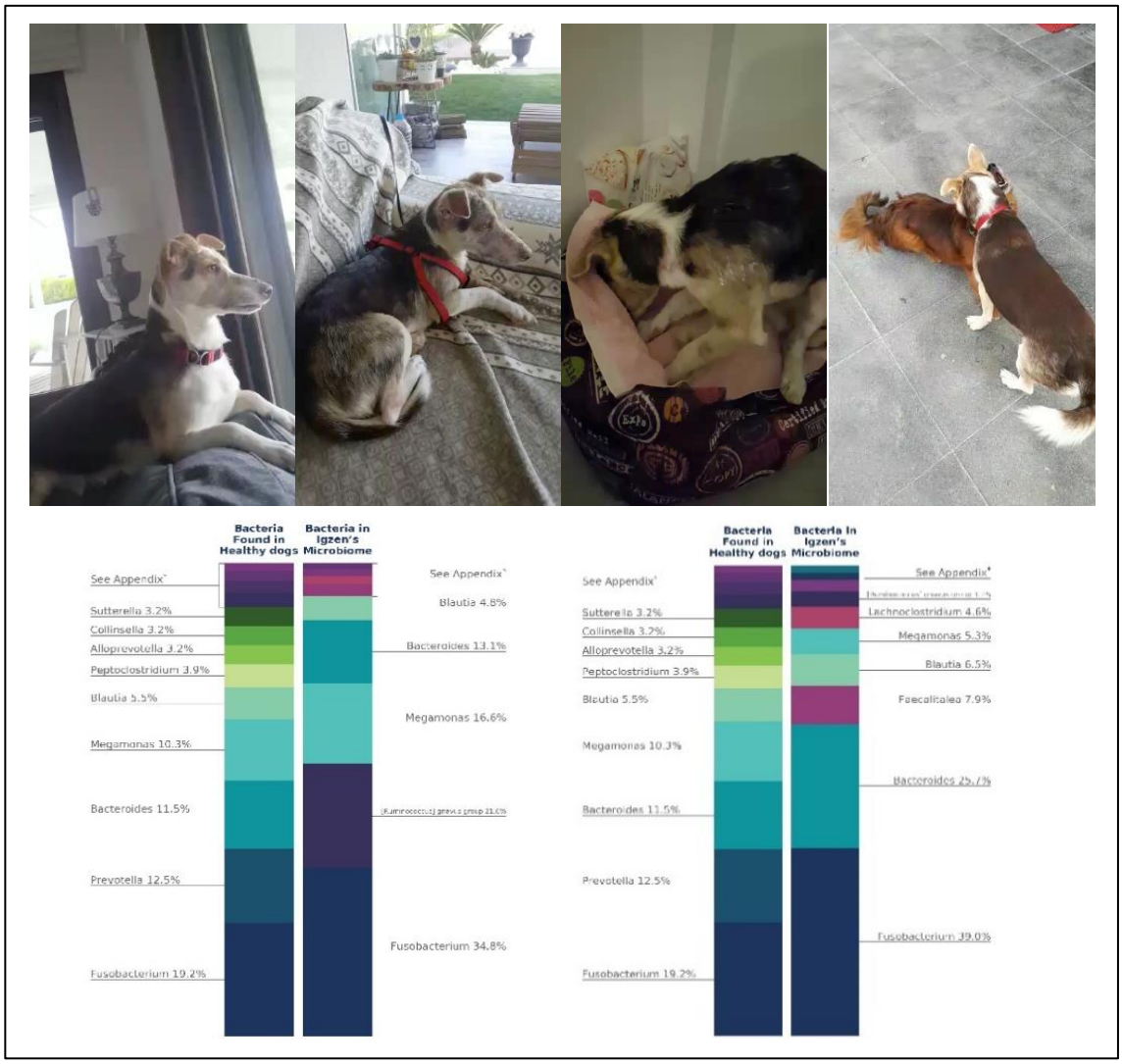

Figure 4. (Case 5) Atopic dog presenting severe pruritus a) prior to treatment, and therafter b, c and d) weeks 2-4, respectively, following fecal microbiota transplantation. Bottom photographs showing gut microbiota alterations comperatively (first bar graphic comperatively presents healthy dog microbiota).

Table 1. Classification, CADESI-04 scores, allergen detection and cornerometric analysis involved within this study apart from microbiota analysis.

Case no	Classification of CAD	CADESI-04 scores		Allergen specific in vitro analysis	Epidermal hydration		Epidermal pH	
		bt	aFMT		bt	aFMT	bt	aFMT
I	Severe	99	12	D.f., R.p	4	100	5	7.8
II	Severe	128	14	D.f.,	8	100	5.3	6.4
III	Severe	76	7	D.f., R.p	24	100	4.2	7.0
IV	Moderate	50	21	D.p.	12	55	4.6	6.7
V	Severe	90	10	D.f.	11	90	4.4	6.0
VI	Severe	101	6	D.f.	16	76	5.0	6.2
VII	Severe	88	8	D.f.	23	80	5.7	7
VIII	Severe	79	4	D.f.	20	78	4.9	6.5

Proposed benchmarks for detecting severity of CAD; 10 (mild), 35 (moderate), 60 (severe atopic dermatitis) - based on skin lesions and CADESI-04 analysis; D.f.: *Dermatophagoides farina*; D.p.: *Dermatophagoides pteronyssinus*; R.p: Rye pollen; CAD: Canine Atopic Dermatitis; CADESI-04: Canine Atopic Dermatitis Extent and Severity Index version 4 bt: before treatment aFMT: after treatment.

Table 2. Statistical analysis of CADESI-04 scores, corneometric analysis and VAS scores shown in Table 1.

	bt (Median ± SD) (min-max)	aFMT (Median ± SD) (min-max)	P value
CADESI-04 scores	89 ± 22.5 (50- 128)	9 ± 5.4 (4 – 21)	0.012
Epidermal hydration	14 ± 7.2 (4 – 24)	85 ± 15.8 (55 – 100)	0.012
Epidermal pH	5.0 ± 0.5 (4.2 – 5.7)	6.6 ± 0.6 (6 – 7.8)	0.012
VAS scores	8 ± 1.7 (6 – 10)	1 ± 0.8 (0 – 2)	0.012

bt: before treatment; aFMT: after treatment; CADESI-04: Canine Atopic Dermatitis Extent and Severity Index version 4 VAS: visual analog scale.

Table 3. Alpha diversity was calculated with respect to operational taxonomic unit (OTU) *richness*, *evenness* and overall diversity to understand the ecological differences within the CAD in which reference ranges for healthy dogs as follows: overall diversity 2.2+; richness 38+; evenness: 0.6+ Firmicutes: Bacteroidetes ratio: < 8 or below (Animal Biome web site).

Case no	Overall diversity		Richness		Evenness		Firmicutes: Bacteroidetes ratio	
	bt	aFMT	bt	aFMT	bt	aFMT	bt	aFMT
I	1.94	2.32	29	44	0.58	0.61	126.44	0.39
II	2.44	2.95	74	113	0.57	0.62	1.09	0.66
III	2.51	2.6	46	48	0.65	0.68	0.88	0.67
IV	1.81	1.85	21	24	0.57	0.61	3.85	1.35
V	1.85	2.44	21	40	0.61	0.45	3.82	1.34
VI	1.92	2.47	62	71	0.55	0.6	2.01	0.72
VII	2.02	2.95	74	113	0.62	0.57	1.33	0.98
VIII	1.87	2.5	34	46	0.57	0.65	46.51	2.64

bt: before treatment; aFMT: after treatment.

Table 4. Selected OTU analysis and deemed available statistical analysis.

	bt (Median ± SD) (min-max)	aFMT (Median ± SD) (min-max)	P value
Overall diversity	1.9 ± 0.3 (1.8 – 2.5)	2.5 ± 0.4 (1.9 – 3.0)	0.012
Richness	40 ± 22.4 (21 – 74)	47 ± 33.8 (24 – 113)	0.012
Evenness	0.6 ± 0.03 (0.6 – 0.7)	0.6 ± 0.1 (0.5 – 0.7)	0.481
Firmicutes: Bacteroidetes ratio	2.9 ± 44.5 (0.9 – 126.4)	0.9 ± 0.7 (0.4 – 2.6)	0.012

bt: before treatment aFMT:after treatment.

Gut microbiota alterations: Alpha diversity was calculated with respect to OTU richness, evenness and overall diversity (Table 3) to understand the ecological differences within the CAD using the Shannon indices. To detect differences in OTU composition for CAD cases enrolled in the present study a comparison was made at time point T_0 (prior to FMT capsule treatment) and there afterwards with the completion of study at time point T28. The OTU distribution was investigated at the phylum, family and genus levels.

Comperative evaluation of gut microbiota analysis indicated changes in Firmicutes: Bacteroidetes ratio among 8 dogs enrolled before (case I: 126.44, case II: 1.09, case III: 0.88, case IV 2.01:3.85, case V:3.82, case VI:2.01, case VII:1.33 and case VIII:3.82) and after completion of treatment (0.39, 0.66, 0.67, 1.35, 1.34, 0.72, 0.98 and 1.34, respectively). Statistical interpretation revealed significant alterations (P=0.012) for overall diversity, richness and Firmicutes: Bacteroidetes ratio, as shown in Table 4.

Dogs were monitorized for a total of 18 months after study was completed. None of them were diseased nor recurrence was detected. All were clinically healthy at the time of writing, after 1.5 years were passed following completion of the present study.

Discussion and Conclusion

Given the microbiome has a pivotal role for immune system homeostasis, alterations in its composition and function, namely dysbiosis both in the skin and the gut, detected to have relationship with CAD. Researches analyzing the influence of microbiome alterations regarding disease process in CAD is awaited. In the present study it was claimed that; i) detecting gut microbiota alterations, and then ii) restoration of gut microbiota within fecal microbiota transplantation (FMT) capsules will significantly decrease both CADESI-04, and the pruritus scores along with maintaining epidermal barrier function after 4 weeks of twice-daily treatment. Available evidence, as proved, in this study showed that FMT capsule therapy via oral route presented clinical cure for CAD, at least for the study duration for 1 month, thereafter 1.5 years monitorization period. Regarding alterations detected within VAS and CADESI-04 scores, FMT capsule therapy resulted in anti-pruritic efficacy and clinical cure was evident to those of all dogs treated, accompanied by withdrawal of clinical signs. All 8 dogs were stable, without appearent itching behaviour, as was in correlation with CADESI-04 scores. Furthermore, a marked correlation was evident for CADESI-04 and pruritus scores, indicating that the FMT capsules resulted with clinical remission might be attributed to i) restoration of gut microbiota, swifting disbiosis (17) to eubiosis, ii) anti-inflammatory effects of probiotics (useful bacteria included in the FMT capsules used, iii) probable impairment of intestinal mucosal barrier (which is involved within the pathogenesis of atopic dermatitis) (17, 18, 25) and is probably due to suppression of small intestinal bacterial overgrowth (12, 16) that has been linked to skin conditions (4). Possible explanation of the relationship between SIBO and skin lesions might be dedicated to damage to gut associated lymphoid tissue, altered lipid metabolism (because of intraluminal bacteria), immune system, elevated intestinal permeability, nutritional insufficiency, systemic extension of lipolysaccharide causing injury against epidermal barrier (12, 16). This may be a foremost factor also within the present study. Altough the precise pathogenesis regarding the communication among gut (microbiota) and skin remains unclear, gut-skin association, namely gut-skin axis, has been associated with several different dermatoses along with AD (2). A preliminary conclusion should be dedicated to AD linked to gut disbiosis and elevated intestinal permeability (10). Thus, restoration of gut microbiota in atopic dogs to those of involved in this study via FMT capsules achieved orally resulted in clinical remission, could support the obtained evidence.

16S rRNA microbiome testing presented alterations in Firmicutes: Bacteroidetes ratio among 8 dogs enrolled before (0.88-126.44) and after completion of treatment (-0.39-3.35). Alterations among Firmicutes: Bacteroidetes ratio (speficically in case 1 from 126.44 to 0.39, respectively through day 0 to 28, might be briefly explained with possible gut restoration and available diversity. All 8 dogs with CAD showed elevated levels of Firmicutes at phylum level. Increased abundance of Firmicutes might indicate exacerbation of CAD symptoms (prior to treatment), in which decreased Firmicutes: Bacteroidetes ratio (and thereof clinical recovery) among all dogs treated with FMT capsules on day 28 supported this explanation involving dogs enrolled in this study. Statistical significance (P=0.012) for overall diversity, richness and Firmicutes: Bacteroidetes ratio, (Table 4) were evident, briefly/additionaly supportive proof of FMT treatment in the present study.

In the present study, the response to FMT capsules for restoration of gut microbiota (as detected by prior to after analysis of 16S rRNA PCR) was rapid, with the majority of the clinical improvement evident by week 3. CADESI-04 scores tended to qucikly improve thereafter in almost all of the cases enrolled. Significant alterations (P=0.012) for CADESI-04 and VAS scores along with corneometric analysis before and after treatment (Table 2) all detected were in favour of FMT treatment modality in the present study. Given the identified role of microbiota in the alterations of several disease processes, there is no doubt that an imbalance of microbiota has a well known connection with gastrointestinal and skin disorders. As an innovative treatment option, as was performed int this study, FMT might be capable of restorating disturbed microbiota evidenced by several different reports and clinical trials. Taking into account emerging data on the probable otential clinical applicability of FMT beyond CDI in both gastrointestinal and nongastrointestinal conditions.

Age related alterations (namely transition) is evident in dogs in which beneficial bacteria species in host animals might present variations (based on the host) (19). In the present study dogs with AD were aged between 24 to 119 months, in which were classified as young to middle aged (young dogs: 2 years- and aged dogs were greater than 10 years old) as was determined previously (19). A question might arouse regarding FMT capsules composition and age of diseased dogs. As FMT capsules were purchased from a commercial company at USA, they claimed that the donors for FMT capsules involved dogs

monitorized for health, age, microbiome composition (based on DNA testing) and other relevant biomarkers, suggesting that age matched treatment (individualized FMT capsules) might be available.

From another point of view, based on "gut-brain-skin axis" gastroenterology and dermatology should met each other at the crossroads (29). Following completion of the present study, it was clearly suggested that dermatological disease should be investigated and an interpretation must be based on underlying gastroenterological issues, as was supported with all cases enrolled. To the present authors knowledge' dogs participate as a role model for atopic diseas in humanbeing, in which obtained results and FMT probably participate as candidate therapeutical armamentarium. As a novel treatment modality FMT capsules might mitigate AD among dogs, in which supported a balanced bridge between the purpose and the results of the present study.

In conclusion, treatment with FMT capsules is an easy and relatively/reasonably priced and resulted within complete efficacy and toleration in 8 dogs with AD. Instead of immunosuppressive treatment, this gut restoration system with potential immunomodulatory, anti-inflammatory and antioxidant formulation may be more likely to benefit by substituting this treatment modality. Further studies are warranted with a larger cases, possessing controlled propective research which should support the present findings. This commercially available FMT gut restoration system product has been licensed in the United States of America for usage in cats and dogs for different diseases. The present author has competing interest within this subject, aimed at performing novel projects focusing on 'gut-brain-skin axis' and gut/skin microbiota.

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Ethical Statement

This study was approved by the Aydın Adnan Menderes University Animal Experiments Local Ethics Committee HADYEK with no: (64583101/2019/022).

Conflict of Interest

I have competing interest to declare, in which development of biomarkers (via projects, PhD studies etc.), microbiome analysis, nutraceutical and functional food (investment and development fields), probiotics (29), synbiotics and "fecal microbiota transplantation capsule (fecal-oral) treatment"are under my arousing attention.

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Case Report / *Olgu Sunumu*

A case of Cheilognathopalatoschisis in a simmental breed calf: A rare case

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Abstract: The subject of this study was a 7-day-old female Simmental calf brought to Firat University Veterinary Faculty Animal Hospital Surgery Clinic with the complaint of cleft in the upper lip and palate. In the clinical examination, it was determined that the calf had respiratory difficulties and the nostrils were not formed normally. The calf was diagnosed with cheilognathopalatoschisis. Palatoplasty and reconstructive surgery were performed on the calf for the treatment of the rarely encountered case. The mucosa of the cleft hard palate was freed by dissecting from the os palatinum on both sides. Then, the hard palate was repaired by suturing the mucosal edges mutually. Palatal and alveolar clefts were repaired using Veau's technique. During the post-operative interview with the patient's owner, it was learned that the calf had difficulty in swallowing during feeding. It was determined that the calf died on the 8th postoperative day due to aspiration pneumonia that developed due to the absence of probable swallowing reflex.
Keywords: Anomaly, calf, cheilognathopalatoschisis, congenital, simmental.

Simental ırkı bir buzağıda Şilognatopalatoşizi olgusu: Nadir bir olgu

Özet: Bu çalışmanın konusunu, üst dudak ve damağında yarık şikâyeti ile Fırat Üniversitesi Veteriner Fakültesi Hayvan Hastanesi Cerrahi Kliniği'ne getirilen 7 günlük, dişi simental ırkı bir buzağı (7 günlük yaşta, dişi) oluşturdu. Yapılan klinik muayenede, buzağının solunum güçlüğünün olduğu ve burun deliklerinin normal olarak şekillenmediği tespit edildi. Buzağıya cheilognathopalatoschisis teşhisi konuldu. Nadiren karşılaşılan olgunun tedavisi için buzağıya palatoplasti ve rekonstrüktif cerrahi uygulandı. Yarık olan sert damağın mukozası her iki tarafta os palatinumdan diseke edilerek serbest hale getirildi. Daha sonra mukozal kenarlar karşılıklı olarak dikilerek sert damak onarıldı. Damak ve alveoler yarıklar ise Veau's tekniği ile karşı karşıya getirilerek onarıldı. Postoperatif dönemde hasta sahibi ile yapılan görüşmede buzağının beslenme sırasında yutma zorluğu yaşadığı öğrenildi. Postoperatif 8. gün buzağının olası yutma refleksinin oluşmamasından dolayı gelişen aspirasyon pnoumonisi sebebiyle öldüğü belirlendi.
Anahtar sözcükler: Anomali, buzağı, şilognatopalatoşizi, konjenital, simental.

Cheilognathopalatoschisis is the simultaneous cleft of the upper lip, maxilla, and upper palate in newborns. Although it is one of the most common congenital anomalies especially in humans, it is rarely encountered in animals (9). Cheilognathopalatoschisis, also known as rabbit-lipped, is an inherited defect with multifactorial genetic predisposition. Although it is hereditary, environmental stimuli are quite important in the etiology of the disease (2, 7, 9, 14). Exposure of cattle during pregnancy to piperidine alkaloids, some poisonings (with wild tobacco (*Nicotiana gluaca*), selenium and lupine species) and some viral diseases (Cache Valley virus, Akabane virus, Aino and Chuzan viruses, and bovine viral diarrhea (BVD) virus) may cause congenital cleft palate and cheliognatopalatoschisis (14).

Cleft palate is classified in different ways according to the presence, location and type of cleft. Some of these classifications are Davis and Ritchie's classification, Veau's classification, Kernahan and Stark's classification and LAHSAL classification. The LAHSAL classification

is an assessment method developed by Kriens in 1989 and accepted by the Royal College of Surgeons in 2005. The abbreviation that was LAHSHAL when Kriens developed it has been used as LAHSAL recently. The abbreviation LAHSAL consists of letters representing the six regions of the mouth. Accordingly, L=right lip, A=right alveoli, H=hard palate, S=soft palate, A=left palate, L=left lip (Figure 1). In the LAHSAL code, capital letters indicate complete cleft formation, and small letters indicate incomplete clefts. The dot mark indicates that the anatomical structure is formed normally (5, 10, 13).

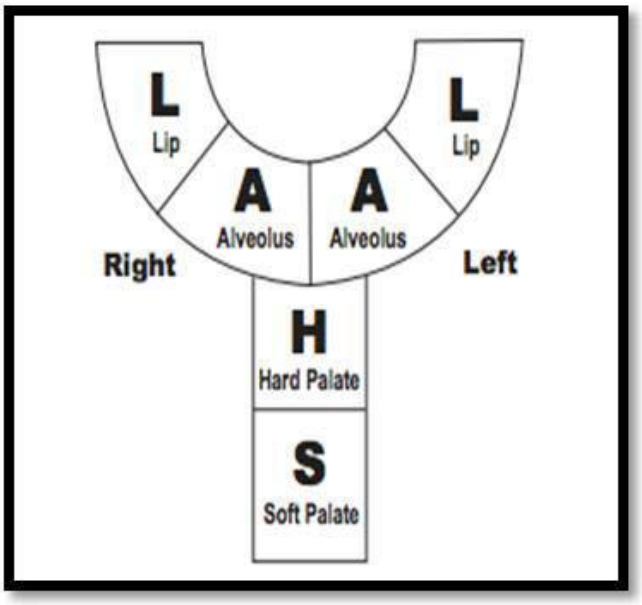

Figure 1. LAHSAL classification.

In cheliognatopalatoschisis cases, due to the connection between the mouth and nasal cavity, ingested food and water may escape into the lungs and cause aspiration pneumonia. This situation thought to be a simple structural disorder, can cause complications that cause the death of the patient (4, 6, 12). Palatoplasty and some reconstructive surgery techniques (such as Millard's technique, Tennison-Randall's technique and Veau technique) can be used to treat cheliognatopalatoschisis cases (1, 4, 6, 11-13, 15). Millard's and Tennison-Randall's techniques are mostly used in unilateral cleft lip (1, 11, 13). The Veau technique is a reconstructive surgical technique that is used more often in bilateral cleft lip, in which the cleft line is repaired in a straight way (13, 15). In this study, the diagnosis, treatment and results of a case of cheliognatopalatoschisis in a Simmental calf were shared.

In this study, a simmental calf (7 days old, female) brought with the complaint of cleft in the upper lip and palate was the subject. In the anamnesis, it was learned that the calf had respiratory difficulties and coughed during food intake. In the clinical examination, it was determined that the right and left nostrils of the calf were not fully formed (Figure 2a). In addition, a wide cleft in the cranial half of the palatum durum was detected (Figure 2b, 2c). It was determined that the calf had respiratory distress. After the examination, the patient was diagnosed with cheliognatopalatoschisis (rabbit lipped). When the diagnosis of the patient was evaluated according to the LAHSAL classification, it was determined that it had LAh.AL type cleft palate and lip (Figure 2).

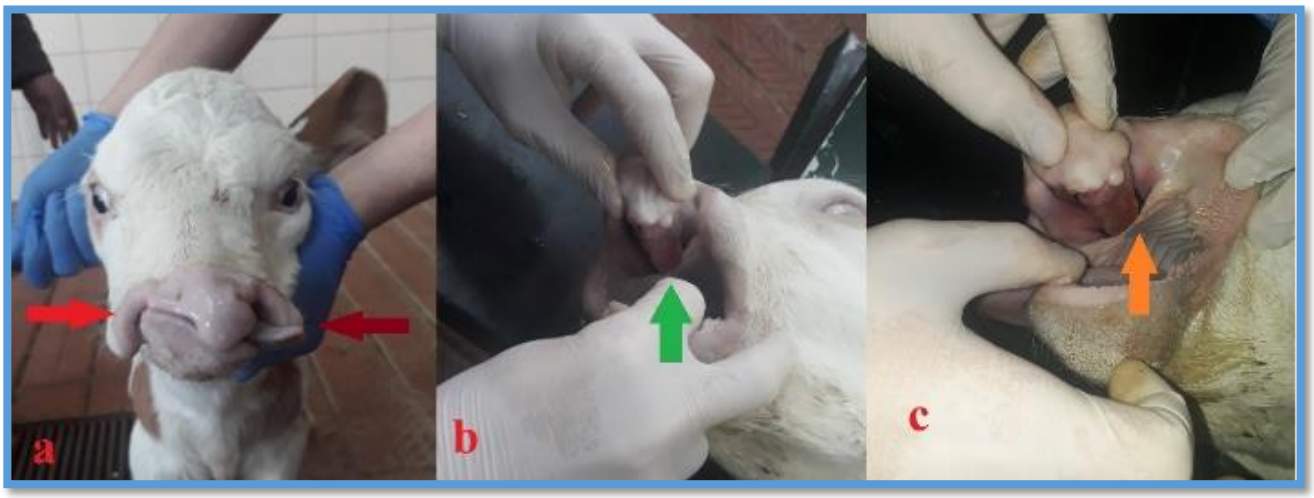

Figure 2. Abnormal position of the nostrils (a), cleft of the hard palate (b, c).

Figure 3. The patient's nostrils in their normal position after the operation (a, b).

It was decided to perform palatoplasty and reconstructive surgery in order to prevent aspiration pneumonia and correct the structural malformation. Before the operation, sedation was provided by intramuscular administration of 0.1 mg / kg dose of xylazine hydrochloride (*Xylazinbio® %2, Bioveta, Czech Republic*). After ten minutes, anesthesia was provided by intramuscular administration of ketamine hydrochloride (*Ketasol® %10, Richterpharma, Austria*) at a dose of 4 mg/kg. After the calf was anesthetized, the hard palate mucosa was freed by dissection from the os palatinum. Then, the free mucous membranes were brought face to face and sutured with non-absorbable suture material. It was then decided to perform reconstructive surgery to create the medial wing of the nose. For this, the bilateral lip and alveolar cleft were repaired by suturing them in a straight line using the Veau technique. For this, a 0.5 cm diameter wound line was created in the medial ends of the upper lips and the philtrum region. The wound lines created on the upper lips were stitched opposite the wound lines in the philtrum region, and the region was optimized for its normal anatomical structure. Stitches were applied using non-absorbable threads (Figure 3). Amoxicillin-clavunic acid (*Synulox, Zoetis, Latina, Italy*) was administered intramuscularly at a dose of 8.75 mg/kg for five days postoperatively. For postoperative pain management, 0.5 mg/kg of meloxicam (*Bavet Meloksikam, BaVET, Istanbul, Turkey)* was administered in two doses, once every three days. In addition, oral feeding of the animal was prevented for 3 days in the postoperative period, and 5% dextrose (*Dekstro-Flex, Eczacıbaşı-Baxter, Turkey*) solution was given intravenously. In the postoperative period, it was reported that the calf died on the 8th day in the interview with the patient's owner. The owner of the patient reported that the calf's breathing and swallowing difficulties continued during the post-operative period when oral feeding was started. According to information obtained from the patient's owner, it was suspected that the patient died due to aspiration pneumonia that developed due to the failure of the swallowing reflex.

Although congenital cleft palate and cheliognatopalatoschisis are hereditary defects, environmental stimuli are very important in their occurrence (2, 7, 9, 14). Exposure to piperidine alkaloids, some poisonings (wild tobacco (*Nicotiana gluaca*), selenium and lupine) and viral diseases (Cache Valley virus, Akabane virus, Aino virus, Chuzan virus, and Bovine Viral Diarrhea virus) during pregnancy are the most important environmental factors for the occurrence of these cases (14). In this case report, it was determined that astragalus was grown intensively in the region where the calf with cheliognatopalatoschisis lived. Selenium accumulates at a high level in the Astragalus plant (16). The reason for the formation of this anomaly is thought to be caused by the mother's consumption of Astragalus during pregnancy. It is thought that selenium toxicity caused by sodium selenite, which is used for protective purposes in the last third of pregnancy, may also cause this anomaly.

Congenital cleft palate can be seen with acroteriasis syndrome in Holstein, chondrodysplasia in Dexter, arthrogryposis and spinal anomalies in Charolais, hydrocephalus, diaphragmatic hernia and freemartins in Simmental cattle (8, 14). In this case, necropsy could not be performed because the owner did not give permission. In the clinical examination, no other anomaly was found in the calf, which was diagnosed with cheliognathopalatoschisis.

Cleft palate and lip clefts are classified by different methods according to their presence, location and type. The most used methods are Veau's technique and LAHSAL technique. The LAHSAL technique was developed by Kriens in 1989. Each letter in the LAHSAL abbreviation represents the anatomical regions of the mouth. (L= right lip, A=right alveoli, H=hard palate,

S=soft palate, A=left alveol, L=left lip). In these anatomical formations, if there is a complete cleft, it is indicated with a capital letter, if there is an incomplete cleft, it is indicated with a small letter (4, 10, 13). In this case report, it was determined that the calf diagnosed with cheliognatopalatoschisis had LAh.AL type palate and lip cleft according to the LAHSAL technique.

Aspiration pneumonia is one of the most common complications in calves with congenital cleft palate and cheliognathopalatoschisis (4, 6, 12). Smolec et al. (12), reported that frequent coughing and dripping milk from the nostrils in a 21-day-old Simmental calf with congenital cleft palate. Although palatoplasty was performed to restore palate integrity, it was decided to euthanize the calf due to aspiration pneumonia that developed in the second postoperative week (12). In this study, as in the study of Smolec et al. (12), it was determined that the calf died due to aspiration pneumonia in the first postoperative week, despite palatoplasty and reconstructive surgery.

The type and width of the clefts are very important when determining the treatment method of cleft palate and lip (3, 4, 6, 12, 13). If the cleft palate is not too wide, it can be closed with a cerclage wire. In some cases, cleft palate can be treated by applying methyl methacrylate, cold acrylic and propylene mesh locally. If the defects are large in cleft palate, autograft skin or fascia graft can be applied (3, 4, 6, 12). In this study, since the cleft in the hard palate was not wide, the hard palate mucosa was freed by dissection from the os palatinum. Then, the free mucous membranes were brought face to face and sutured with non-absorbable suture material. In the treatment of lip and alveolar clefts, it is very important whether the clefts are unilateral or bilateral. While Millard's technique or Tennison-Randall's technique is applied mostly in unilateral clefts, Veau technique is applied in bilateral clefts (1, 11, 13, 15). In this study, the lips were sutured to the philtrum region in a simple straight line by applying the Veau technique in a calf with bilateral lip and alveolar clefts.

In conclusion, cheliognatopalatoschisis is an anomaly that can have fatal complications and can be seen in all animal species. The most important and fatal complication is asphyxia due to aspiration pneumonia. This study revealed that the classification of cleft palate and lip is very important in determining the treatment method and possible complications. This study contributes to the literature on the management of the operative and postoperative process in similar cases.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Case Report / *Olgu Sunumu*

Artificial insemination in a cat: Report of first successful performance resulted with parturition in Turkey

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Abstract: In this case, semen that handled by urethral catheterization was deposited into the deepest point of the vagina of an anesthetized queen. Intravaginal artificial insemination was applied on the 2nd day of onset of the oestrus and only single insemination was performed. Insemination time was determined by behavioural and cytological evaluations. Two healthy kittens were obtained 62 days after insemination. The case has important reports in terms of being the first artificial insemination performance in cats in Turkey, resulted in parturition.
Keywords: Artificial insemination, queen, tomcat, urethral catheterization.

Bir kedide suni tohumlama: Türkiye'de ilk defa doğumla sonuçlanan başarılı bir uygulama raporu

Özet: Bu vakada bir erkek kediden üretral kateterizasyon yöntemiyle alınan sperma, bir dişi kedinin vaginal kanalının en derin noktasına bırakılarak suni tohumlama işlemi gerçekleştirildi. Intravaginal suni tohumlama, östrusun 2. gününde yapılan tek tohumlama şeklinde gerçekleştirildi. Tohumlama zamanı, davranışsal ve sitolojik değerlendirmeler sonucunda belirlendi. Suni tohumlamadan 62 gün sonra iki adet sağlıklı yavru doğumu gerçekleşti. Bu vaka raporu, Türkiye'de canlı yavru doğumu ile sonuçlanan kedilerdeki ilk suni tohumlama uygulaması olması dolayısıyla önemli bilgiler içermektedir.
Anahtar sözcükler: Dişi kedi, erkek kedi, suni tohumlama, üretral kateterizasyon.

While artificial insemination (AI) and reproduction techniques have been widely applied in other species in the world, they are not commonly applied in cats. Although AI studies in cats are very limited, there is only one study conducted on this subject in Turkey (1). Studies are still continuing to improve the effectiveness of AI by scientists and clinicians today, even after approximately 50 years from the first kittens obtained by artificial insemination (9). There are many practical limitations for AI in cats such as behavioral or physical indications, proestrus aggression, semen collection techniques, semen volume, determining the optimal time for insemination, ovulation induction and the contraindication of sedation procedures (7, 10). The primary reasons for using assisted reproductive techniques in cats are to preserve the genetic material of the valuable species that can be protected and used in different conditions and breeding the cats without physical disabilities or behavioral disorders (10). On the other hand, the knowledge and experience gained from domestic cats could be used in attempts to protect wild cat species (7, 12, 13). Domestic cats may also be a useful model for research into human diseases (5).

The aim of the case is to report the first kittens obtained from the first successful AI of Turkey.

A queen and a tomcat were referred to the Reproduction and Andrology Clinic by the owners with a history of a queen that did not allow males to mate or even approach to her, even though she showed estrus behaviors. Thus, performing AI with fresh semen was decided.

Queen was a British Shorthair, 22 months old, 3 160 g indoor cat with unknown fertility. She had no recent or previous diseases reported. On the first examination, the

queen was bright, responsive with normal vital parameters, good body condition score, and body temperature.

Tomcat was a British Short Hair, 25 months old, 3 960 g indoor cat, living together with the queen. He had mating experiences for many times with different queens and had kittens from these matings. On the first examination, the tomcat was bright and responsive with normal vital parameters, good body condition score, and body temperature.

Queen and tomcat were presented to the Reproduction and Andrology Clinic on the second day after onset of the oestrus behaviors. Oestrus behavior histories were taken by the owners such as vocalization, lordosis, rolling, rubbing, etc. Vaginal cytology was performed by using a cotton swab to confirm the oestrus. Smear was prepared and stained with Diff-Quick stain. The vaginal cells were evaluated at 200x magnification under a light microscope. Oestrus was confirmed by detecting a vaginal smear with a rate of superficial cells greater than 80%.

Tomcat was anesthetized using Medetomidine 0.32 ml + Propofol 1.0 ml for semen collection (7, 13). Urethral catheterization technique was used for semen collection (13). Urethral catheter (1.0 x 130 mm, 3FG) was lubricated and gently inserted into the urethra. The catheter was kept in the urethra for a minute to allow the semen to fill up into it. After catheter removal, 0.8 ml Atipamezole hydrochloride was used to arouse the tomcat (Figure 1). After removing the catheter, the semen was examined for its color and immediately marked on the catheter with a marker pen to determine the semen volume (Semen filled length [2.5 cm] x ∏ [3.14] x radius of the catheter [0.5 mm]). Then the catheter was filled up with a heated isotonic solution until the catheter was full.

On the same time with the tomcat, queen was anesthetized by medetomidine 0.20 ml + Propofol 1.0 ml (7, 8). The semen filled catheter was inserted into the vagina and 27.5 µL extended semen is deposited. After the catheter was removed, vaginal stimulation was performed by using a glass rod to induce ovulation provocation. No antisedatives were used to arouse the queen to prevent from return of the semen from the vagina (Figure 1).

To authorize Reproduction and Andrology Clinic to perform an AI in cats practice and its requirements, informed consent form was signed by owners.

After the AI procedure was completed, semen was examined in terms of volume, color and motility. The semen had creamy color. Semen volume was calculated approximately as 6.25 µL (Lenght of native semen was 2.5 cm). The semen remaining in the catheter after the insemination was examined subjectively in terms of motility by a light microscope. The percentage of sperm motility was around 60%. Concentration could not be determined because only a small amount of semen remained in the catheter.

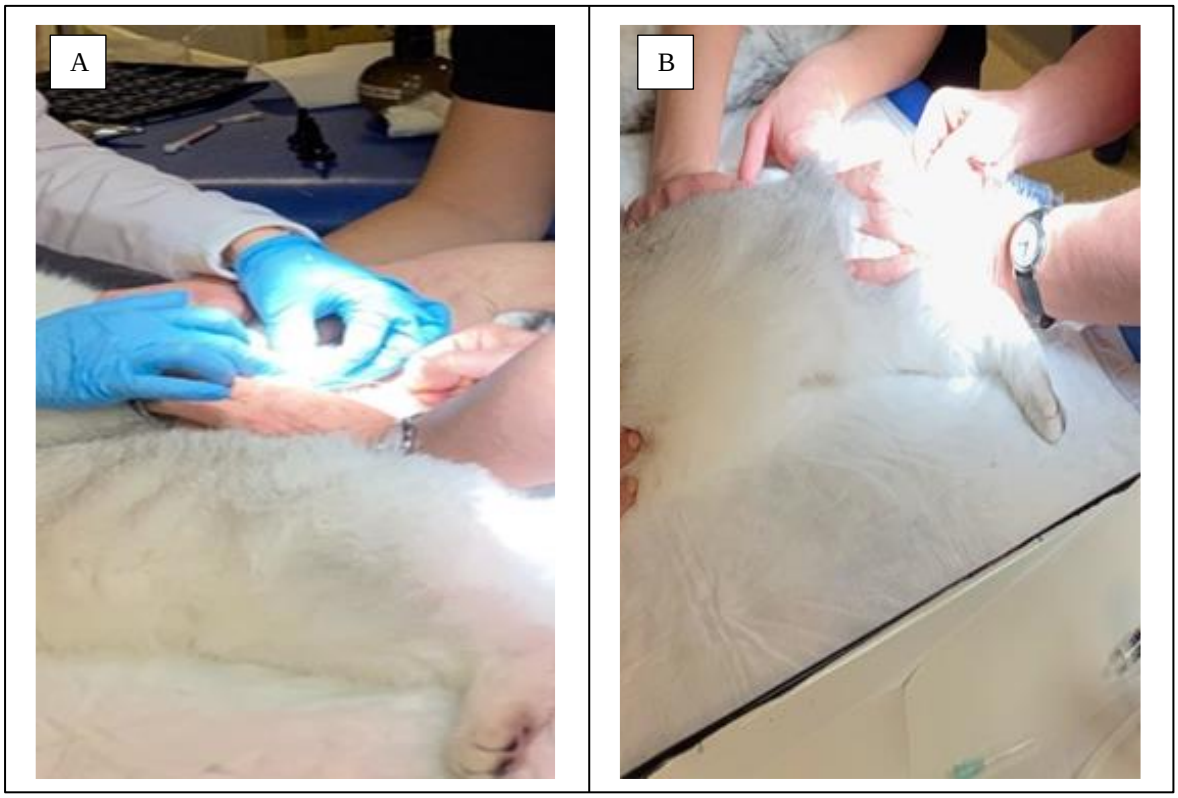

Fig 1. A. Semen collection by urethral catheterization from tomcat. **B.** Vaginal insemination in queen.

Two healthy kittens were born 62 days after the AI procedure.

AI for domestic cats is not a common clinical application as it is in dogs. However, AI studies in domestic cats will lead to the conservation of wild cat species and especially will bring new points into human medicine in the light of new studies (4, 5, 7, 12). There is only one study conducted on AI in domestic cats reported from Turkey (1). Although a wide range of researches and performances have been done in the previous study, they have reported only 19th day pregnancy diagnosis (1). The case report has high importance because it has reported the first successful parturition (two healthy kittens) obtained from AI in cats, in Turkey.

Researchers suggest that the AI in cats should be performed on the 2nd day of the onset of oestrus determined by behavioural inspection or cytological determination, and repeated 2 days later (2, 3, 7). In our case, AI was applied on the 2nd day after the onset of oestrus behaviours, however, second repetition did not be performed.

Although there are different types of anaesthesia protocols, Medetomidine+Propofol combination preferred for its fast recovery, less risky and giving option to use antisedatives for unexpected situations. Besides, medetomidine administration is obligatory in tomcats if urethral catheterisation technique is used. Also, medetomidine is known as the α2-adrenergic receptor stimulator and it allows the release of a small volume of highly concentrated sperm from the cauda epididymis into the urethra (7, 8, 11).

According to reported studies (3, 6, 7, 11, 12) fresh semen is more appropriate for intravaginal inseminations and intravaginal insemination with fresh semen is more suitable for clinical applications, because it must be rapid and unstressed. In the case, intravaginal AI by urethral catheter was applied. This is a practical approach in veterinary clinic conditions for performing intravaginal AI in cats.

Success of AI in domestic cats depends on good knowledge of the anatomical structure, determining the appropriate insemination period correctly, using appropriate equipment, depositing the semen in suitable conditions (frozen or fresh) and a suitable area of the vaginal tract (intrauterine, intravaginal). The case demonstrated that if the correct time for insemination is determined correctly with the appropriate methods, single insemination will be sufficient to produce a pregnancy and healthy parturition. The case is significant because it is the first AI performance in Turkey that resulted in parturition.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Review / *Derleme*

What has been done in the fight against *Varroa destructor*: from the past to the present

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Abstract: Bees are the major pollinators in natural ecosystems and in the agricultural production of several crops used for human consumption. However, they are exposed to multiple stressors that are causing a serious decline in their population. We highlight a major one among them, the *Varroa destructor* mite (Varroa) that causes severe impacts on the health of honey bee colonies, transmitting a variety of viruses that can affect the survival ability of individual bees and entire colonies. Diagnosis and mite control methods have been intensively studied in recent decades, with many studies in different areas of knowledge having been conducted. This overview summarizes these studies with a focus on colony defense systems, biological characteristics of the parasite Varroa, diagnostic methods used to establish the infestation level of colonies, and currently used control methods.
Keywords: Diagnosis, Honey bee disease, mite, parasite, *Varroa* control.

Geçmişten günümüze *Varroa destructor* mücadelesi

Özet: Arılar, doğal ekosistemlerde ve insan tüketimi için kullanılan çeşitli mahsullerin tarımsal üretiminde başlıca tozlayıcılardır. Bununla birlikte, popülasyonlarında ciddi bir düşüşe neden olan çoklu stres faktörlerine maruz kalırlar. Bal arısı kolonilerinin sağlığı üzerinde ciddi etkilere neden olan ve tek tek arıların ve tüm kolonilerin hayatta kalma kabiliyetini etkileyebilecek çeşitli virüsler bulaştıran *Varroa destructor* akarı (Varroa) üzerinde duruyoruz. Teşhis ve akar kontrol yöntemleri, son yıllarda yoğun bir şekilde araştırılmış ve farklı bilgi alanlarında birçok çalışma yapılmıştır. Bu derleme, koloni savunma sistemlerine, parazit Varroa'nın biyolojik özelliklerine, kolonilerin istila düzeyini belirlemek için kullanılan tanı yöntemlerine ve şu anda kullanılan kontrol yöntemlerine odaklanarak bu çalışmaları özetlemektedir.
Anahtar sözcükler: Akar, Bal arısı hastalığı, Parazit, Teşhis, Varroa kontrolü.

Introduction

Bees are exposed to numerous stressors and the decline of their populations on different continents has been reported in recent decades (13, 84, 87). According to Goulson et al. (42), there is evidence of a combination of factors causing this decline, such as: pesticides, habitat degradation and fragmentation, increase of monocultures in agriculture, decline in flower diversity and abundance, as well as parasites and diseases. Among the stressors, *Varroa destructor* mite the main pest that affects the health of young and adult bees and, consequently, the colony (11, 28, 47, 95).

In accordance with Milani (78), in the second half of the last century, the mite *V. destructor* spread across the European continent and since 1980 several studies have been conducted in order to find methods to identify and control the mites (16, 69). This review aims to compile knowledge about the *V. destructor* mite from recent literature, such as its biological characteristics, impact on bee populations, and updated control mechanisms.

Honey bee colony defense systems

Honey bees have evolved a collective anti-parasite defense system which is allo-grooming behavior in order

to remove parasites from group members (101). Social defense systems in honey bees can be activated on demand, based on collective action or altruistic behaviors of infected individuals that benefit the colony (23). Individual bees evolved physiological, immunological, and behavioral defense systems toward pathogens and parasites. The colony-level response, as opposed to the immune responses within an individual bee, presents coordinated behavioral cooperation among individual bees in a nest. The collective defense against parasites is termed "social immunity" (24). We can observe continuous simple interaction between adult honey bees in a colony. Social immune responses against parasites are initiated on a colony level when several individual adult honey bees interact with each other. Social immunity with a defense effect is a type of behavioral mechanism believed to contribute to reduced Varroa survivability in honey bee colonies. The system targets the mites at the phoretic stage, during their feeding on adult host bees. It occurs when adult worker bees remove mites from adult bee bodies via auto-grooming and allo-grooming. An important target of social immunity is during the mite reproduction phase on bee pupae sealed in comb cells (49).

Tolerance and resistance characteristics

When we select and rear honey bees two professional terms need to be considered and potentially introduced into breeding practice. They are tolerance and resistance, which refer to different mechanisms to enable bees in reducing the effects of the parasite Varroa or any pathogenic or other physiological factors, especially with repeated exposure. The body thus becomes less responsive and develops the ability to overcome the effects without exhibiting disease appearance (27). In a honey bee apiary, tolerance is the ability of bees to live in association with Varroa. When Varroa transfers secondary infections, bees may be developing resistance to these pathogenic organisms and the final effect is developing tolerance to Varroa infestation (14, 59). The ability of organisms to remain unaffected or slightly affected is thus considered as resistance. Resistant bees are able to maintain low levels of Varroa infestation or other pathogens due to known or unknown bee characteristics. In absolute resistant bees, Varroa or other pathogens would not infest or infect the individual bee or the entire colony.

Grooming behavior of honey bees

The ability to remove foreign particles from the honey bee body surface can be removed by performing self-grooming behavior (auto-grooming). The phenomenon when bees groom another bee it is known as allo-grooming. This behavior was described in *Apis cerana* as a defense mechanism against Varroa (15), and by Africanized *A. mellifera* in the tropics (79). One of the possible mechanisms of resistance to Varroa in Africanized bees is "auto- and allo-grooming" behavior, where bees brush particles from themselves or from their nest mates. It is demonstrated that Africanized bees, which are important hybrids of *A. mellifera scutellata* nowadays bred in Brazil, appear to have more resistance than European strains. Another example of grooming behavior efficacy as a defense mechanism to Varroa is described in *A. cerana* (79). Adult workers grooming behaviors can explain reduced *V. destructor* reproductive success in the colony and this phenomenon has an impact on colony survival. Varroa resistant colonies with expressed adult bees grooming behavior have lower mite population rates (up to 15-fold) and a higher percentage of damaged mites (up to 9-fold) in comparison to colonies with weak grooming behavior. It has been established that grooming behavior and corresponding Varroa mite populations growth is an important component in the resistance of some honey bee genotypes (49).

Hygienic behavior of honey bees

Disease resistance is known to correlate with the "hygienic behavior" of worker bees. This is a genetically controlled collective response by adult workers to recognize and then remove the dead, infected or damaged brood (larvae and pupae) (43). Hygienic behavior was originally described in honey bee colonies resistant against *Paenibacillus larvae*, where bees may uncap comb cells containing dead, sick or damaged brood and remove this brood from the colony (99). It was found that hygienic behavior is also a significant defense mechanism against Varroa mites parasitism (10, 61, 108, 124). The hygienic behavior of honey bees is therefore responsible for the ability to identify and remove Varroa infested pupae from comb cells in the honey bee colony nest. *A. cerana* worker bees in their colonies regularly detect Varroa-infested pupae. Workers may either make a hole in the wax capping or may remove the pupa and subsequently release the mite confined in the brood cell (10). This resistance mechanism is well known in *A. cerana* and may also be activated in heavily parasitized host *A. mellifera* colonies. Varroa reproduction preferably takes place in drone broods and it is thought that *A. cerana* colonies resistance largely depends on the seasonal nature of their drone development (35, 97).

Rapid population growth of Varroa in colonies of European honey bee races is due to Varroa’s ability to reproduce in both drone and worker brood of *A. mellifera*. Hygienic behavior based on the workers olfactory stimuli is responsible for their ability to remove mite-infested worker brood. This trait is a highly desirable selection criterion against varroosis (77, 107). By killing any mite offspring during the reproductive cycle and reducing Varroa reproductive success there is a negative cumulative

effect on the Varroa population dynamic in colonies. In selected colonies, with highly Varroa infested pupae, workers were able to remove up to 60% of the experimentally infested brood (106). Adult worker bees detecting and removing mite-infested brood have been defined as displaying Varroa sensitive hygiene (VSH) and this has been identified as a trait in specific bee strains (53).

These colonies were selected to effectively remove pupae that were mite-infested (52). The VSH selection procedure based on brood removal is more effective in comparison to the freeze-killed brood assay procedure (62). The removal of infested brood inhibits individual mite reproduction and therefore reduces the in-colony entire mite population (54-55). VSH selected bees expressed the specific characteristic that Varroa is removed from comb cell opening after worker's bee hygienic activities (53). There is a need to study the variety of potential resistance mechanisms in honey bee colonies that can contribute to colonies having a lower level of Varroa infestation. In beekeeping practice, specific phenotypic characteristics have been observed and through selection activities more Varroa tolerant honey bee lines can be reproduced.

Impact of Varroa on colonies health and survival of honey bees

Varroa as an external parasite has physical and pathological effects on individual bee and on the whole colony level. Varroa attacks both adult bees and developing larvae. Parasitized brood is injured with reduced larval protein content and subsequently bee body weight is reduced, organ development is affected and finally, worker or drone life is shortened (11). Emerging bees may be deformed with missing legs or wings and together with deformed wing virus, microbes, and reduced immune competency, adult bees' survival is significantly affected in untreated colonies. Highly infested colonies that are not examined for mites, and effectively treated may die or contribute to increased winter mortality or queenlessness. Varroa mites are also a vector in transmitting a number of viruses from infected to healthy bees. Viruses associated with Varroa mites in colonies are: DWV, Acute Bee Paralysis Virus (ABPV), Chronic Bee Paralysis Virus (CBPV), Slow Bee Paralysis Virus (SBPV) (76), Black Queen Cell Virus (BQCV), Kashmir Bee Virus (KBV), Sacbrood Virus (SBV) (75, 76, 111).

Varroa parasitization, together with viruses as secondary infections, influences the weakened bee's immune system and contributes to an increased risk of colony mortalities. *Varroa* infestation has been shown to induce increased DWV abundance in parasitized bees and also result in increased immunosuppression (82). Consequentially ABPV, BQCV and DWV also appear in workers and queens during queen rearing procedures (127). The increased incidence of viruses present in bees has resulted also in the appearance of an epidemic that is demonstrated by increased colony mortalities and this has been contributed to by *V. destructor* parasitizing (123). Bees are thus directly damaged by mites feeding on them, and additionally efficiently inoculated with harmful viruses. It was also shown that highly pathogenic DWV and ABPV associated with Varroa in highly infested honey bee colonies contribute to winter colony losses (8). Moreover, it has been found that the mites are activators of several mechanisms in honey bees to induce immune-suppressive action and subsequently increase virus replication (126). Therefore, proper Varroa diagnosis and timing of colony treatment together with sufficient efficacy is imperative for beekeepers to preserve their honey bee stock.

Reproduction and infertility of Varroa

Varroa mites reproduce more in done brood than in worker brood, which may be because of physiological factors of non-reproduction mites observed only in worker cells. A female Varroa has on average 1.5 - 2 reproductive cycles in its life (37) with a range of 0 - 7 cycles (100). It was found that Varroa oviposition after entering into brood cell might be stimulated by prior feeding on adult bees or the bee larvae respectively and a shorter feeding period has slightly reduced fertility potency in female mites.

The phoretic mites are more attracted to nurse than forager bees probably because they carry them to their reproduction site (25). Duration of the phoretic phase is variable and could depend on the type of bee carrying the mite having an impact on the mites' life cycle and reproduction. The phoretic mites stay on adult bees for a variable amount of time, from one to ten days (7). Multiply-infested cells also have an impact on reduced Varroa reproduction as a result of the existence of chemical factors in female Varroa and subsequently, the number of daughters per mite decreases in multiply-infested cells (83). It is also evident that Varroa has lower reproduction potential in tropical Brazil where bees expressed approximately two times greater proportion of non-reproductive mites in comparison to honey bees in a temperate climate in Europe (94). In addition, mite population dynamics depends also on the type of brood (i.e., worker drone). The honey bee drone brood is more attractive to *V. destructor*, in comparison to worker brood. Drone brood takes more time to develop and therefore leads to the higher production of Varroa offspring (39). Mother mites are able to choose nurse bees over foragers and newly emerged bees as their optimal host in the phoretic phase to quickly infest new brood cells (125). Varroa mite population can increase in honey bee colonies

up to ten times during the beekeeping season and thus demonstrate a high degree of adaptation (105).

Over a long period of time, with the help of selected breeding activities, the host has the opportunity to develop resistance mechanisms to its pest. There is some evidence in beekeeping operations indicating variability between honey bee colonies in resistance of honey bees to Varroa. In addition to resistance traits of individual larvae, VSH behavior of workers has been demonstrated as a useful indicator for developing honey bee resistance to Varroa mites in breeding stocks. This behavior can be considered in breeding activities where selected honey bees show the ability to detect infested capped brood and destroy Varroa. Strains of VSH bees have been developed and are now successfully used in beekeeping operations in the United States of America and are available for individual beekeepers (51, 121).

It was also found that *A. mellifera carnica*, *A. m. mellifera*, and *A. m. ligustica* have developed no adaptations in terms of the reproductive success of Varroa. In all subspecies groups mother mites reproduce equally successfully and are potentially able to cause detrimental damage to their host when not treated sufficiently. Furthermore, it was also established that a population once Varroa tolerant does not necessarily pass on this trait to following generations. Established tolerance to Varroa parasitism is also not evident in offspring (F1) (86). This phenomenon could be of particular interest for beekeepers when selecting populations for resistance breeding.

Breeding better bees

Numerous parameters have been considered and applied in apiculture practice in order to contribute to increased Varroa resistant bees such as: the initial population of mites, duration of brood developmental and the capped period of workers and drones. Additionally Varroa phoretic period, Varroa preference to drone brood, Varroa infertility level, the number of Varroa reproductive cycles, and winter mortality of Varroa are also considered for selection (35). Beekeepers can also incorporate into their breeding program additional variables associated with Varroa parasitism, such as colonies survival during winter period, monitoring natural Varroa mortality and Varroa population growth in the host colony. In North America breeding programs have been performed in order to produce Varroa resistance stocks using imported honey bee races (60). In European beekeeping conditions, the rich diversity of natural honey bee races (subspecies) and local varieties (ecotypes) offers enormous genetic resources for selection with native honey bees on Varroa resistance. The most important autochthonous subspecies employed for selection programs are *A. m. carnica*, *A. m. ligustica*, and *A. m. macedonica*, among others widely spread throughout Europe. Furthermore, in addition to selection on traits affecting the reproductive rate of Varroa, which is considered as the most important trait, selection on grooming and hygienic behavior would contribute to a reduction in Varroa population. Varroa resistant stock need to be continuously maintained with a sufficient gene pool and controlled mating is required for achieving progress in developing better honey bee colonies. Selected honey bee colonies normally require fewer acaricide treatments for Varroa control, and they demonstrate longer survival without Varroa control. Some honey bee populations in Europe have been found to naturally survive mite infestations without treatment for several years. Surviving colonies originating from Varroa infested colonies were able to survive several years without treatment (36, 71, 85). The mechanisms of naturally developed resistance are not described yet. Potentially reduced mite reproduction in naturally surviving colonies may be monitored in normal beekeeping conditions (71, 92).

Varroa control

The world's honey bees are in a huge decline, with millions of hives disappearing in recent years (44). This decline is of growing concern on account of the critical role honey bees play in maintaining natural plant communities and sustaining human and livestock food sources. Today, it is linked to multiple environmental stressors including but not limited to virus, bacteria or a parasitic infection, pesticide exposure, and poor nutrition, environmental variation, and the synergistic effects between these factors (42, 103, 112). It is known that Varroa can be devastating for honey bee colonies and no other honey bee pathogen or parasite has had a comparable impact on managed honey bees. Therefore, Varroa control has been an important part of maintaining the colony's health. Determination of Varroa infestation in honey bee colonies with an appropriate method that beekeepers can use to measure Varroa infestations in their hives is important for efficient mite control. There are various methods including genetic, mechanical, biological, and chemical for Varroa control. However, there is no single best method to control the Varroa population (Figure 1) (5, 98).

Mechanical control

Mechanical controls (Cultural or physical treatments) involve hive manipulations and interruption of the brood cycle in the management practices of beekeeping. These methods could be used as an alternative method based on not using chemicals to reduce mite levels. Due to their low effectiveness against Varroa, they must be used in combination with other control techniques each working in different ways (chemical treatment) in the

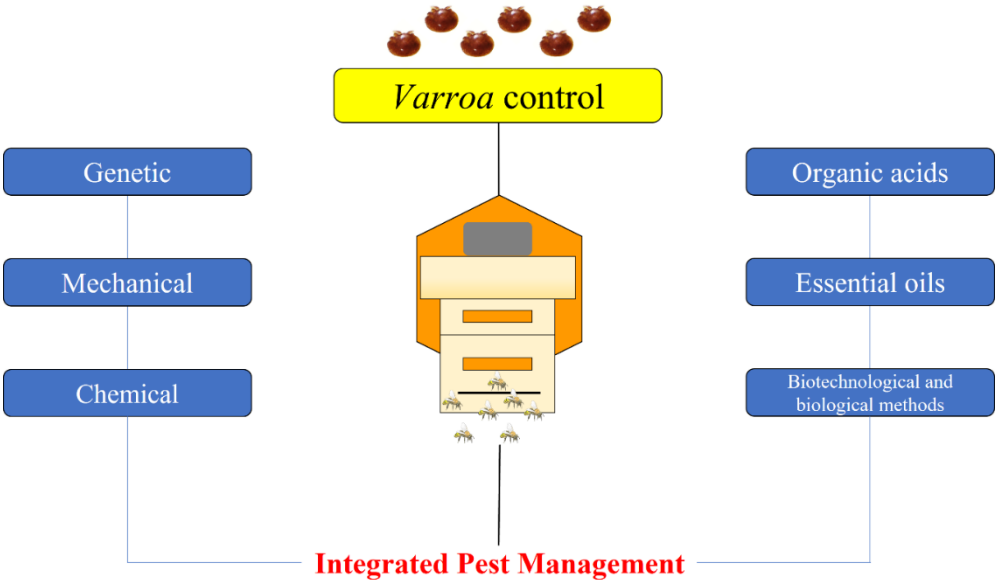

Figure 1. The design of different methods of Varroa control.

management practices of beekeeping. Mechanical controls include drone brood removal, screened bottom boards, dusting bees with powdered sugar, and manipulating brood (47). The most effective cultural and physical method is the partial removal of drone brood, where mites reproduce more often in comparison to worker broods, with average differences between five and 12-fold (21, 22). However, this method may not be practical for beekeepers who manage a large number of beehives as it is labor intensive. Also, this method can be only used in spring and summer when drone brood is present in the beehive (117). Drone brood removal significantly reduces the population of mites in colonies. However, it may not be as effective as chemical-based methods. Thus, it can serve as a useful component in an integrated Varroa control program and may reduce the need for other treatments on a colony-by-colony basis (17).

Other mechanical methods include screened bottom boards, and powdered sugar dusting. Sprinkling several non-toxic powdered materials, including glucose powder, ground pollen, wheat flour and baby powder, on honey bees stimulates grooming behavior which enables them to bite adult mites, and remove the mites from their bodies, resulting in the reduction of Varroa population in bee hives. However, it requires large quantities of high-priced materials and intense manipulation in beehives and leads to increased bee mortality. Also, it is not sufficient Varroa mite control on its own but can be used in conjunction with screened bottom boards for higher effectiveness (30, 32). Screen bottom board (sticky bottom board or open mesh floor) tray inserted from the back of the bee hive is used to reduce mite populations by preventing the return of Varroa, that fall from the bee cluster as a result of grooming or any other reason (73). This may be due to providing improved hive ventilation or the loss of mites falling to the bottom of the hive through the screened floor (98). Replacing solid wooden floor (Solid surface) with screened bottom boards (non-solid surface) in bee hives makes Varroa less likely to climb back or invade brood cells, resulting in a reduction in Varroa reproduction in brood cells (70). As a result, these mechanical methods show little effectiveness and should only be used as a supplement to more effective methods like chemical treatments.

Chemical control

Today, chemical control is of great importance to reduce the Varroa mite population in honey bee colonies and can be achieved by the use of various acaricides. All acaricidal chemicals used for controlling Varroa mites are called “Varroacides”. Synthetic acaricides including pyrethroids (tau-fluvalinate, flumethrine), formamidines (Amitraz), and organophosphates (Coumaphos) have been the major effective method used for years in the control of Varroa (1, 5). As a result of the widespread use of chemical-based drugs for Varroa control the beekeeping industry is facing two important public health issues. Firstly, the Varroa mites develop resistance to these chemicals when used repeatedly. The second major problem is the presence of chemical residues in bee hive substrates and products (97, 104). An increase in these problems has raised interest in treatment with non-synthetic substances. These control strategies are mostly based on the use of organic acids (formic acid, oxalic acid, lactic acid) and volatile oils (thymol, carvacrol and menthol) (88, 115). Due to their hydrophilic and volatile properties, they are unlikely to accumulate in the stored

honey, where they are able to migrate from the wax comb. Also, mites are unlikely to develop resistance to them (50, 97). However, single applications of organic acids may be insufficient for adequate Varroa control. Whereas chemical control, combined with mechanical control, can increase the efficacy of Varroa control (47).

Synthetic acaricides: In the last 20 years, the most commonly used synthetic acaricides against *V. destructor* are the organophosphate coumaphos, the pyrethroids tau-fluvalinate and flumethrin, and the formamidine amitraz (72). Coumaphos has been widely used for many years as an active ingredient to control ectoparasites on cattle, goats, sheep, and honey bees. Veterinary medicinal products (Checkmite, Perizin) containing coumaphos impregnated in plastic strips have used to control Varroa in the beehives by hanging them between frames for the allotted time, which is approximately 45 days. It is a phosphonothioate proinsecticide requiring in vivo bioactivation by cytochrome P450 monooxygenases to its active phosphate metabolite coroxon, which is selectively toxic to insects through inhibition of acetylcholinesterase (AChE) but far less toxic to mammals (65, 120). It had high varrocidal activity against Varroa in the first years of its use, but recently drastic decreases in varrocidal activity have occurred. The reduction in its efficacy has been proposed to be related to the development of resistant strains of Varroa (56).

The synthetic pyrethroid acaricides flumethrin (Bayvarol) and tau-fluvalinate (Apistan) are registered treatments against Varroa in most countries in the world (2, 104). They affect both the peripheral and central nervous system of the insects by modifying the ion channels (especially sodium channels) in neuronal membranes. They also induce excessive sensory hyperactivity of the peripheral nervous system and muscle spasms (57, 110). They have relatively low toxicities to honey bees and are easy to use. Plastic strips impregnated with tau-fluvalinate and flumethrin are inserted between combs in bee hives. While synthetic pyrethroids initially showed high acaricide performance, individuals resistant to these acaricides appeared due to the adaptation potential of the mite (41, 56, 67, 109). Amitraz, a formamidine acaricide proposed to activate octopamine/tyramine receptors, is widely used to control mite and tick infestation of domestic animals. Amitraz impregnated strips have been used for Varroa control in honey bee colonies for over twenty years (48, 68). Extensive use of amitraz has resulted in high levels of resistance in some areas and their treatment failures (93).

Organic acids: Overuse and misuse of synthetic acaricides have caused the development of resistance in Varroa populations, therefore, beekeepers have turned to alternative treatments (104). As alternatives to conventional treatment methods using acaricides, organic acids such as formic acid and oxalic acid, and essential oils such as thymol are available for Varroa treatment (46, 115). Organic acids such as acetic, citric, formic, oxalic and many others are present in honey's composition in small quantities and can play an important role in controlling Varroa infestation (102). Oxalic acid and formic acid are the most widely used organic acids for Varroa control with lactic acid use less common. There are significant differences between these acids in terms of application, concentration and amount used. Although many commercial preparations are available, beekeepers apply them empirically using different methods whose efficacy has not been tested. Therefore, care should be taken when applying untested and non-commercial methods (116, 118).

Oxalic acid, which is an organic compound with the formula C2H2O4, in the form of crystals, gelatin capsules or tablets is heat-evaporated and used predominantly during the broodless period (91). Using a syringe or similar applicator, oxalic acid dihydrate is normally trickled directly on the bees in the spaces between the combs. The application is quick, cheap, and easy (113). Different concentrations of oxalic acid have been used for control of Varroa mites due to the climatic conditions of the European regions. Notably, a higher concentration of oxalic acid was shown to be more effective in a southern climate (80, 81). In a northern climate, a lower concentration of oxalic acid was found to be more suitable (38, 91).

Formic acid, which a colorless liquid that fumes, is effective both against phoretic and reproductive phases of the mites (90). It was approved for use on mites in gel-pack formulation (pad) and liquid formulations being administered in bee hives using different evaporators. The pad containing formic acid is applied by placing it on the upper bars of the brood box. As a fumigant, formic acid vapors generated by heating to a high temperature are released into the beehive (4, 33). The effectiveness of formic acid for the control of Varroa varies considerably based on several factors such as the distance from the place where formic acid is applied to the honeycombs, the place where it is placed in the hive, the amount of brood in the hive, time of year, and the ambient temperature (18, 29, 116).

Essential oils: Essential oils, extracted from various parts of medicinal and aromatic plants using several methods such as hydrodistillation or steam distillation, are complex mixture of volatile aromatic compounds (6). Essential oils represent an alternative and useful tool to control Varroa due to having high toxicity to mites, low toxicity to bees and mammals, and a low environmental impact. Essential oils can be incorporated into an Integrated Pest Management program, reducing the use of synthetic drugs (62). While many essential oil

components, including thymol, carvacrol, citral, menthol, and Tau-ßuvalinate, have varroacidal activity, thymol is an ingredient that has rapidly risen in popularity (115). Active ingredient Thymol (Thymovar, Apiguard), is a product of thyme oil. It is an essential-oil based product effective against Varroa and exerts its bioactivity in a way that is most likely mediated by neurological mechanisms (9). It can be used both alone or in combination with other varroacidal agents and biotechnological applications such as brood removal (19, 45). Thymol is used in the form of tray (coated slow-release gel) and impregnated cloth sponge strip for the control of Varroa.

Biotechnological and biological methods

There are several biological ways for control and treatment of *V. destructor*. These are investigated for biocontrol of honeybees against some ectoparasites including *V. destructor*.

Biotechnological approach: This method is a combination of mechanical technique and application of organic molecules. The main aim is to reduce the population burden of *V. destructor* with using total interruption of brood, removing all drone brood, caging the queen and adding trapping combs (16, 40, 91). Unfortunately, these methods are not effective in every region in the world. For instance, interruption of brood has different results for Europe and USA (63). The methods should be reconsidered according to the regions. Sugar shake, which is another method used for diagnosis, decreases the pressure of the number of mites on the colony without any serious harm. Although these are effective methods to reduce the population pressure of *V. destructor*, they are not desirable because they can lead to honey losses and are difficult to apply.

Predator Animals: Predators in nature can be another alternative in the treatment of *V. destructor* biologically. For this, various species have been investigated for their potential benefit in treatments. One of these beneficial species is pseudoscorpions which feed on ectoparasites in hives causing no harm to honeybees (31, 119). They use their venom to paralyse and kill the mites (122). Different kinds of species have been identified, such as *Nesochernes gracilis* and *Chelifer cancroides*. Although some of them are beneficial to bees, this does not mean that all can have beneficial effects on the colony. Pseudoscorpions can be considered as a long-term solution for treatment against the *V. destructor*. However, their actual benefit is not entirely known and investigated thoroughly, and it is required to conduct field experiments. Another candidate for controlling *V. destructor* is another mite called *Stratiolaelaps scimitus*. However, research has shown that they sometimes prefer feeding on the honeybee eggs to the mite (96). Furthermore, there are also some field experiments revealing that they did not have enough effect to decrease the mites during early and late fall in the colonies. An efficient and desirable natural predator would have to eat the eggs of ectoparasites or the larvae directly to become a potential biological control candidate (20).

Microbiota: In the case of honeybees, worker guts from *V. destructor*-infested colonies have a high increased population of *Snodgrassella alvi* and a lower reduced population of *Lactobacillus spp.*, in comparison to un-infested colonies, showing that the acari has altered their microbiome (58, 74). Furthermore, un-infested larvae have an increased population of Enterobacteriaceae, whereas infected larvae have a diverse microbiota comparable to that of ectoparasites (26). As a result, the microbiota provides a new avenue for combating the ectoparasite. One of them believes that transgenic gut bacteria should be used for biocontrol. The symbiotic bacterium, *S. alvi*, from the honeybee gut can produce genes (12) that are transferred by conjugation to gut microbiota. Varroa feeding with designed bacteria expressed higher mortality in comparison to controls. It is, therefore, demonstrated that bees' gut bacteria can contribute to the better survival of parasitized honey bees.

Pathogens: *Bacillus thuringiensis*, an entomopathogenic microbe, is commonly employed as a bioinsecticide in agriculture (12, 66). It infects the host by ingesting a protein termed Cry, which creates crystals and vegetative poisons. *B. thuringiensis* was found on *V. destructor* corpses in *in vitro* investigations, and it was removed to assess its pathogenicity in mites and honeybees. It was found that *V. destructor* shook, regurgitated, suffered intestinal inflammation, and died after being treated with *B. thuringiensis* for 24 hours. Short-term exposure to *B. thuringiensis* had no lethal effects on *A. mellifera* adults or larvae, and it may have reduced vertical displacement, whereas chronic exposure to *B. thuringiensis* caused precocious mortality in both adults and larvae bees.

Entomopathogenic fungi, which destroy acarine species, were used in a number of experiments in addition to bacteria. Conidia are specialized spores that entomopathogenic fungi use to proliferate into their hosts. A lack of nutrients, water stress, toxic effect, and mechanical disruption require 3 to 10 days to kill the host. The fundamental disadvantage of fungus and bacteria, despite the lack of long-term data, is the low specificity of their toxins and the difficulty in colonizing and surviving in the hive ecology. Furthermore, from an evolutionary standpoint, the honeybee and the ectoparasite are related, making it difficult to isolate one from the other (3, 114).

Integrated Pest Management (IPM)

V. destructor is one of the most serious biological hazards to the health of western honey bees (*A. mellifera*

L.) globally. IPM is a method of keeping a pest or parasite population under control. Through the coordinated application of one or more procedures, an economic threshold can be reached. If the parasite density reaches a certain level, economic damage (loss of honey output or colony death) can be expected. Using chemical treatments and antibiotics need to be reduced in IPM programs, and they are avoided wherever possible. Minimizing chemical treatments ensures the integrity of hive products, increases the time it takes for parasites to develop resistance to treatments, and reduces the risk of detrimental effects on bees and the environment. The basic purpose of cultural control is to alter the hive environment to make it less conducive to the pest or illness while causing the honey bees as little harm as possible. Mechanical control includes pest control by physical methods or mechanical devices such as equipping the honeycomb with a sieve bottom plate, trapping broodstock with drones or heat treatment. These nonchemical approaches are considered essential for long-term, sustainable solutions to Varroa control (97).

The traditional definition of biocontrol is a pest management tactic that involves antagonistic organisms to reduce pest population densities (89). Varroa control is most commonly attempted using chemical treatment, though, within an IPM paradigm, the chemical application should be used in combination with other pest management methods to keep pest populations below economic injury levels or nuisance thresholds (34). Varroa mites continue to be a major issue for beekeeping despite efforts to control the problem. Sustainable control of Varroa is difficult to achieve using a single control approach, instead, it can be achieved by integrating multiple control approaches for maximum efficiency. However, because our understanding of how Varroa/virus transmission affects honey bees is low and our current economic threshold is narrow (2 vs > 3 mites/100 bees), it should be fair to consider IPM even as a viable approach to controlling Varroa (64).

Conclusions

V. destructor mite is the main pest that affects the health of developing and adult honey bees and, consequently, the entire honey bee colony. Honey bees have evolved individual and collective anti-parasite defense system including behavior systems in order to remove parasites from their body surface or from the parasitized brood. A variety of diagnostic and control methods including integrated Varroa control management practices are implemented in beekeeping operations. Diagnostic methods are used to establish the infestation levels in honey bee colonies in order to apply proper control methods, to minimize the use of chemically based acaricides. Sustainable control measures can be applied in organic and conventional beekeeping operations. The treatment methods include organic acids and essential oils. Applied IPM programmes combine a controlled and measured use of chemical treatments in order to ensure the integrity of hive products, lessen the risk of parasite resistance against acaricides developing and reduce the risk of detrimental effects on bees and the environment. To achieve sufficient efficacy of control treatments, applied biotechnical methods and the acaricidal substances need to be used in accordance with control protocol including, optimal time. Using recommended control methods, beekeepers avoid damage to adult bees and brood. In order to maximize mite control efficacy and to ensure production quality and safety of honey bee products, beekeepers need to consider seasonal treatment effects, medicinal product rotation and efficacy in Varroa control.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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