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RESEARCH ARTICLE

The Role of Antral Follicle Count in OPU/IVEP Donor Selection in Holstein Cattle

Muhammed Furkan Çiftçi[1*], Maide Gölbaşı[2], Ömer Faruk Yeşilkaya[1], Ayşe Sarı[3], Sakine Ülküm Çizmeci[1], Dursun Ali Dinç[1]

[1]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Selcuk University, Konya, Türkiye
[2]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Muğla Sıtkı Kocman University, Muğla, Türkiye
[3]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Necmettin Erbakan University, Konya, Türkiye

ABSTRACT

The aim of this study was to determine the effect of antral follicle number on oocyte yield and IVEP in Holstein breed donors. The study groups were constituted following ultrasonographic examinations conducted on 23 Holstein heifers at one-week intervals. The Low-AFC group (n = 8) comprised animals with an average antral follicle counts in the ovary of ≤ 15, as determined by ultrasonographic examination. The High-AFC group (n = 9) comprised animals with an average antral follicle number of ≥30. Oocytes were collected from animals in both groups four times, one week apart, any day of the estrous cycle. The oocytes were classified according to their morphological characteristics and subsequently incorporated into the in vitro embryo production process. The total and viable oocyte numbers were found to be significantly greater in the High-AFC group than in the Low-AFC group ($P < 0.01$). The number of blastocysts obtained per session was also greater in the High-AFC group ($P < 0.01$). No statistically significant difference was observed between the blastocyst and viable oocyte rates in the two groups. Consequently, oocytes and blastocysts were obtained from Holsetin donors with high antral follicle counts in greater numbers. It was thought that the number of antral follicles in Holstein breed donors could be employed as a parameter in donor animal selection to increase the success of OPU/IVEP.

Key Words: Antral follicle counts, Donor selection, OPU/IVEP.

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Holstein Sığırlarda OPU/IVEP için Donör Seçiminde Antral Folikül Sayısının Rolü

ÖZ

Bu çalışmada Holstein ırkı donörlerde antral folikül sayısının oosit verimine ve in vitro embriyo üretimine etkisinin belirlenmesi amaçlandı. Çalışma grupları 23 baş Holstein ırkı düveye 1 hafta arayla yapılan ultrasonografik muayeneler sonrasında oluşturuldu. Ultrasonografik muayene sonrasında ovaryumundaki ortalama antral folikül sayısı ≤ 15 olan hayvanlar Düşük-AFS grubuna (n=8) alındı. Ortalama antral folikül sayısı ≥ 30 olan hayvanlar ise Yüksek-AFS grubuna (n=9) alındı. Her iki gruptaki hayvanlardan östrüs siklusunun rastgele bir gününde birer hafta arayla 4 kez oosit toplandı. Toplanan oositler morfolojik özelliklerine göre sınıflandırılarak in vitro embriyo üretim sürecine alındı. Toplam ve in vitro embriyo üretimine uygun oosit sayısı Yüksek-AFS grubunda Düşük-AFS grubundan yüksek bulundu ($P < 0.01$). Seans başına elde edilen blastosist sayısı da Yüksek-AFS grubunda daha fazlaydı ($P < 0.01$). Blastosist oranı ve in vitro embriyo üretimine uygun oosit oranı iki grup arasında istatistiksel farklılık göstermedi. Sonuç olarak Holstein ırkı donörlerde yüksek antral folikül sayısına sahip hayvanlardan daha fazla sayıda oosit ve blastosist elde edildi. Holstein ırkı donörlerde antral folikül sayısının OPU/IVEP başarısını artırmak için donör hayvan seçiminde bir parametre olarak kullanılabileceği düşünüldü.

Anahtar Kelimeler: Antral folikül sayısı, Donör seçimi, OPU/IVEP

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ORCID ID; *MFÇ: 0000-0001-8333-6500 MG: 0000-0002-0218-8313 ÖFY: 0000-0002-7721-2576 AS: 0000-0003-4181-9509 SÜÇ: 0000-0003-2939-8019 DAD: 0000-0002-9597-227X*

*Corresponding author e-mail:: mf.cftc@gmail.com

INTRODUCTION

Reproductive biotechnology methods are often used in cattle breeding to increase the number of animals with superior genetic characteristics (Berglund 2008). Embryo technologies make it possible to rapidly change the genetic composition of a herd by reducing the generation interval (Mapletoft and Hasler 2005). Bovine embryos are produced in vivo and in vitro. In recent times, there has been a gradual decline in the number of embryos produced in vivo, while there has been a corresponding increase in the number of embryos produced in vitro (Watanabe et al. 2018; Viana 2022). The advent of the OPU (ovum pick-up) technique has been a significant factor in the increased production of embryos in vitro (IVEP). The OPU technique allows oocytes to be retrieved from highly productive donors on multiple occasions within a short period of time (Pieterse et al. 1988; Boni 2012).
A number of studies are currently being conducted in different areas with the objective of increasing the oocyte yield obtained from cattle (Demissie et al. 2022; Saleem et al. 2022). The selection of donor animals is of significant importance in order to collect more oocytes in a short time. To increase the number of oocytes retrieved, donors with a high oocyte yield should be used. In embryo production, criteria such as genetics and productivity characteristics are generally focused on for donor animal selection (Mebratu et al. 2020). However, genetically superior donors may not have high oocyte yields. Therefore, evaluating parameters related to oocyte yield in donor selection for OPU/IVEP may increase success rates. It has been found that the anti-Müllerian hormone (AMH) level and the antral follicle count (AFC) provide an indication of the oocyte yield of donor animals (Garcia et al. 2020; Feres et al. 2024). AMH and AFC are positively correlated, making them useful criteria for donor selection (Ireland et al. 2010; Zangirolamo et al. 2018). There is considerable variation in AFC and serum AMH levels between cattle, but these reproductive parameters are highly consistent within the same animal. While AMH measurement requires lab analysis, AFC can be evaluated through ultrasound of both ovaries by a experienced operator (Silva-Santos et al. 2014; Morotti et al. 2018). For this reason, AFC is a more economical parameter for donor animal selection. The AFC represents the number of follicles with a diameter of at least 3 mm that can be observed in the ovary (Morotti et al. 2018). The primary source of oocytes in OPU applications is follicles with a diameter of 3–8 mm (Dieleman et al. 2002, Hendriksen et al. 2004). Accordingly, it is postulated that the oocyte yield of animals with a high AFC will be higher. Therefore, AFC emerges as a critical parameter in the selection of donors for OPU/IVEP procedures (Lollato et al., 2022).

It has been demonstrated that the AFC is one of the most effective techniques for evaluating reproductive efficiency in Bos taurus cattle breeds. Although AFC reveals individual differences between animals, it is of great importance in assessing the future reproductive performance of the same animal (Burns et al. 2005). In addition, there is research indicating that the effect of AFC on reproductive efficiency differs between Bos taurus and Bos indicus cattle breeds. Therefore, further studies are needed to determine the suitability of AFC for the selection of donors for OPU/IVEP in a variety of bovine breeds (Dos Santos et al. 2016, Garcia et al. 2020).
The aim of this study was to determine the effect of AFC on the yield of oocytes and blastocysts in Holstein breed heifers.

MATERIALS and METHODS

The study animals were a selection of 23 Holstein heifers from a farm that had genomic selection in place. The animals were between 14 and 18 months old and exhibited body condition scores ranging from 3.0 to 3.5. The animals did not have any health problems. The animals were kept under the same conditions and fed ad libitum. The ration comprised alfalfa silage, hay, corn silage, concentrate feed and alfalfa.

Experimental Design

Ultrasound scans were performed on 23 Holstein heifers 4 times at 1-week intervals to form the study groups. The AFC in the animals' ovaries was recorded during ultrasound scans. Animals with an average number of antral follicles in the ovaries ≤ 15 were included in the low antral follicle count (Low-AFC) donor group. Animals with an average number of antral follicles in their ovaries ≥ 30 were included in the donors with high antral follicles (High-AFC) group. The study excluded other animals.
Oocytes were retrieved from donors in the Low-AFC (n=8) and High-AFC (n=9) groups by transvaginal ultrasound 4 times, starting any day of the estrous cycle, 1 week interval. The experimental design is schematized in Figure 1.

Ovum-Pick Up

For oocyte collection, a catheter-aspiration device (230 V, Minitube), 4.0-9.0 MHz microconvex probe (Esaote, SC3123 VET) and real-time ultrasonography device (Esaote MyLab TwiceVet, 5001) were used. Aspiration vacuum pressure was 80-90 mm/Hg during oocyte retrieval. A catheter and a microconvex vaginal probe was used to aspirate all follicles larger than 2 mm from the ovary (20-gauge needle).

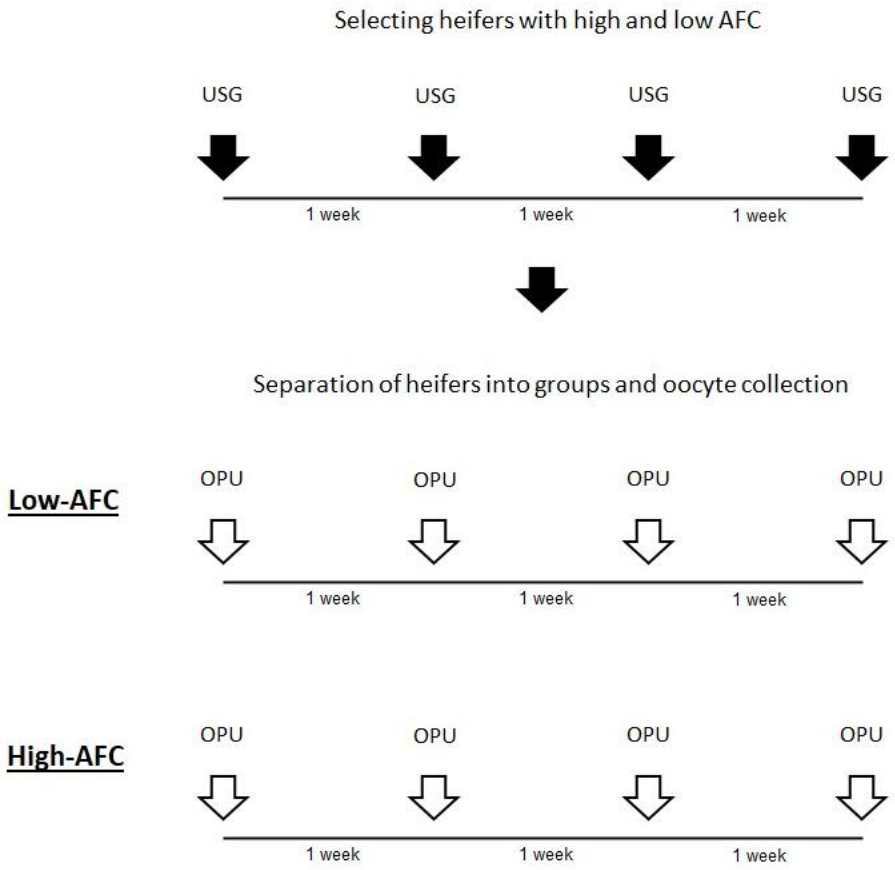

Figure 1. Separation of the heifers into groups and design of the experiment (AFC: Antral Follicle Count, USG: Ultrasound-Guided Antral Follicle Counting, OPU: Ovum - Pick Up).

Cumulus Oocyte Complex Classification

Cumulus oocyte complexes (COCs) were examined under a stereomicroscope (S ApoE, Leica). Morphological features were used to classify COCs. COCs were evaluated for morphological characteristics, including cytoplasm homogeneity, number of cumulus cell layers, and cumulus cell density. COCs were classified according to their quality. It was rated as very good, good, fair, or poor (respectively Grade A, B, C and D) (Petyim et al. 2003; Gordon 2003). A, B and C quality oocytes were included in the IVEP process. COCs with homogeneous cytoplasm, uniform appearance and at least one layer of compacted cumulus cells were classified as viable (Hayden et al. 2022).

In vitro Embryo Production

In vitro embryo production was performed using media produced by a commercial company (IVF Bioscience). After oocyte collection, the COCs that were suitable for IVEP were washed three times in the oocyte washing medium (BO-Wash). The COCs were then incubated in an in vitro maturation medium (BO-IVM) at 38.5 °C and 5.5 % CO_2 for 20 - 22 hours. For in vitro fertilization (IVF), semen from the same sire was used throughout the experiment. Sperm washing was performed with semen preparation medium (BO-SemenPrep). The number of spermatozoa was calculated as 1 million per mL. Spermatozoa were then added to in vitro fertilization medium containing COCs (incubated at 38.5°C, 5% CO_2 for 20 hours). After IVF, prospective zygotes were vortexed and transferred to in vitro culture medium (BO-IVC) coated with mineral oil (BO-Oil). It was cultured in culture medium at 38.5°C, 6% CO_2 and 6% O_2 for 7 days. Embryo quality and developmental stages were assessed according to the International Embryo Transfer Society (IETS) (Bó and Mapletoft 2013; Alkan et al. 2023).

Statistical Analysis

The data were analysed with the SPSS 25.0 statistical software (IBM Corp. Released 2017. IBM SPSS Statistics for Windows). *Shapiro–Wilk* test was used for the prerequisites of homogeneity of variances and normality of variables. The mean ± standard deviation (SD) of normally distributed variables was presented. The variables were evaluated with t-test. Blastocyst rate were analysed by Chi-square test. At the 95% confidence level ($p<0.05$), the differences were considered statistically significant.

RESULTS

Oocyte and blastocyst yield from donors with high and low AFC are shown in Table 1. The oocytes counts of the highest quality (Grade A) obtained following oocyte retrieval was significantly greater in the High-AFC group than in the Low-AFC group ($P < 0.01$). Furthermore, the number of oocytes exhibiting quality grades B, C, and D was also greater in the High AFC group ($P < 0.01$). Additionally, the study revealed that the number of viable oocytes suitable for IVEP was statistically higher in the High AFC group than in the Low AFC group. Nevertheless, no statistically significant difference was observed in the rate of viable oocytes between the two groups. Oocytes counts of all qualities was found to be statistically higher in the High-AFC group, as demonstrated by the results of the study. Accordingly, the total number of oocytes obtained from animals with high AFC was also higher ($P < 0.01$). Blastocysts counts obtained per OPU was approximately twice as high in the High-AFC group as in the Low-AFC group ($P < 0.01$). No statistically significant difference was observed in the blastocyst rate between the two groups.

Table 1. Post-OPU oocyte and blastocyst data of Holstein heifers with high and low AFC (mean ± standard error of mean, Viable oocyte rate: viable oocyte number/total oocyte number, Blastocyst rate: blastocyst number/viable oocyte number, OPU: Number of OPU sessions).

Variables	Low-AFC	High-AFC	P Value
Grade A oocytes / %	1.28 ± 0.24 / 21.13	3.58 ± 0.49 / 24.42	< 0.0001
Grade B oocytes / %	1.50 ± 0.25 / 24.74	3.19 ± 0.52 / 21.61	0.007
Grade C oocytes / %	1.75 ± 0.29 / 28.86	3.66 ± 0.38 / 24.81	< 0.0001
Grade D oocytes / %	1.53 ± 0.29 / 25.25	4.33 ± 0.63 / 29.32	< 0.0001
Viable oocytes/OPU	4.53 ± 0.46	10.52 ± 1.06	< 0.0001
Viable oocytes rate (%)	74.74	70.67	-
Total oocytes/OPU	6.06 ± 0.51	14.86 ± 1.52	< 0.0001
Blastocysts/OPU	1.34 ± 0.18	2.58 ± 0.29	0.001
Blastocyst rate (%)	29.65	24.46	-

DISCUSSION

Counts and quality of collected oocytes affect IVEP success rates. Increasing oocyte yield increases the number of blastocysts obtained per OPU. Oocyte yield varies considerably even within the same breed. It is therefore crucial to select suitable donors in order to increase the success of OPU/IVEP. One of the most practical methods for evaluating the oocyte yield AFC was higher when synchronization was applied prior to OPU. In a separate study examining the impact of AFC on oocyte yield, it was demonstrated that there was a positive correlation between the total and viable oocyte numbers and AFC (Dos Santos et al. 2016). In a comparable study, Monteiro et al. (2017) of donor animals is antral follicle count. A study investigating the association between AFC and reproductive efficiency found a positive correlation between the two variables (Mossa et al. 2012). García et al. (2020) found in their study that the total and viable oocyte numbers obtained from donors with high AFC were higher. Furthermore, the study revealed that the oocyte yield of donors with a high reported that they collected a greater number of oocytes from donors with a high AFC. Viana et al. (2003) posited that ovarian damage and the formation of scar tissue may be heightened in donors with a high AFC due to the increased number of follicle punctures. It has been stated that this situation may negatively

affect oocyte yield in animals with high AFC in repeated OPU applications. The results of the presented study indicate that a greater number of total and viable oocytes were obtained from Holstein breed heifers than from donors with a high AFC. Furthermore, oocytes counts of all qualities in the high AFC group was observed to be higher than in the low AFC group. The results were in line with those of previous studies, which indicated that a greater number of oocytes could be harvested from donors with a higher AFC.

For IVEP, blastocysts counts obtained per OPU and the blastocyst rate are as important as oocyte yield. A study evaluating the effect of antral follicle population on in vitro embryo production (IVEP) found that the number of blastocysts obtained per OPU from donors with a high AFC was higher than from donors with a low AFCAdditionally, the study demonstrated that there was no statistical difference in the rates of oocytes collected from animals with high and low AFC reaching the blastocyst (Guerreiro et al. 2014). Monteiro et al. (2017) reported that the antral follicle population influences the number of blastocysts obtained per OPU. The study by Silva-Santos et al. (2014) exhibited that the number of embryos obtained per OPU and vitrifiable embryos were greater in donors with high AFC. Furthermore, the study demonstrated that a greater number of blastocysts were obtained in the group with a high AFC, yet the cleavage rate remained comparable. Ireland et al. (2007) observed that embryos obtained from animals with a high AFC was four times greater than that obtained from animals with a low AFC. In the presented study, a higher number of blastocysts per OPU was obtained in the high AFC group. But there was no statistical difference in blastocyst rates between the groups. It was thought that this discrepancy was due to the fact that, although a greater number of viable oocytes were collected in the high AFC group, the rate of viable oocytes was similar.

CONCLUSION

Antral follicles counts affected the oocyte yield of donor animals. In OPU applications, more oocytes were collected from donors with high AFC. Viable oocytes counts collected in the high-AFC group was also greater. However, viable oocyte rate and blastocyst rate did not show any statistical difference between the two groups according to AFC. It was thought that using of donors with high AFC could increase the success of OPU/IVEP. It was concluded that AFC in Holstein breed donors can be used as a parameter in donor animal selection.

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Ethics approval and consent to participate: This study was approved by the Local Ethics Committee (Selcuk University Local Ethics Committee for Animal Experiments, Approval Number: 2024/02/30). All applied methods are in accordance with the ARRIVE guidelines.

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RESEARCH ARTICLE

Comparison of *İn-Vitro* Digestibility of Commonly Used Forage and Concentrate Feeds in Dairy Buffalo, Cow and Sheep by Using Daisy Incubator

Aamir IQBAL[1]*, Ümit ÖZÇINAR[2], Syed Rizwan Ali SHAH[2], Abdul QUDOOS[2], İbrahim Sadi ÇETİNGÜL[2], E. Eren GÜLTEPE[2], İsmail BAYRAM[2]

[1]Department of Livestock and Poultry Production, Faculty of Veterinary and Animal Sciences, University of Poonch Rawalakot, Azad Kashmir, Pakistan

[2]Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Türkiye

ABSTRACT

In this study was evaluated in-vitro true dry matter (DM) digestibility of 8 different feedstuffs by using in vitro system. Eight different feeds were grouped as roughage and concentrate and tested by using Buffalo, cow and sheep inoculums.. The experiment was replicated on three different timelines for all feeds and the three inoculum sources. The incubation time for the digestibility was 48 hours for each species inoculum. In the study, rumen fluids from different animals did not have a significant effect on the in vitro true? dry matter (DM) digestibility of different feedstuffs during the 48-hour incubation period (P>0.05). The pellet wheat bran showed the lowest mean value in sheep among all feedstuffs. It was concluded that the daisy incubator method could be used to predict the true digestibility of different feedstuffs in different species animals.

Keywords: Concentrate, daisy, dry matter, digestibility, feed, ruminant

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Sütçü Manda, İnek ve Koyunlarda Yaygın Olarak Kullanılan Kaba ve Konsantre Yemlerin *İn-Vitro* Sindirilebilirliğinin Daisy İnkübatör Kullanılarak Karşılaştırılması

ÖZ

Bu araştırmada, manda, inek ve koyunlarda in vitro sistem kullanılarak 8 farklı yem maddesinin in vitro gerçek (NDF) sindirilebilirliği değerlendirilmiştir. Yemler, kaba ve konsantre olarak gruplandırıldı ve manda, sığır ve koyun rumen sıvıları kullanılarak test edildi. Araştırmada, tüm yemler için, üç tekerrür, üç farklı inokulum kaynağı (rumen sıvısı) kullanıldı. Sindirilebilirlik için inkubasyon süresi her tür rumen sıvısı için 48 saatti. Araştırmada, farklı hayvanlara ait rumen sıvılarının, 48 saatlik inkubasyon süresi boyunca yem maddelerinin in vitro gerçek? kuru madde sindirilebilirliği üzerinde önemli bir etkisi olmamıştır (P>0.05). Pelet buğday kepeği, sindirilebilirlik değeri açısından, diğer yem maddelerine göre koyunlarda en düşük değeri vermiştir. Sonuç olarak, in vitro sindirilebilirlik deneme yönteminin, farklı hayvan türlerinde yemlerin gerçek sindirilebilirliğini tahmin etmek için kullanılabileceği kanısına varılmıştır.

Anahtar kelimeler: Daisy, kuru madde, sindirilebilirlik, ruminant, yem

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ORCID ID; *AI: 0000-0003-4473-2329, ÜÖ: 0000-0002-1143-1215, SRAS: 0000-0002-2752-4854, AQ: 0000-0002- 7112-329X, İSÇ: 0000-0002-7608-6176, EEG: 0000-0002-2404-1232, İB: 0000-0002-9993-7092*

*Corresponding author e-mail: aamir_vet@yahoo.com

INTRODUCTION

The rumination and peristalsis of the gastrointestinal tract are triggered by the fibers included in the animal's diet. Chewing lessens the particle size of the engulfed feed, enhances the microbial attachment by improving the surface area, and maintains the rumen pH by affecting saliva production (Van Soest, 1994). Better peristalsis ensures the polite rumen movements for effective digestibility of the feedstuffs. It provides a homogenous environment for good bioavailability by particle retention and efficient gut motility by the outflow from the rumen (Van Soest, 1994). The particle size, density, palatability, freshness, and digestibility of fibers are physical parameters that control the rumen fill and dry matter intake (DMI) (Conrad et al., 1964). The DMI and gut filing are a more conscious determinant when *ad-libitum* feed is offered and during the first lactation phase (Allen & Piantoni, 2014). Rumen fill promotes rumination by stimulating the pressure and stretching receptors in the reticulum and rumen wall (Allen & Piantoni, 2014). Moreover, the effect of amylase and sodium-treated with ash correction ($aNDF_{om}$) digestibility on dry matter intake was studied by Kendall et al. (2009). The main fibrous components in feedstuffs are cellulose, hemicellulose, and pectin. Even after a long time, the protein that remains indigested by ruminal microorganisms is subtracted from the NDF to measure the potentially digestible NDF (Nousiainen et al., 2004). It is very important to measure the total tract digestibility (Huhtanen et al., 2006), rumen fill (Krizsan & Huhtanen, 2013), and DMI (Cotanch et al., 2014). For some dynamic rumen models such as Cornell Net Carbohydrate and Protein System (CNCPS), indigestible NDF is a helping tool (Fox et al., 2004; Tylutki et al., 2008; Van Amburgh et al., 2015). Lignin is understood as a major fraction of indigestible fiber (Besle et al., 1994).

With the advancement in human population, climatic circumstances, and scarcity of water resources, animal feed is being sold at high prices in many countries (Ajila et al., 2012). The agricultural by-products found after processing fruits, vegetables, crops, and nuts are valuable resources that overwhelm this threat (Rojas-Downing et al., 2017). By-product feedstuffs are more abundant and economical energy and fibrous sources for livestock (Devendra & Sevilla, 2002). Subsequently, the animals could be fed effectively without disturbing the human need for food (Odum et al., 2018). The in vitro methods are efficiently used to evaluate the quality and digestibility of the different feedstuffs offered to the ruminants (Getachew et al., 1998). The most precise and applied research technique available for accessing ruminant digestibility (Goldman et al., 1987). The strategy has been modified and adjusted for starch feedstuff examination (Holden, 1999). A few analysts have value-added its calculation precision (Mabjeesh et al., 2000). Distinctive dilution buffers for the rumen alcohol have been created to alter the pH of the inoculum (Tylutki et al., 2008).

The display considers assessed the in-vitro genuine $aNDF_{om}1$ digestibility of diverse feedstuffs for buffalo, cattle, and sheep after 48 hours of incubation (% of NDF/dry matter). This study was carried out to measure their in vitro (real) digestibility of different by-product feeds by using different ruminant inoculants.

MATERIAL and METHODS

The study was conducted at the dep. of Animal Nutrition, Faculty of Veterinary Medicine, University of Afyon Kocatepe. All procedures were approved by the local ethics committee (No: 495337002-07, Date: 14/01/2019).

Feedstuff Collection and Sample Preparation

Eight different feedstuffs were selected for the study, including dried tomato leaves (coarse), dried tomato leaves (fine), hazelnut, pellet wheat bran, grape pulp silage, biscuits, bulgur bran pellet, and poppy seed meal. The feedstuffs were obtained from the local livestock farms in Afyonkarahisar, Turkey. The rumen fluid for the in vitro incubation was obtained from the cannulated dry dairy cattle and dry dairy buffalo in the Education Research and Practice Farm, Faculty of Veterinary Medicine, Afyon Kocatepe University. Rumen fluid of sheep was obtained from the local slaughterhouse after immediately slaughtering.

The small part of the feeds was grounded separately by using Variable Speed Rotor Mill Pulverisette 14 Premium. All of the samples were dried in a hot air oven. Grape pulp silage was put in the oven at 65-70 °C 48 h, and for other concentrate samples, 100-105 °C for overnight was provided. The dry matter (DM) values of the feedstuffs are determined by gravimetric analysis according to the Affiliation of Official Expository Chemists, 1997 (Official Strategies of Examination, 16th ed. (AOAC Universal: Washington, D.C.)

Daisy Incubator Filter Bags Preparation

After calculating the dry matter, F57 filter bags (ANKOM, Macedon, Unused York; U.S) were pre-rinsed in filtered acetone for three to five minutes and air-dried to maintain a strategic distance from underestimation of NDF assimilation of scrounge tests in ANKOM F57 packs (Adesogan 2005). Moreover, the acetone wash evacuates a surfactant that represses microbial assimilation. Each pack was labeled with a dissolvable solvent-resistant marker. The empty weight of each F57 bag was recorded. Each samples was weighed and recorded (0.25g~0.5 g) and heat-sealed employing a 200 mm Parker IS/7300H motivation sealer. One purge fixed clear sack was utilized for the redress calculation.

Buffer arrangement was based on that of Tilly and Terry (1963). (Table 1) with McDougall-manufactured spit modifications (last pH 6.8 at 39°C). In partitioned holders, 266 ml of arrangement B and 1330 ml of arrangement A were arranged agreeing to the equation concentration of the reagents. A rise to the sum of Buffer arrangements A and B was included in all of the four assimilation jugs with tests. Eight samples were picked for the … (IVNDF) digestibility, and two tests were included for each jolt.

Table 1. Solutions

Buffer Solution A reagents	**Quantity, g/ liter**
KH2PO4	10
MgSO4•7H2O	0.5
NaCl	0.5
CaCl2•2H2O	0.1
Urea (reagent grade)	0.5
Buffer Solution B reagents	
NA2CO3	15.0
NA2S•9H20	1.0
Neutral Detergent Solution	Quantity for 2 liter
In	120 gm
Triethyl Glycerol	20 gm
Sodium Sulfite	20 gm

After obtaining, the rumen fluid for each animal species (~2 L) was transported to the lab by storing in a tightly closed thermos that pre-warmed (38°C) with distilled water. Each of the jars was continuously gassed with CO_2 before and during the placement of samples. The incubator was operated at 39°C temperature. After 48 hours of incubation, the samples were removed from the jars and were left to dry in a room overnight. After the samples were dried, $aNDF_{om}$ values of samples with heat-stable α-amylase and sodium sulfite were determined according to Van Soest et al. (1991) using the FibreTherm apparatus (Fibretherm®, C. Gerhardt GmbH & Co. KG, C., Konigswinter, Germany). The fiber values were expressed without residual ash (Mertens 2002). The in vitro 48h NDF digestibility ($IVNDFD_{48}$) was calculated with the following formula:

$IVNDFD_{48}$ (DM basis) = 100 – (W3 - (W1 x C1)) x 100/ (W2 x DM, %)

Where:

W1 = Dried bag tare weight

W2 = Sample weight

W3 = Dried final bag weight after in vitro and sequential NDF treatment

C1 = Blank bag correction (final oven-dried weight/original blank bag weight)

Statistical Analysis

In vitro digestibility data of each feedstuff were analyzed using the Kruskal-Wallis nonparametric test with MedCalc statistical software (v 19.0.3; MedCalc Software bvba, Ostend, Belgium). Data were expressed in tables as $\pm SEM$. Statistical significance was declared at $P<0.05$.

RESULTS

The results showed that the digestibility of all the feedstuffs under trial had a non-significant effect. Moreover, it could be seen that in sheep, the mean value of the pellet wheat bran was the lowest among all species. In differentiation, the penetrability of the sacks and the test weight per sack surface range may disturb the (IVTD) values. The starch degradability from distinctive feedstuffs was higher when the benefactor dairy animals were encouraged a proportion containing 1:1 feed: concentrate (on a DM premise) than when eat less was based only on roughage. In-vitro true digestibility of feedstuffs for water buffalo, cattle and sheep after 48 hours of incubation were shown in Table 2.

Table 2. In-vitro true $aNDF_{om}$[1] digestibility of different feedstuffs for water buffalo, cattle and sheep after 48 hours of incubation, % (DM basis)

Item	**Feedstuffs**	**Cattle**		**Buffalo**		**Sheep**		**P-Value**
		Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	
No.1	**Coarse Tomato leaf meal**	36.70	0.53	36.43	1.34	35.43	2.33	0.999
No.2	**Fine Tomato leaf meal**	29.24	2.03	23.64	2.93	17.61	0.25	0.102
No.3	**Hazelnut Meal**	24.97	0.13	27.59	7.45	20.52	0.49	0.368
No.4	**Pellet Wheat Bran**	35.39	2.33	39.47	1.99	12.62	3.16	0.156
No.5	**Grape Silage**	68.44	0.01	65.53	0.53	65.74	0.58	0.151
No.6	**Biscuit Bran**	46.68	0.56	44.24	1.41	37.48	1.19	0.101
No.7	**Bulgur Bran Pellet (cracked Wheat)**	29.49	2.35	25.59	0.45	21.35	1.29	0.102
No.8	**Poppy Seed Meal**	35.78	3.56	35.70	1.58	33.59	4.01	0.867

[1] NDF with heat-stable amylase treated and without residual

DISCUSSION

The in vitro method of surveying the digestibility of ruminant feedstuffs is utilized universally. The strategy is less demanding than in vivo studies and avoids the prerequisite of surgically planning creatures in totally different positions within the gastrointestinal tract. The IVTD decided by the Daisy strategy can be influenced by a few flows related to the packs utilized, nourish characteristics, and the device itself. One apparent advantage of the Daisy instrument over the in vivo strategy is the nonstop revolution of the maturation vessels, which efficiently mixes the assimilation inoculum amid the hatching period and eradicates the prerequisite for a time-consuming centrifugation step after brooding (Hogan & Flinn, 1999). These perspectives have been broadly surveyed for the in-situ strategy /DESI Hatchery (Nocek, 1988; Vanzant et al., 1998). Cone et al. (1989) found that the assortment of diets encouraged to the benefactor creature influences the values of in vitro degradability. In any case, the adaptation of the concentrated blend had as it were an immaterial impact on degradability values (Devendra & Sevilla, 2002; Richards et al., 1995). (Cone et al., 1989) appeared that the activity of rumen microflora, measured at diverse times after feeding, was higher in alcohol taken from dairy animals bolstered the next level of DM when bolsters were hatched for 6 hours. The pore measure of the sacks (50±15 μm) was inside the extent summarized by (Vanzant et al., 1998) for numerous considerations detailed within the writing. Test handling, especially crush measure, interatomic with pore estimate of the pack and influences the degree of nourish vanishing (Michalet-Doreau & Ould-Bah, 1992).

CONCLUSION

The daisy system is a more straightforward, less time-consuming method of measuring IVTD of ruminant feed. However, in the current study, the results for the IVTD % of eight feedstuffs were non-significant. Moreover, it is advised to conduct more trials to develop appropriate sample sizes and estimate the appropriate feedstuffs for the livestock according to their percentage of digestibility so that the economics for feed of livestock is improved.

Conflict of Interest: The authors declare no conflict of interest.

Authors' Contributions: Conceptualization, A.I. and I.B.; Methodology, Ü.Ö and E.E.G.; Investigation, S.R.A.S.; Resources, I.S.C. and I.B.; Data curation, A.Q Writing—original draft, A.I. and Ü.Ö.; Writing—review & editing, İ.B; Visualization, E.E.G.; Supervision, İ.S.Ç. and I.B. All authors have read and agreed to the published version of the manuscript.

Ethical Approval: This study was carried out at Afyon Kocatepe University Reserch Animals Application Center. This research was approved by The Ethics Committee of the Faculty of Veterinary Medicine, Afyon Kocatepe University (AKUHADYEK, Ref No: 49533702/07, Tarih: 01/2019). This study involves protecting the welfare of animal subjects.

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RESEARCH ARTICLE

Comparison of Median and Flank Laparotomy Approaches of Ovariohysterectomy Surgery in Cats

Merve YARDIMCI[1], Ece KOLDAŞ ÜRER[1*]

[1]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Mustafa Kemal University, Hatay, Türkiye

ABSTRACT

The study aimed to compare the operative and postoperative effects of flank laparotomy and traditional median approach in ovariohysterectomy in healthy female cats. Cats divided into median (MOHE, n=17) and left flank approach (FOHE, n=15) groups. In the intraoperative period, surgery duration, incision length, intraoperative complications and skin suture numbers were monitored. Various variables (aggression, agitation, onset of voluntary food and water intake, overall recovery from anesthesia effect, end of the stumble waking, voluntarily using the litter box, going to food bowl behavior) were monitored to monitor recovery in the early-postoperative period. On the 3rd postoperative day, pain/tenderness, erythema, edema, discharge in the wound and the cats' licking behavior on the wound were monitored. To evaluate the surgical stress response, Interleukin-6 concentration was measured in blood serum at 0 and 2 hours post-surgery. In order to evaluate muscle damage/recovery between groups, Lactate dehydrogenase, Aspartate transaminase and Creatinine kinase enzyme levels were determined in blood serum on day 0, postoperative day 3 and day 10. The incision length in the FOHE group was significantly shorter than the MOHE group ($P<0.05$). The change in Creatinine kinase between the sampling days of each group was significantly different ($P<0.05$). The difference between the other parameters monitored was not significant in the groups ($P>0.05$). In conclusion, lateral laparotomy and median ovariohysterectomy did not differ in terms of operative complications, surgical stress response, postoperative recovery, and complications. Since the methods do not have superiority over each other, it was concluded that the approach to be chosen in elective sterilization according to the vet's preference.

Keywords: Intraoperative Complication, Queen, Spaying, Surgery

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Kedilerde Medyan ve Flank Laparotomik Ovaryohisterektomi Operasyonlarının Karşılaştırılması

ÖZ

Çalışma, sağlıklı dişi kedilerde ovaryohisterektomide flank laparotomik ve geleneksel medyan yaklaşımın operatif ve postoperatif etkilerini karşılaştırmayı amaçladı. Kediler medyan (MOHE, n=17) ve sol flank yaklaşım (FOHE, n=15) gruplarına ayrıldı. İntraoperatif dönemde ameliyat süresi, kesi uzunluğu, intraoperatif komplikasyonlar ve cilt sütür sayıları takip edildi. Ameliyat sonrası erken dönemde iyileşmeyi izlemek için çeşitli değişkenler (saldırganlık, ajitasyon, gönüllü yiyecek ve su alımının başlangıcı, tam uyanma süresi, uygun yürüme süresi, yiyecek ve çöp kutusuna gitme davranışı) izlendi. Ameliyat sonrası 3. günde yarada ağrı/hassasiyet, kızarıklık, ödem, akıntı ve kedilerin yarayı yalama davranışları izlendi. Cerrahi stres yanıtını değerlendirmek için ameliyattan 0 ve 2 saat sonra kan serumunda İnterlöykin-6 seviyesi ölçüldü. Cerrahi yaklaşımlar arasında kas hasarının ve iyileşmesinin değerlendirilmesi amacıyla 0. gün, postoperatif 3. ve 10. gün kan serumlarında Laktat dehidrogenaz, Aspartat transaminaz ve Kreatinin kinaz enzim seviyeleri belirlendi. FOHE grubundaki insizyon uzunluğu MOHE grubuna göre anlamlı derecede kısaydı ($P<0.05$). Her grubun numune alma günleri arasındaki kreatinin kinaz değişimi anlamlı derecede farklıydı ($P<0.05$). Gruplarda izlenen diğer parametreler arasındaki fark anlamlı değildi ($P>0.05$). Sonuç olarak, lateral laparotomi ve medyan ovariohisterektomi ameliyat komplikasyonları, cerrahi stres yanıtı, ameliyat sonrası iyileşme ve komplikasyonlar açısından farklılık göstermedi. Yöntemlerin birbirine üstünlüğü bulunmadığından elektif kısırlaştırmada seçilecek yaklaşımın veterinerin tercihine göre şekillenebileceği kanısına varıldı.

Anahtar kelimeler: Ameliyat, Dişi Kedi, Kısırlaştırma, İntraoperatif Komplikasyon

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ORCID ID; *MY: 0000-0002-6668-3655 EKÜ: 0000-0002-9631-8501*

*Corresponding author e-mail:: ecekoldas@gmail.com

INTRODUCTION

Cats can give birth to an average of 4 kittens (Kustritz, 2006), complete 2-3 pregnancies during a breeding season (Park, 2015), unwanted estrus symptoms are frequent and intense, and the incidence of accidents increases if they are not sterilized (Gagnon et al., 2020). For these reasons, owners' request for sterilization is the most common reason for coming to the clinic. In Turkey, ovariohysterectomy (OHE) is used as a routine surgical method for spaying female cats and dogs, and basically three different approaches are used: ventral midline open surgical method, laparoscopic method and flank approach. The right or left flank approach is recommended to be applied in wild cats in cases where the mammary glands are overdeveloped and postoperative care and observation are not possible (McGrath et al., 2004; Reece, 2018). Nevertheless, OHE with the flank approach is also widely applied for routine spaying purposes.

Creatinine kinase (CK) has been used mainly as a marker of skeletal muscle damage (Aktaş et al., 1993). Muscle injury causes the release of intracellular enzymes and subsequent increase in CK activity in serum (Shelton, 2010). While CK activity is markedly increased in necrotic (Wells et al., 2009) and inflammatory (Evans et al., 2004) myopathies, it is commonly normal or slightly increased in non-inflammatory muscle diseases. However, its diagnostic specificity in minor extrinsic muscle damage is low due to the short half-life of serum CK activity, approximately 2 hours. It has been stated that following intramuscular drug injections, CK activity reaches its highest level at the 4th hour, shows a consistent increase in the first 24 hours, but decreases to the initial level on the 3rd day. On the other hand, CK that increases steadily or is persistently high may indicate the severity of muscle lesions Although it varies depending on the anesthetic used and the type of surgery, it has been reported that there is an increase in CK activity in serum within 6-12 hours after surgical interventions (Shelton, 2010).

Aspartate aminotransferase (AST), another serum enzyme that reflects muscle damage, is found in all cells and is used as a diagnostic enzyme for liver and muscle diseases due to its high activity in tissues. High CK along with high AST in serum analysis suggests that it is of muscle origin. Its serum concentration increases in case of hereditary myopathy, malignant hyperthermia, hypothyroidism, vitamin E-selenium deficiency, prolonged decubitus, intramuscular injections, surgery. Values of AST have been reported to increase between 12 and 24 hours after muscle damage and remain elevated for 1 or 2 weeks (Alves et al., 2009).

The other serum enzyme associated with muscle damage is Lactate dehydrogenase (LDH), a cytoplasmic isoenzyme that catalyzes the conversion of lactic acid and pyruvic acid (Nagy et al., 2013).

Because LDH is an enzyme found in all organ systems, its serum activity is abnormal in many diseases. LDH is a cell death marker released from cells as a result of necrosis or apoptosis (Johnson-Davis and McMillin, 2010). Increased levels are observed in heart, liver, skeletal muscle and kidney diseases, as well as in various hematological and neoplastic disorders. Increased LDH levels have also been reported in skeletal muscle disorders and some leukemias (Johnson-Davis and McMillin, 2010).

Considerable variation in serum values of various cytokines can be observed in studies comparing surgical methods or different anesthesia protocols. After a surgical procedure, the immune system may be activated or suppressed depending on the type of stress. This response is mediated by cytokines or interleukins (IL), which are low molecular weight immunological proteins produced by the activity of leukocytes, fibroblasts, and endothelial cells (Alazawi et al., 2016). Serum concentrations of proinflammatory cytokines increase within a few hours after the first stimulation and rapidly reach target organs and tissues (Eckersall and Bell, 2010). The pro-inflammatory response is mediated by numerous cytokines, including IL-1, IL-6 and tumor necrosis factor-alpha (TNF-α) (Palmer et al., 2019). It has been reported that IL-6 concentration changes significantly at the 2nd hour after midline OHE in cats (Marinov et al. 2018).

The aim of this study were to clinical and hematological compare the intraoperative and postoperative complications, muscle damage and healing, suture complications, surgical stress response and recovery period of median line and flank laparotomic OHE surgeries, which are commonly used for OHE in cats. Thus, it was aimed to reveal possible differences by making a comprehensive comparison between two frequently applied surgical methods.

MATERIALS and METHODS

The study was conducted with the approval of the Hatay Mustafa Kemal University Local Ethics Committee on Animal Experimentations decision (18/08/2021, no: 2021/05-02).

Study Population

Study animal consisted of female cats that were brought to Hatay Mustafa Kemal University Veterinary Health Practice and Research Hospital with a request for spaying. A total of 32 cats requesting spaying surgery were randomly allocated

into two groups: median OHE (MOHE, n=17) and left flank approach (FOHE, n=15).

Preoperative Interventions and Anesthesia Protocol

Age (months), breed and body weight of the cats were noted before the surgery. Regardless of the surgical method to be applied, cats were premedicated with subcutaneous atropine (Atropin 0.2%, Vetaş ®, Turkey) at a dose of 0.045 mg/kg, and 10 minutes later with intramuscular xylazine (Basilazin 2%, Bavet®, Turkey) at a dose of 2 mg/kg. Then, cats were intubated orotracheally with appropriate endotracheal tubes (no: 3-4). Anesthesia was maintained with isoflurane (1%, Isoflurane, Piramal Critical Care Inc., USA) in oxygen throughout the surgery. Isoflurane dose was 4-5% initial the anesthesia, and continued with 2-3% as the anesthesia deepened. The surgical area was shaved in accordance with the surgical approaches and aseptically prepared for surgery with povidone-iodine solution.

Surgical Methods

In the left flank approach, a skin incision was made by dissecting the skin with tissue scissors from the midpoint of the area between the tuber coxa-last rib-columna vertebralis in the left fossa paralumbalis region. Abdominal muscles and peritoneum were passed through blunt dissection with curved hemostatic forceps, and reached the abdominal cavity. A three-headed retractor (tracheostomy retractor) was used to observe the abdominal cavity clearly. The ovary was reached by observing and removing either the left ovary or the left horn. The suspensory ligament was ligated (PGA, USP: 2/0, Katsan, Turkey) over the left ovary, was dissected, and removed. Subsequently, the left horn was palpated up to the bifurcation area and the right horn was revealed. The right horn was palpated up to right ovary, and the ovary was removed as described for the left ovary. The uterus was exenterated by ligating the cervix from the bifurcation area as close as possible to the uterus. Passive ligatures were used for hemostasis during tissue incisions. The abdominal cavity was closed with the reverdin suture (muscles and peritoneum together and the subcutaneous connective tissue separate). Simple interrupted sutures placed to skin by absorbable suture material (PGA, USP:2/0, Katsan, Turkey).

In the median approach, the skin was incised with a scalpel, starting approximately 3 cm below the belly button and ending 2-3 cm in front of the pubic bone. The muscles were incised from the midline. If the peritoneum was not attached to the abdominal muscles, it was further incised and the abdominal cavity was reached. Any of the uterine horns were captured and the ovaries were subsequently revealed. The ovary was pulled out and the ligamentum suspensorium was ligated from the ovary. The same procedure was applied to the ipsilateral ovary. After the ovaries were dissected, the uterus was ligated and dissected from the junction of the corpus uteri and the cervix uteri. The surgery was completed by closing the peritoneum, abdominal muscles and skin with the method and suture material described in the left flank approach.

Supportive Treatments and Postoperative Care

All cats received intravenous (iv) isotonic saline solution (0.9% NaCl, 50 ml/kg/day, once), iv cefazolin sodium (Eqizolin 1gr, im/iv, 20 mg/kg, once) and meloxicam (Bavet meloxicam, 0.3 ml/5 kg, SC, once) during the intraoperative period. Surgical wounds were covered with dressing bandages. After surgery, all cats were kept under observation in the intensive care unit for 3 hours. In order to prevent possible aspiration in the early postoperative period, a 10-12 hour food and water restriction was recommended for cats. Antibiotic syrup treatment containing Amoxicillin + clavulanic acid was recommended for 1 week postoperatively.

Telephone calls were made to the patient owners at 24 and 48 hours after surgery to evaluate possible complications and overall health. Cats with routine postoperative procedures were given a follow-up appointment on the 3rd postoperative day to check their wounds and change dressings. In case of a non-routine postoperative process (extreme pain, rejection of oral antibiotics, dressing slippage, severe emesis, apathy), patient owners were informed that they should come to the clinic immediately so that appropriate replacement treatments could be applied. Skin sutures were removed on the 10th postoperative day.

Monitored Parameters

Variables monitored during and immediately after surgery in cats are given in Table 1.

Table 1. The parameters monitored during the intraoperative period

Surgery duration (min)	……
Incision length (cm)	…….
Number of skin sutures	…….
İntraoperative complications	☐ No complication ☐Bleeding ☐ Tissue rupture (any of ovary, uterus or suspensory ligaments)

Data collected in terms of clinical recovery, recovery from the effects of anesthesia and surgical stress response of cats housed in their routine places in the early postoperative period (first 48 hours) were obtained through telephone conversations with the patient owners at the 24th and 48th postoperative hours. The information obtained was recorded as shown in Table 2.

If signs of aggression such as hissing, scratching, attacking other pets in the house, and signs of agitation such as shouting, rolling around and hiding behavior were not observed, Score: 0 was noted. If they were seen, they were noted as Score 1a, Score 1b and Score 1c according to the duration of presence (Table 3).

On postoperative day 3 (mid-postoperative period), the surgical wound was examined during dressing change. During the examination, the presence/absence of pain/tenderness in the incision, erythema, edema, discharge and licking behavior were noted. Skin sutures of all cats were removed on the 10th day after surgery.

Collection, Storage and Analysis of Blood Samples

Blood samples were taken from the cats a total of 4 times at the beginning of anesthesia (Hour 0/Day 0), at the 2nd postoperative hour, on the 3rd postoperative day, and on the 10th postoperative day. Plain blood collection tubes were used to collect blood samples taken from V. saphena. Sera of blood samples were removed as quickly as possible by centrifuging at 3000 rpm for 7 minutes. Serum samples were kept in a freezer at -20 ºC until relevant analyses.

Serum IL-6 levels were measured in the 0th and 2nd hour samples of 10 randomly selected cats from each group using the Cat Interleukin 6 ELISA kit (MyBioSource, San Diego, CA, Cat no: MBS284478).

Serum enzyme concentrations (LDH, CK, AST), which may reflect muscle damage, were measured (GESAN, Chem 2000, Automatic Chemistry Analyzer) in blood samples on the 0th, 3rd and 10th days in the MOHE (n=14) and FOHE (n=12) groups

Statistical Analysis

Statistical comparison of 24-48 hour observation results, postoperative 3rd day observation results, and complication rates in MOHE and FOHE groups was made using Fisher's Exact Test. Comparison of LDL, CK, GOT, IL-6 values of MOHE and FOHE groups was made with Mann Whitney U Test. Changes in IL-6 values within the group were determined by Wilcoxon Signed Rank Test; Changes in LDL, CK, AST values were made according to the Friedman Test. The significance level for all analyzes was determined as 0.05.

Table 2. Follow-up parameters of cats in the early postoperative period

	Score 1	Score 2	Score 3	Score 4
	(Hours)			
Overall recovery from anestehesia effect	6-12	12-18	18-24	>24
End of the stumble waking	0-6	6-12	12-24	>24
Onset of voluntarily using the litter box	0-6	6-12	12-24	>24
Going to food bowl behavior	0-6	6-12	12-24	>24
Onset of water consumption without emesis	12-18	18-24	24-48	>48
Onset of voluntary food intake	12-18	18-24	24-48	>48

Table 3. Scoring aggression and agitation in cats

	Score 0	Score 1		
		a	b	c
Aggression	None	0-12	12-24	24-48
Agitation	None	0-12	12-24	24-48

RESULTS

The number of skin sutures, surgery duration (Table 4) and intraoperative complications (Table 5) during the intraoperative period were not statistically different between MOHE and FOHE (P>0.05). The average incision length in the MOHE was significantly longer than the average incision length in the FOHE (P=0.008). Aggression, agitation, overall recovery from anesthesia effect, end of the stumble waking, voluntarily using the litter box, going to food bowl behavior, onset of water consumption without emesis, onset of voluntary food intake parameters

evaluated in the MOHE and FOHE groups by the 24-48 hour postoperative observation of the patient owners, were not significantly different between the groups (Table 6).

The variables monitored in the surgical wound in the mid-postoperative period (postoperative day 3) were not significantly different between the groups (P=0.999). No erythema or discharge was observed in the wound of any cat included in the study (Table 7).

The mean LDH values on day 0 (P=0.936), day 3 (P=0.820), and day 10 (P=0.527) were not significantly different between the FOHE and MOHE groups. LDL values did not change significantly (P = 0.576) in the FOHE (P=0.441) and MOHE groups between the 0th day, 3rd day, and 10th day (Figure 1).

There was no significant difference between the mean CK values between surgical methods on day 0 (P=0.742), day 3 (P=0.595), and day 10 (P=0.212) (Figure 2). In the FOHE group, CK values on day 0, day 3, and day 10 showed significant changes (P=0.002). Similarly, in the MOHE group, CK values on day 0, day 3, and day 10 showed significant changes (P<0.001), (Figure 2).

The mean AST values on day 0 (P=0.940), day 3 (P=0.860), and day 10 (P=0.317) were not significantly different between the FOHE and MOHE groups (Figure 3). While the change within the group between different sample days in the FOHE group was not statistically significant (P>0.05), in the MOHE group, AST values on the 0th day, 3rd day, and 10th day showed a statistically significant change (P<0.05).

The mean IL-6 levels at preoperative (0th hour) (P=0.940) and postoperative 2nd hour (P=0.860) were not significantly different between the FOHE and MOHE groups (Figure 3). Furthermore, there was no difference between the postoperative 2nd hour mean IL-6 level and the preoperative mean IL-6 level in the FOHE and MOHE groups (Figure 4).

The data were analyzed with the SPSS 25.0 statistical software (IBM Corp. Released 2017. IBM SPSS Statistics for Windows). *Shapiro–Wilk* test was used for the prerequisites of homogeneity of variances and normality of variables. The mean ± standard deviation (SD) of normally distributed variables was presented. The variables were evaluated with t-test. Blastocyst rate were analyzed by Chi-square test. At the 95% confidence level (P<0.05), the differences were considered statistically significant.

Table 4. Monitored parameters during the intraoperative period in groups

	FOHE			MOHE			
	Med.	25%	75%	Med.	25%	75%	P
Surgery duration (min)	35,00	28,00	45,00	34,00	30,00	40,00	0,910
Incision length (cm)	2,60	2,10	3,00	3,50	3,00	3,60	0,008
Number of skin sut	5,00	4,00	6,00	5,00	5,00	6,00	0,448

Table 5. Intraoperative complications in groups

	FOHE		MOHE		
	n	%	N	%	P
No Complication	11	73,3	16	94,1	0,158
Bleeding	1	6,7	1	5,9	
Tissue rupture*	3	20,0	0	0,0	

*uterus, ovary or ligaments

Table 6. Monitored early postoperative parameters in cats

		FOHE		MOHE		
		n	%	n	%	P
Aggression	None	9	64,3	11	64,7	0,438
	0-12 h	4	28,6	2	11,8	
	12-24 h	1	7,1	4	23,5	
Agitation	None	8	57,1	12	70,6	0,101
	0-12 h	5	35,7	2	11,8	
	12-24 h	0	0,0	3	17,6	
	24-48 h	1	7,1	0	0,0	
Onset of voluntary food intake	12-18 h	9	64,3	7	41,2	0,440
	18-24h	3	21,4	7	41,2	
	24-48 h	2	14,3	3	17,6	
Onset of water consumption without emesis	12-18 h	4	28,6	8	47,1	0,379
	18-24h	5	35,7	2	11,8	
	24-48 h	4	28,6	4	23,5	
	>48 h	1	7,1	3	17,6	
Overall recovery from anesthesia effect	6-12 h	8	57,1	12	70,6	0,924
	12-18 h	2	14,3	2	11,8	
	18-24h	3	21,4	2	11,8	
	24-48 h	1	7,1	1	5,9	
End of the stumble waking	0-6 h	5	35,7	8	47,1	0,938
	6-12 h	6	42,9	6	35,3	
	12-24 h	2	14,3	2	11,8	
	>24 h	1	7,1	1	5,9	
Going to food bowl behavior	0-6 h	2	14,3	9	52,9	0,053
	6-12 h	7	50,0	7	41,2	
	12-24 h	3	21,4	0	0,0	
	>24 h	2	14,3	1	5,9	
Voluntarily using the litter box	0-6 h	5	35,7	9	52,9	0,157
	6-12 h	6	42,9	7	41,2	
	12-24 h	3	21,4	0	0,0	

	>24 h	0	0,0	1	5,9	

Table 7. Wound Complications in mid-postoperative period in groups

		FOHE		MOHE		
		n	%	N	%	P
Pain	No	11	78,6	14	82,4	0,999
	Yes	3	21,4	3	17,6	
Licking	No	13	92,9	16	94,1	0,999
	Yes	1	7,1	1	5,9	
Edema	No	13	92,9	15	88,2	0,999
	Yes	1	7,1	2	11,8	

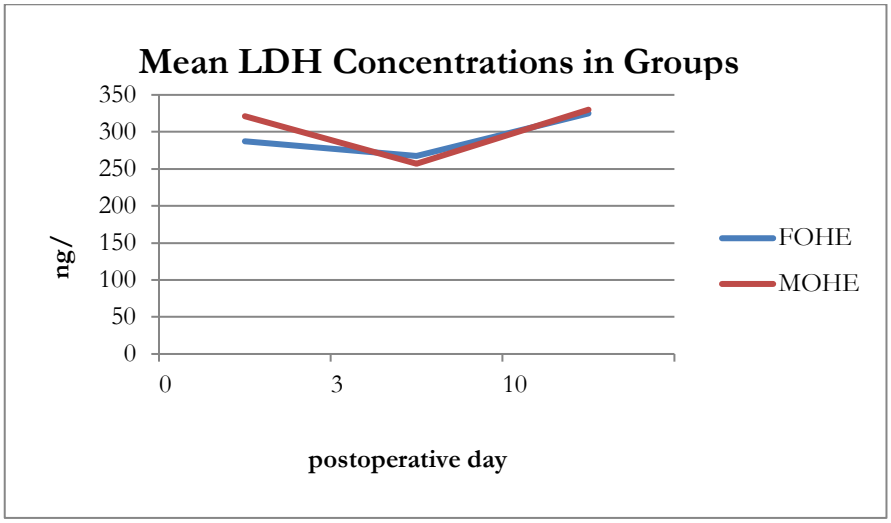

Figure 1. Mean serum LDH concentrations in MOHE and FOHE

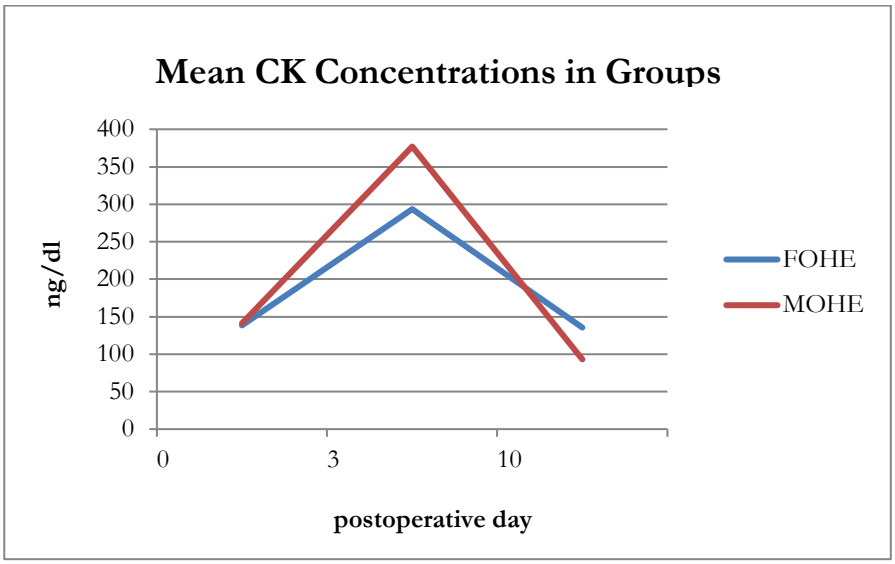

Figure 2. Mean serum CK concentrations in MOHE and FOHE

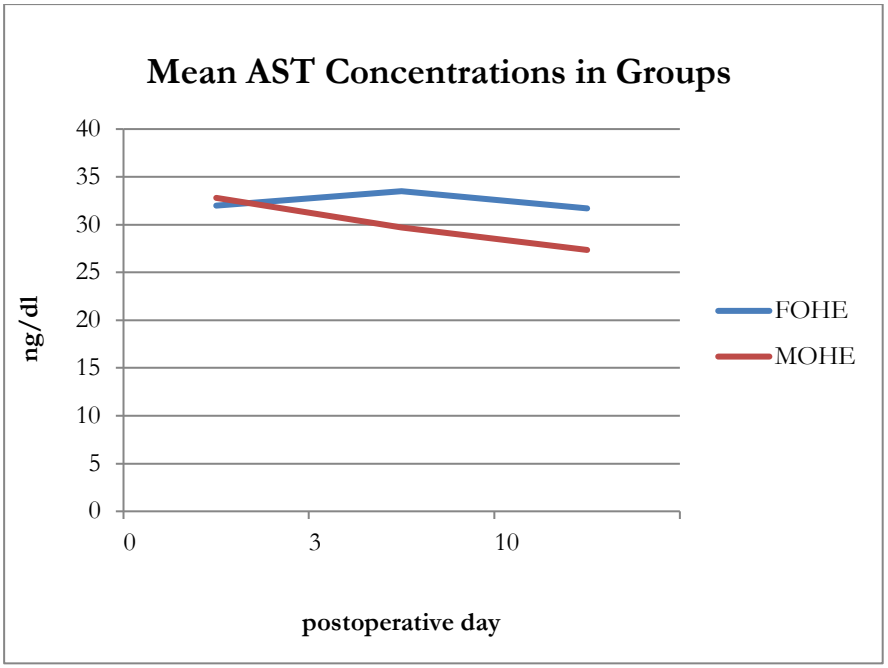

Figure 3. Mean serum AST concentrations in MOHE and FOHE

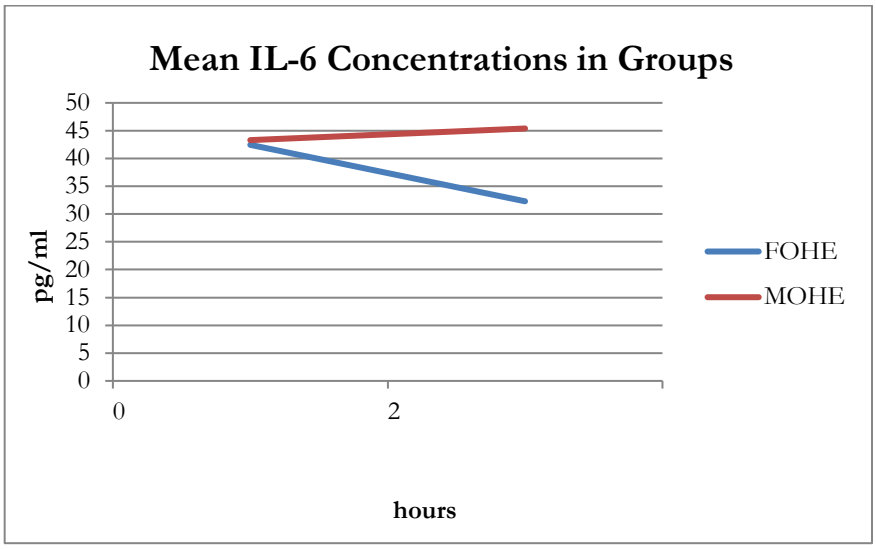

Figure 4. Mean serum IL-6 concentrations in MOHE and FOHE

DISCUSSION

Studies comparing the duration of spaying procedures using the conventional midline and flank approaches have produced conflicting findings. Kiani et al. (2014) reported that the flank approach (23 min) took less time than the median line (31 min) surgery. Similarly, Rana (2007) reported that the flank approach (24±2.65 min) was completed in a shorter time than the median line approach (29±3.51 min) in cats. Conversely, Swaffield et al. (2020) stated that there is no difference between the two methods in terms of completion time of the surgery. Coe et al. (2006) reported that the skin incision and opening the peritoneum phases took longer in the flank approach, while finding the uterus phase took longer in the midline approach. In this study, spaying took an average of 35 minutes in the FOHE group and 34 minutes in the MOHE group (P>0.05). Unlike Coe et al. (2006) results, finding the uterus in the flank approach and completing the closure sutures in the midline approach were probably the stages that required the longest time. In fact, this study did not analyze the spaying surgeries step by step. However, the authors of this study believe that the reason the two procedures took relatively similar times to be completed was due to differences in the amount of time spent at each stage of the procedure. To clarify this issue, studies evaluating steps such as skin incision, muscle incision, identification of the uterus, and ovarian ligation during lateral and midline neutering procedures in cats are needed.

The length of the flank incision was typically significantly shorter than the midline incision length in studies comparing these two methods (Ghanawat and Mantri, 1996; Shuttleworth and Smythe, 2000; Rana, 2007). According to Coe et al. (2006), typical incision lengths for midline approach of OHE was 3.1±0.6 cm, while for lateral approach of OHE was 2.6±0.2 cm in cats. In this study, the average length of the FOHE incision (2.6 cm) was shorter than the average length of the MOHE incision (3.5 cm) and was found to be compatible with previous studies (P>0.05, Table 4). According to Rana (2007), the reason for the short incision length of the lateral approach is the ease of access to the uterus and ovary. In this examination, after approximately half a centimeter of skin dissection in the lateral approach, the incision line was passively widened with hemostatic forceps. In fact, no surgical incisions are actually made in FOHE cats unless there is intraoperative bleeding. As a result, it is not possible to discuss an actual surgical incision in this study. Regarding this, in addition to the justification stated by Rana (2007), it was thought that a factor that significantly contributed to the statistical difference between the groups was the application that benefited from the natural elasticity of the skin. The length of the skin incision differed between groups, but the average number of skin sutures was the same in both groups (P>0.05), which may be due to more frequent skin sutures in the flank approach.

The rate of surgical complications of OHE in healthy cats and dogs varies between 6.2-20.6% depending on the surgeon's experience (Samojlović et al., 2015). Tektepe (2019) reported that bleeding occurs at a rate of 10% in cats and 20% in dogs during surgery. However, significant bleeding complication that resulted in mortality occurred in just one in every 1450 cats undergoing elective spaying (Adin, 2011). Swaffield et al. (2020) reported that there was no difference in intraoperative complications including hypotension, hemorrhage, and ligature opening in OHE performed from the flank (n = 37) and median line (n = 38) in cats. The difference between intraoperative complications in FOHE and MOHE cats was not significantly different (P>0.05, Table 5). Although "rupture of tissues", one of the

intraoperative complications, occurred only in the FOHE group, since it was possible to control bleeding, the intraoperative bleeding complication was not found to be different between the groups (1 cat in both groups, P>0.05). Many studies classify the complication resulting from the ligament puncturing the tissue (which in this case was classified as tissue tear) as a bleeding complication. The bleeding complication in this study refers to a condition that typically presents as leakage and originates from the broad ligament ovary, or abdominal muscles. In both groups, bleeding was easily controlled by placing additional ligatures. The complication, which is considered as tissue rupture, includes situations that occur before the tissue is removed, easily controlled and re-ligated with the help of hemostatic forceps, and is often caused by excessive tension that occurs during traction at the junction of the ovary and the uterus. Similar complication was reported by Coe et al. (2006) in both the median and lateral flank approaches (opening of the ovarian ligature, cutting the uterine body with the ligature, accidentally cutting the uterus), but the possible cause was not explained. Extreme care should be taken to maintain hemostasis during the estrus period due to increased vascularity and edema of the ovarian and uterine tissues (Bushby, 2012). It was thought that the complication of tissue rupture, which occurred due to the deterioration of tissue strength and the ligatures cutting the tissue, may be caused by differences in the sexual cycle period of cats. However, the sexual cycle periods of cats were not evaluated in this study.

Behavioral changes (general activity level, time spent sleeping, play behaviors, aggressive behaviors, the cat's desire to be closer to a person, the desire to be held, hiding, vocalizations other than purring or hissing) and appetite changes have been reported to occur for several days following surgery in cats recovering at home after ovariohysterectomy (Väisänen and Tuomikoski, 2007). When cats are in pain, they are in a hunched posture, with their noses tense, their eyes narrowed, their ears flattened outward, their whiskers tense, and their heads held below the line of their shoulders. They also exhibit behaviors such as freezing behavior, biting the wound, wagging the tail, hiding behaviors, vocalization when approached (groan, hiss, or growl), and spending extended amounts of time lying down (Steagall and Monteiro, 2019; Brondani et al. 2011). In this study, observational parameters that may reflect pain did not differ between the FOHE and MOHE groups over the 24- to 48-h home monitoring period (P>0.05, Table 6). This finding would suggest that the effects of both surgical techniques on surgical stress and postoperative pain in cats are comparable. However, the validity and reliability of the monitored parameters in reflecting the postoperative process have not been demonstrated, and this makes it difficult to reach a definitive conclusion.

IL-6 levels are significantly increased in various pathological conditions associated with pain and hyperalgesia. Therefore, it has been stated that IL-6 levels can be an objective indicator in the evaluation of pain in animals (Sommer and Kress, 2004). It is known that the serum IL-6 level, which has a short half-life, begins to increase within 30–60 minutes after surgery, and a considerable increase occurs after 2-4 hours. However, it has been stated that it can reach its maximum level at the 24th postoperative hour after invasive surgeries, and this high serum level might last for up to 48–72 hours postoperatively (Desborough, 2000). In this study, it was found that the IL-6 level did not increase considerably by the second hour postoperatively (Figure 4). Moreover IL-6 concentration measured preoperatively and at the 2nd hour postoperatively did not differ significantly between the FOHE and MOHE groups and within the groups themselves (P>0.05). In the study, postoperative meloxicam was applied to both groups at equal doses and at the same times. Meloxicam affects cyclooxygenase activity and blocks prostaglandin synthesis, as well as causing a decrease in cAMP, which is responsible for IL-6 regulation (Mahdy et al. 2002). However, since pain management is a necessity after spaying surgeries, analgesics are routinely applied. Since the aim of this study was to compare two routinely applied OHE approaches within routine practices, the possible effects of analgesics on IL-6, and therefore on surgical stress response and pain, were ignored.

Wound complications (inflammation and suture dehiscence) have been reported to occur 2.95 times more frequently with the median line approach than the flank approach (Robert et al., 2015). Swaffield et al. (2020) noticed that the pain score was higher in the flank group at the first postoperative hour, whereas it was higher in the midline group on the third and tenth postoperative days, and that edema was present in the wound in the median line group in all controls. Another study comparing right flank and median line OHE (Murugesan et al. 2020) found that edema and discharge at the suture line were seen in 5 cats with the flank approach and 3 cats with median line surgery, but there was no difference in postoperative pain scores between the groups. Coe et al. (2006) compared midline (n = 24) and flank approach (n = 17) OHE surgeries and classified wound complications (discharge, excessive licking behavior, edema, suture dehiscence) on the seventh postoperative day as mild, moderate, or severe based on information provided by owners. As a result, significant discharge occurred in five animals in the flank group and one in the midline group; wound edema occurred in three cats in the midline group but not in any of the animals in the flank group. In this study, the presence of pain and/or tenderness, erythema, edema at the suture line, and licking behavior at the time of dressing removal were evaluated in cats in both groups on the 3rd

postoperative day. No discharge or erythema occurred at the suture line in any cats. Pain, edema, and licking behaviors were present at a rate of 35.71% (5/14) in the FOHE group and 35.29% (6/17) in the MOHE group but the difference was not significant (P> 0.05, Table 7). These findings are compatible with those of Murugesan et al. (2020) and Coe et al. (2006). When combined with previous data obtained from the study, it was decided that there was no difference in intraoperative, early, and mid-postoperative complications in cats with OHE performed with median line and flank approach. This research indicates that both methods have comparable effect on clinical recovery in cats.

In cases of muscle damage caused by any reason, there is a significant increase in serum LDH, AST, and, most specifically and strikingly, CK levels. Even in relatively minor muscle damage, such as intramuscular injections or minimal exercise, serum CK levels can increase two or threefold due to its high specific activity in the muscle (Kraemer et al. 2009). Therefore, an increase in serum CK activity is used in the diagnosis of neuromuscular diseases and in the confirmation of muscle damage (Fascetti et al. 1997; Auguste 1992). Although increases are most noticeable in cats with anorexia, they have also been reported to variable levels in heart illness, trauma, bite wounds, bacterial infections, prior general anesthesia, and after intramuscular injections (Aroch et al. 2010). In this study, while CK levels changed within the group on days 0, 3, and 10 in both FOHE ($P<0.05$) and MOHE groups ($P<0.01$), the difference between the groups was not significant ($P>0.05$, Figure 2). This result shows that a similar muscle healing process was formed in both groups.

A study comparing median line and laparoscopic ovariectomy surgeries in cats found that AST levels in the laparoscopic approach were higher than those in the traditional approach, and these high values decreased at the 12th postoperative hour in the median line and at the 24th postoperative hour in the laparoscopic approach (Alves et al. 2009). In this study, the difference in mean serum AST levels on the sampling days was not statistically significant in FOHE and MOHE groups ($P>0.05$). On the other hand, the difference in AST concentration within each group was not significant either ($P>0.05$, Figure 3). It has been reported that the half-life of AST in cats is approximately 1.5 hours (Chapman and Hostutler, 2013) and that the increase in serum AST occurs later than the increase in serum CK (Alves et al. 2009). Serum AST levels increase 12–24 hours after muscle injury and remain elevated for 1 or 2 weeks (Alves et al. 2009). In this study, serum AST levels remained constant despite an increase in blood CK levels on the third postoperative day. The reason for this effect remains unclear. Serum AST levels may have increased earlier in the postoperative period but may have decreased by the day of sampling. When muscle damage occurs, first LDH, then CK, and finally AST are expected to rise in serum (Billings, 2013). In the study, the difference in serum LDH levels between the MOHE and FOHE groups and within each group was not significant ($P>0.05$, Figure 1). Although it is one of the suggested serum enzymes for assessing muscle injury, there is insufficient evidence on the change in serum LDH levels following surgical intervention in cats. In this context, it is believed that more research is needed, particularly in cats.

In conclusion, this study found no difference between flank laparotomy and median line OHE in terms of intraoperative complications, operative time, number of skin sutures, muscle damage, muscle recovery, surgical stress response, postoperative wound healing. The FOHE incision was shorter than the MOHE incision, but this does not appear to affect the healing process. In cats, only serum CK levels change significantly, indicating muscle injury. Serum CK should be monitored as part of the muscle recovery process. Monitoring serum CK is recommended to monitor muscle recovery in cats.

In light of the findings of the study, it is thought that further research is needed to investigate the potential of AST, LDH and CK enzymes, which can evaluate muscle damage in cats, to reflect muscle damage. In order to evaluate the enzymes mentioned above together, the times when they are elevated in serum in cats should be reported. In this context, similar studies are expected to be effective in evaluating serum samples taken at more frequent intervals starting from the first hours after surgery. In addition, examining IL-6 together with other cytokines in the evaluation of surgical stress may highlight the differences between techniques more clearly.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: MY and EKÜ contributed to the project idea, design and execution of the study. MYB and EKÜ contributed to the acquisition of data. MY drafted and wrote the manuscript. EKÜ reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was approved by Local Ethics Committee on Animal Experimentations of the Hatay Mustafa Kemal University (Ref no: 2021/05-02, Date: 18/08/2021).

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RESEARCH ARTICLE

Evaluation of Testicular Arterial Doppler Ultrasonography and Spermatological Parameters in Pomeranian Dogs with Alopecia X

Cumali KAYA[1*], Burcu ESİN[1], Çağatay ESİN[2]

[1]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Türkiye
[2]Department of Veterinary Internal Medicine, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Türkiye

ABSTRACT

The present study investigates the effects of vascular parameters in testicular vessels on sperm quality in Pomeranian dogs with alopecia X. In the study, Doppler parameters such as peak systolic velocity (PSV, cm/s), end-diastolic velocity (EDV, cm/s), resistive index (RI), and pulsatility index (PI) of testicular arteries were measured in the healthy control group and dogs in the alopecia X group, and motility, progressive motility, and abnormal morphology analyses were performed in semen collected from dogs. When the obtained results were examined, it was determined that RI and PI parameters were higher, and the examined spermatological parameters were significantly lower, in dogs in the alopecia X group compared to the healthy control group ($p < 0.05$). It is thought that increased RI and PI values may negatively affect spermatogenesis by causing insufficient blood flow in the testicles and that a decrease in sperm motility, progressive motility, and morphological parameters may be related to this situation. The results obtained provide important data on the negative effects of alopecia X on reproductive health and emphasize that this disease should be addressed in male dogs not only in terms of dermatological but also reproductive functions.
Keywords: Alopecia X, Doppler ultrasonography, Sperm evaluation, Testicular artery

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Alopecia X'li Pomeranian Köpeklerde Testiküler Arteriyel Doppler Ultrasonografi ve Spermatolojik Parametrelerin Değerlendirilmesi

ÖZ

Sunulan çalışma, alopecia X'li pomeranian ırkı köpeklerde testis damarlarındaki vasküler parametrelerin sperm kalitesi üzerindeki etkilerini araştırmayı amaçlamaktadır. Çalışmada sağlıklı kontrol grubu ile alopecia X grubunda bulunan köpeklerde testis arterlerinin pik sistolik hızı (PSV, cm/s), son diyastolik hız (EDV, cm/s), rezistif indeks (RI) ve pulzatilite indeksi (PI) gibi doppler parametreleri ile köpeklerden alınan spermadan motilite, progresif motilite ve anormal morfoloji analizleri yapılmıştır. Elde edilen sonuçlar incelendiğinde alopesi X grubunda bulunan köpeklerin sağlıklı kontrol grubuna kıyasla daha yüksek RI ve PI parametreleri, incelenen spermatolojik parametrelerde ise alopesi x grubunda bulunan köpeklerin sağlıklı kontrol grubuna kıyasla anlamlı derecede daha düşük olduğu belirlenmiştir ($p<0,05$). Artan RI ve PI değerlerinin testislerde yetersiz kan akışına yol açarak spermatogenezi olumsuz etkileyebileceği, sperma motilitesi, progresif motilite ve morfolojik parametrelerdeki düşüşün de bu durum ile ilişkili olabileceği düşünülmektedir. Elde edilen sonuçlar alopecia X'in üreme sağlığı üzerindeki olumsuz etkilerine dair önemli veriler sağlamaktadır ve bu hastalığın erkek köpeklerde yalnızca dermatolojik değil, aynı zamanda üreme fonksiyonları açısından da ele alınması gerektiğini vurgulamaktadır.
Anahtar Kelimeler: Alopesi X, Dopler ultrasonografi, Sperma analizi, Testiküler arter

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ORCID ID; *CK: 0000-0002-0666-5359, BE: 0000-0002-5728-1478, ÇE: 0000-0002-7029-9066*
*Corresponding author e-mail: cumali.kaya@omu.edu.tr

INTRODUCTION

The skin is the largest organ in the animal body and reflects the functionality and general health of the structures beneath it. Although the skin has many important functions, its primary role is to protects living beings from mechanical damage, chemicals, pathogens, ultraviolet radiation, and dehydration. Dermatological problems are among the most common health problems in pets. Since the skin is the organ most vulnerable to external factors and infections, such diseases can usually be easily noticed by pet owners (Weller et al. 2008).

Fertility can be affected by both hereditary and acquired skin diseases as well as their clinical management. The impact of skin diseases on fertility varies depending on the age of onset, severity, and chronicity of the disease. Although the prevalence of skin diseases negatively affecting fertility is relatively low, treatment strategies for the male reproductive system in these cases must be carefully evaluated (Abdel-Naser and Zouboulis 2016).

Alopecia refers to partial or complete hair loss. "Alopecia X" is a type of alopecia caused by a progressive hair cycle disorder without obvious systemic symptoms or triggering factors (Gökalp and Kırbaş 2021). This condition is most commonly seen in young adult dogs such as Pomeranian, Siberian Husky, Malamute, Samoyed, Keeshond, and Poodle, especially in unneutered males (Scott et al. 2001). Alopecia is commonly seen in young adult dogs, both males and females, regardless of their infertility status. Clinical symptoms are characterized by partial or complete alopecia on the neck, tail, caudo-dorsal region, perineum, base of tail, and eventually on the trunk, excluding the head and forelegs. In addition, skin hyperpigmentation may be observed in areas with alopecia (Frank et al. 2004; May et al. 2019). Although the etiology of alopecia X is not fully known, several theories have been proposed, including hyposomatotropism in mature dogs, gonadal sex hormone imbalance in intact males, and adrenal sex hormone disorders (Frank et al. 2003; Gökalp and Kırbaş 2021).

This study aimed to evaluate testicular arterial Doppler ultrasonography and spermatological parameters in male Pomeranian dogs diagnosed with alopecia X. The goal was to understand the effects of alopecia X on fertility and reproductive functions, to reveal vascular changes and spermatological parameters in these dogs and to gain new perspectives on the disease in terms of reproduction.

MATERIAL and METHODS

Animal Selection and Experimental Design

This study was approved by the Local Ethics Committee for Animal Experiments of Ondokuz Mayıs University (approval number: E-68489742-604.01-2400217735). Ten Pomeranian dogs were examined in the study. All dogs included in the study were owned dogs. The study design consisted of two groups: a healthy control group (n=5; Pomeranian breed, unsterilized dogs with no health problems) and an Alopecia X group (n=5; Pomeranian breed, unsterilized dogs diagnosed with Alopecia X). The mean age and weight in each experimental group were as follows: mean age in 2.40±1.14 years, kg 2.74±0.59 in the healthy group, mean age in 2.80±1.30 years, kg 2.66±0.61 in the alopecia x group. All dogs were regularly vaccinated, fed with commercial dry food, and had free access to water. Only dogs whose owners consented to participate the experiment were selected for ethical reasons. All animal owners signed a written consent form before participating in the study and were informed.

Within the scope of the study, male Pomeranian dogs of different age groups and body weights brought to Ondokuz Mayıs University Veterinary Faculty Animal Hospital with complaints of alopecia were evaluated. According to the anamnesis obtained from the owners brought to the internal medicine clinic, it was reported that hair loss was observed in the tail and back region for an average of 2 to 3 months. In the physical examinations, the areas with hair loss were examined with a Wood's lamp and deep skin scraping samples were taken for fungal and scabies diseases; however, no diagnosis was reached for these samples. In addition, routine hemogram examinations were performed on blood samples taken from the vena cephalica antebrachii of the dogs and collected in tubes containing ethylenediaminetetraacetic acid (EDTA), and it was determined that the obtained data were within the reference range. It was determined that cortisol, cholesterol, ALT, ALP, AST, and GGT values were within reference range. As a result of all these evaluations, the patients were diagnosed with alopecia X (Figure 1).

In this study, the dogs' testicles in both groups were examined in detail using arterial Doppler ultrasonography techniques. In addition, sperm samples were collected from each dog in the study and control groups and evaluated in terms of motility, progressive motility, and morphology.

Measurement of Testicular Arterial Hemodynamics by Doppler Ultrasonography Technique

Ultrasound examinations of testicular blood flow were performed in all dogs in the healthy control and alopecia X groups before sperm collection. To avoid the negative effects of anesthetic agents on TBF, the dogs were immobilized in a ventrodorsal position without sedation. An ultrasound device (Vetus 9, Mindray) equipped with a microconvex probe (6.5–7.5 MHz) was used for all evaluations. In this study,

testicular blood flow was evaluated in the supratesticular (Fig. 2), intratesticular (Fig. 3), and marginal testicular (Fig. 4) arteries. A more detailed and comprehensive analysis of the distribution and waveform of blood flow was performed in the study; Doppler parameters such as peak systolic velocity (PSV, cm/s), end-diastolic velocity (EDV, cm/s), and resistive index (RI) and pulsatility index (PI) of the testicular arteries were recorded. The angle between the Doppler beam and the examined vessel was kept 45-60°, parallel to the blood flow direction. The Doppler probe was placed in a central region of the vessels, and at least three consecutive waves were monitored to ensure the automatic determination of spectral curves and vascular indices.

Figure 1. Dogs 1 and dogs 4 belong to the alopecia X group, respectively.

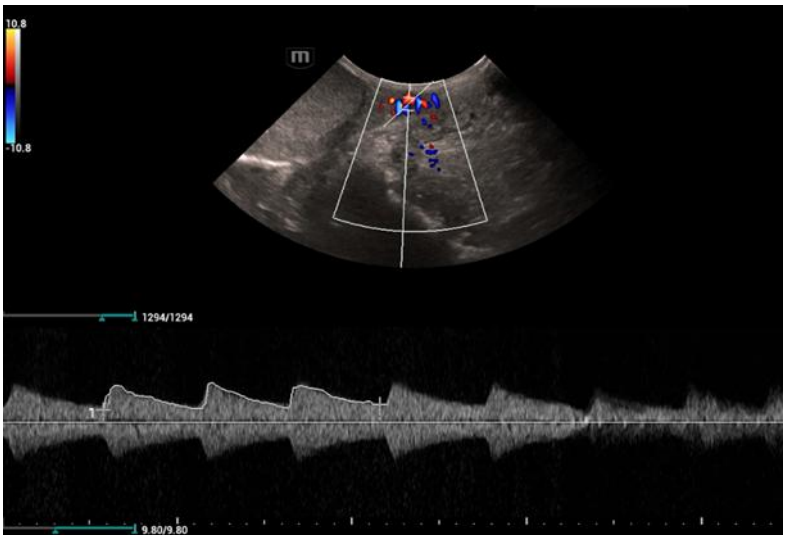

Figure 2. Hemodynamics of the left supra-testicular artery in dog number 1 belonging to alopecia X group

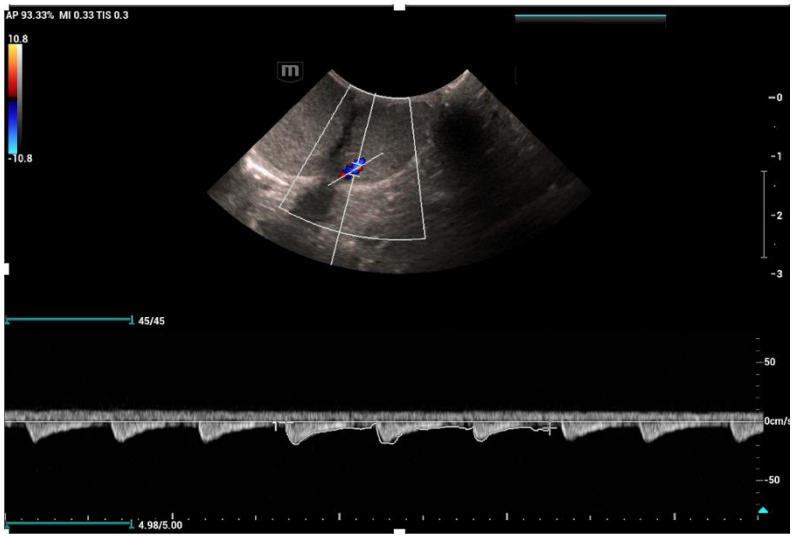

Figure 3. Hemodynamics of the right intratesticular artery in dog number 3 belonging to the alopecia X group

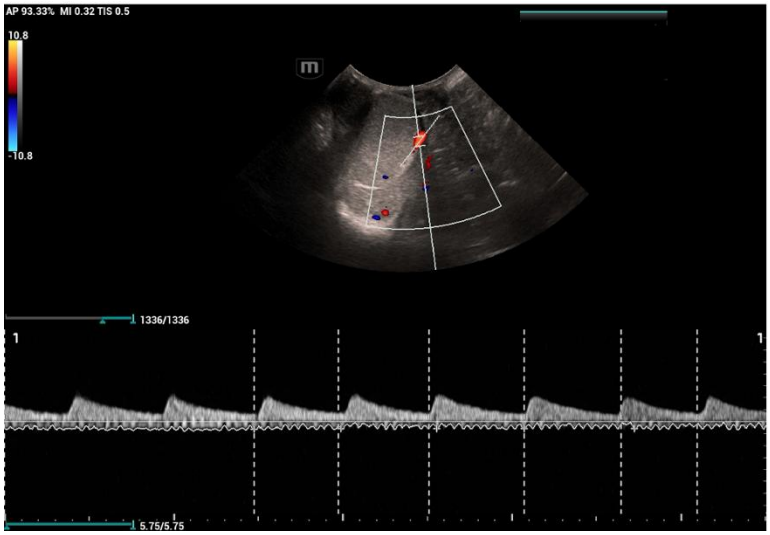

Figure 4. Haemodynamics of the right testicular marginal artery in dog number 5 belonging to the alopecia X group

Sperm Collection and Evaluation

Sperm samples were collected by digital manipulation from all dogs in healthy control and alopecia X group. The fresh spermatozoa obtained were diluted to a final concentration of 50-100x106/ml with a tris-based extender (3.025 g tris, 1.7 g citric acid, 1.25 g fructose, 5% v/v glycerol, and 20% v/v egg yolk with 100 mL distilled water) to ensure uniformity and then evaluated under the Computer Aided Sperm Analyzer (CASA) (SCA, Sperm Class Analyzer, Version 6.5.0.91; Microptic, Barcelona, Spain).

For the evaluation of spermatozoa motility, the obtained sperm were transferred to an Eppendorf tube. 10 µl of sperm was taken from each sample, placed on a slide, and covered with a coverslip. The prepared samples were examined using a phase-contrast microscope (Nikon, Eclipse, Tokyo, Japan), a heating stage at 37°C, 10x objective, and a camera recording at 60 frames/sec. As a result of the analyses, total motility (%) and progressive motility (%) parameters were obtained by measurements made in at least five different microscope fields after the dilution rate of the sperm sample was entered into the software. The results were recorded for statistical analyses.

For the morphological evaluation of spermatozoa, the morphology module of the CASA system and the SpermBlue® stain kit (Microptic, Spain) were used. 10 µl of the sperm sample was taken, and a smear was prepared on the slide and left to dry. After the drying process, the slides were dipped into a jar containing SpermBlue dye at room temperature and kept for 2 minutes. After the staining process, the slides were left to dry at an angle of 60-80 degrees. Once dried, at least 200 spermatozoa were evaluated morphologically using the CASA system phase-contrast microscope

with a blue filter and a heating table. Spermatozoa were evaluated in terms of head, midpiece, tail, and total abnormal spermatozoon rate.

Statistical analysis

All analyses were performed using a commercially available software (SPSS®, IBM Inc.). All data are expressed as mean ± standard deviation (SD). Data normality checking was carried out using the Shapiro-Wilk test. Statistical analysis of normally distributed parameters between groups was evaluated using the Student's t-test for independent samples. Comparisons between measurements in the study group at different times were assessed with a paired sample t-test. P<0.05 value was considered significant.

RESULTS

The study groups' mean values of total motility (TM), progressive motility (PM), and morphological evaluations (morphological disorders of the head, midpiece, and tail and total abnormal spermatozoa) were analyzed. Comparative results of these values are presented in Table 1

.

Table 1 Comparisons of spermatological parameters in healthy control and alopecia X groups

Parameters	Healthy control group				Alopecia X group				
	Mean	SD	Min	Max	Mean	SD	Min	Max	p value
TM (%)	94.75	2.11	92.25	97.23	84.78	4.48	80.21	91.88	**0.002**
PM (%)	58.17	6.26	51.69	68.53	39.86	9.81	23.88	48.62	**0.008**
Abnormal head	2.80	1.64	1.00	5.00	4.00	1.00	3.00	5.00	0.201
Abnormal midpiece	2.20	0.83	1.00	3.00	5.40	1.51	3.00	7.00	**0.003**
Abnormal tail	3.80	1.30	2.00	5.00	7.60	1.81	5.00	10.00	**0.005**
Total abnormality	8.80	1.92	6.00	11.00	17.00	3.93	11.00	21.00	**0.003**

Mean: Mean SD: Standart deviation Min: Minimum Max: Maximum p: Significance value

When these differences are analyzed, revealed that there is a significant decrease in the healthy control group TM (94.75±2.11%) and PM (58.17±6.26%) values of semen compared to TM (84.78±4.48%) and PM (39.86±9.81%) values of alopecia X group semen (p<0.05). In addition, there was a significant increase in the mean values of the head, midpiece, tail, and total abnormal sperm morphology values of the semen alopecia X group (p<0.05).

The mean values of PSV, EDV, RI, and PI for supratesticular, intratesticular, and marginal arteries in the study groups were evaluated by Doppler ultrasonography. The comparative results of these values are presented in Table 2, Table 3, and Table 4, respectively.

When Table 2 is examined, except for the statistical difference observed in the increase in the PI value of the alopecia X group (p<0.05), no statistical difference was found in other parameters. In Table 3, the increase in PSV, RI, and PI values, which are values other than EDV, was found to be statistically significant (p<0.05). In Table 4, which includes the measurement results of the marginal artery, the statistical difference is observed in the EDV and RI parameters (p<0.05).

Table 2. Comparisons of the Supratesticular artery hemodynamic parameters in healthy control and alopecia X groups

Supratesticular Artery	Healthy control group				Alopecia X group				
	Mean	SD	Min	Max	Mean	SD	Min	Max	p value
PSV (cm/s)	17.20	1.30	16.00	19.00	19.40	5.02	14.00	25.00	0.371
EDV (cm/s)	8.00	0.70	7.00	9.00	7.80	0.83	7.00	9.00	0.694
RI	0.52	0.05	0.43	0.57	0.57	0.10	0.42	0.68	0.368
PI	1.09	0.67	1.03	1.19	1.27	0.99	1.12	1.39	**0.009**

Mean: Mean SD: Standart deviation Min: Minimum Max: Maximum p: Significance value

Intratesticular Artery	Healthy control group				Alopecia X group				
	Mean	SD	Min	Max	Mean	SD	Min	Max	p value
PSV (cm/s)	6.20	1.30	5.00	8.00	13.40	6.26	6.00	21.00	**0.036**
EDV (cm/s)	4.00	0.70	3.00	5.00	4.20	0.83	3.00	5.00	0.694
RI	0.35	0.06	0.25	0.40	0.63	0.13	0.50	0.80	**0.002**
PI	0.70	0.07	0.60	0.81	1.30	0.18	1.10	1.53	**0.000**

Mean: Mean SD: Standart deviation Min: Minimum Max: Maximum p: Significance value

Table 3. Comparisons of the Intratesticular artery hemodynamic parameters in healthy control and alopecia X groups.

Marginal Artery	Healthy control group				Alopecia X group				
	Mean	SD	Min	Max	Mean	SD	Min	Max	p value
PSV (cm/s)	12.20	1.30	11.00	14.00	16.60	4.44	12.00	22.00	0.067
EDV (cm/s)	7.80	0.83	7.00	9.00	6.00	1.00	5.00	7.00	**0.015**
RI	0.35	0.06	0.27	0.46	0.59	0.17	0.41	0.77	**0.020**
PI	0.82	0.08	0.70	0.91	1.01	0.21	0.80	1.30	0.107

Mean: Mean SD: Standart deviation Min: Minimum Max: Maximum p: Significance value

Table 4. Comparisons of the Marginal artery hemodynamic parameters in healthy control and Alopecia X groups.

DISCUSSION

Alopecia X disease may be caused by many systemic diseases such as hypothyroidism, hyperadrenocorticism, and follicular dysplasia, and researchers have reported that sex hormone endocrinopathies such as testosterone and estrogen may also play a role in its etiology (Frank et al. 2004; Crawford et al. 2024). Dihydrotestosterone (DHT) is a more potent androgen than testosterone and is an end-organ effector that directs growth and differentiation in specific tissues. DHT is usually formed by the reduction of testosterone in target cells, and this peripheral conversion occurs primarily in tissues such as the prostate, male genitalia, and skin. Testosterone conversion in the skin supports the normal development of sexual characteristics at different stages of life. However, this process can occasionally increase abnormally, leading to undesirable effects (Price 1975).

Considering the existence of these mechanisms in dogs, similar excessive accumulation of DHT in the skin and other tissues may cause clinical signs such as hair loss. The role of DHT in alopecia X cases in dogs should not be ignored. This disease has been defined as a hair loss problem that is seen especially in certain breeds and develops due to abnormal hormonal activity in the skin. Considering the effects of DHT on the prostate, skin, and genital tissues, the effect of this type of alopecia seen in dogs on spermatogenesis, sperm quality, testicular functions, and fertility is not yet fully understood, but considering the role of testosterone and related hormones, it is thought that this condition may also affect spermatological parameters and testicular functions.

Our study shows that spermatological parameters examined in dogs with alopecia X are significantly lower than in the healthy control group ($p<0.05$). Systemic hormonal changes observed in dogs with alopecia X may explain the negative effects on sperm parameters. It is thought that these hormonal changes negatively affect spermatogenesis, and as a result, decreased sperm motility, decreased progressive motility, and increasing morphological disorders are observed (Safarinejad et al. 2011; Rosety et al. 2014). Alopecia X is a condition encountered in dogs and shares some clinical features with androgenic alopecia (AGA) in humans. AGA is a condition that leads to a hair loss process in men via dihydrotestosterone (DHT) and is characterized by a decrease in the anagen phase (Ntshingila et al. 2023). Güngör et al. (2016) found lower motility and higher morphologically disordered sperm rates in patients with androgenic alopecia when they examined spermatological parameters compared to the control group of healthy individuals. It is thought that oxidative stress-induced hair loss in the inguinal region where the testicles are located leads to decreased spermatogenesis and sperm damage (Omu 2013). The data we obtained in our study suggest that, as in humans, hormonal imbalance, oxidative stress, and systemic proinflammatory conditions may be associated with deterioration in sperm quality.

The vessels entering the testicles provide stable blood flow, necessary for metabolic processes and sperm production. Testicular blood flow is key in transporting nutrients, regulatory hormones, and secretory products to and from the testicles (Bergh 1993). Due to the low oxygen concentration in the seminiferous tubules, regulating blood flow in the testicles is critical (Setchell 1990). Inadequate blood flow can lead to disruptions at different stages of the spermatogenesis process. Doppler measurements are a crucial tool for assessing the effectiveness of this vascular regulation. In addition, detecting abnormal blood flow can be an important indicator in determining fertility problems (Samir et al. 2018). PSV and EDV values used in Doppler measurements indicate arterial blood flow in the standard cardiovascular cycle. In the Zelli et al. (2013) study, no correlation was found between spermatological parameters and PSV and EDV values. However, it has been stated that RI and PI values provide more robust

data on the anatomical structure and blood flow velocity of the testicular vessels (Biagiotti et al. 2002). RI is a measure that reflects the resistance to blood flow caused by the microvascular bed distal to the measured area. PI measures the oscillations in the waveform of blood flow. It is assumed that RI and PI values are inversely proportional to blood flow perfusion and that the lower these indices are, the more efficient spermatogenesis can be qualitatively (Zelli et al. 2013). Higher RI and PI parameters in the three vessels examined in dogs with alopecia X indicate increased resistance and low blood perfusion in the testicular vessels. This situation may lead to insufficient blood flow in the testes and, thus, a decrease in oxygen and nutrients, which may negatively affect spermatogenesis. Increased vascular resistance disrupts the microenvironment required for spermatozoon production, which can be directly linked to the observed decreases in sperm motility, progressive motility, and morphological parameters. The high RI and PI values suggest that vascular abnormalities in the testes may be linked to adverse outcomes in sperm quality in dogs with alopecia X.

CONCLUSION

In conclusion, the findings of our study showed that alopecia X, which is characterized by hormonal changes, may adversely affect in testicular arterial hemodynamics and sperm motility, progressive motility, and morphological parameters. These findings demonstrate that there is a relationship between increased vascular resistance in testicular vessels (high RI and PI values) and decreases in sperm quality in dogs with Alopecia X. The obtained results reveal that alopecia X is not only a dermatological problem but also may negatively affect male reproductive health. These data are important in evaluating reproductive functions and optimizing treatment processes in dogs with alopecia X. Future studies may contribute to developing specific treatment approaches by examining the effects of this disease on reproductive health in more detail.

Conflict of Interest: The authors have no conflicts of interest to report.

Authors' Contributions: CK, BE, and ÇE contributed to the project idea, design, and execution of the study. CK, BE, and ÇE contributed to the acquisition of data. CK, BE, and ÇE analyzed the data. CK, BE, and ÇE drafted and wrote the manuscript. CK, BE, and ÇE reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical Approval: This study was carried out at Ondokuz Mayıs University Animal Hospital. This research was approved by the Animal Experiments Ethics Committee of the University Ondokuz Mayıs (approval number: E-68489742-604.01-2400217735).

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RESEARCH ARTICLE

Investigation of As, Cu, Fe, Ni and Zn Concentrations of Some Potentially Toxic Elements in Water, Sediment and Gill Tissues of Different Trout Species (Salmo Trutta and Oncorhynchus Mykiss) and Shabut (Tor Grypus) Fish in Atatürk Dam Lake

Ozan AYGÜN[1], Füsun TEMAMOĞULLARI[1]*, Zozan GARİP[1]

[1]*Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Harran University, Şanlıurfa, Turkey*

ABSTRACT

In this study, the accumulation concentration of copper (Cu), iron (Fe), nickel (Ni), zinc (Zn), and arsenic (As) in water, sediment, and gill tissues of brown trout (Salmo trutta), rainbow trout (Oncorhynchus mykiss), and shabut (Tor grypus) fish in Atatürk Dam Lake were evaluated by ICP-MS. Regarding Fe and Ni accumulation, the difference between rainbow trout and other species was found to be statistically significant ($p<0.05$). In terms of Cu and Zn accumulation, it was determined that the difference between Brown trout and other species was statistically significant, and the concentration of As accumulation was less and statistically significant ($p<0.05$). In shabut fish, it was determined that Fe accumulation in terms of weight was statistically significant ($p<0.05$) and As and Cu accumulated more than other fish gills ($p<0.05$). In water samples, it was determined that the concentration of Cu and Fe (1. and 3. regions) were above the reference values according to the reference values of the surface water quality regulation of the Republic of Turkey, and the concentration of Fe was above the reference limits set by the World Health Organization (2022). In sediment samples, Cu and Fe concentration were found to be above the serious and toxic effect reference values. As a result, it was observed that metals accumulated in gill samples of different fish species living in Atatürk Dam at different rates depending on the species, and the concentration of Fe and Cu were high in sediment and Fe in water samples.

Keywords: Fish, ICP-MS, PTEs (Potentially Toxic Elements), sediment, water

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Atatürk Baraj Gölü'nde Su, Sediment ve Farklı Alabalık Türleri (Salmo Trutta ve Oncorhynchus Mykiss) ve Şabut (Tor Grypus) Balıklarının Solungaç Dokularındaki Bazı Potansiyel Toksik Elementlerin As, Cu, Fe, Ni ve Zn Konsantrasyonlarının Araştırılması

ÖZ

Bu çalışmada, Atatürk Baraj Gölü'nde su, sediment ve kahverengi alabalık (Salmo trutta), gökkuşağı alabalık (Oncorhynchus mykiss) ve şabut (Tor grypus) balıklarının solungaç dokularındaki bazı metallerin; bakır (Cu), demir (Fe), nikel (Ni), çinko (Zn) ve arsenik (As) birikim konsantrasyonları ICP-MS cihazıyla değerlendirilmiştir. Gökkuşağı alabalıklarında uzunluk bakımından sadece Cu birikiminin istatistiki açıdan önemli olduğu görülmüştür ($p<0,05$). Birecik bölgesinden toplanan gökkuşağı alabalık solungaçlarında Fe miktarının diğer bölgelere göre daha yüksek olduğu belirlenmiştir ($p<0,05$). Fe ve Ni birikimi bakımından Gökkuşağı balıklarında diğer türler ile arasındaki farkın istatistiki olarak önemli olduğu tespit edilmiştir ($p<0,05$). Cu ve Zn birikimi bakımından Kahverengi alabalıklarında diğer türler ile arasındaki farkın istatistiki olarak önemli olduğu ve As birikim miktarının ise daha az olduğu ve istatistiki olarak önemli olduğu tespit edilmiştir ($p<0,05$). Şabut balıkları solungaçlarında ağırlık bakımından Fe birikiminin istatistiki açıdan önemli olduğu ($p<0,05$) ve As ve Cu'ın diğer balık solungaçlarından daha fazla biriktiği belirlenmiştir ($p<0,05$). Su örneklerinde; Türkiye Cumhuriyeti yer üstü su kalitesi yönetmeliğinin referans değerlerine göre Cu ve Fe miktarlarının referans değerlerinin üzerinde olduğu, Fe miktarının ise Dünya Sağlık Örgütü (2022) ve Avrupa Birliği Komisyonu (2008) 'nun belirlediği referans limitlerinin üzerinde olduğu belirlenmiştir. Sediment örneklerinde; Cu ve Fe miktarlarının ciddi ve toksik etki referans değerlerinin üzerinde olduğu belirlenmiştir. Sonuç olarak; Atatürk barajında yaşayan farklı alabalık türlerine ait solungaç örneklerinde metallerin türe göre farklı oranlarda biriktiği, su ve sediment örneklerinde de Fe ve Cu miktarlarının genel olarak yüksek çıktığı görülmüştür.

Anahtar kelimeler: ICP-MS, balık, su, sediment, PTEs (Potansiyel Toksik Elementler)

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ORCID ID; *OA: 0000-0001-9268-6254, FT: 0000-0001-7738-1145, ZG: 0000-0002-8971-7918*

*Corresponding author e-mail: fkaracal@harran.edu.tr

INTRODUCTION

Water pollution is becoming an important problem today due to the increase in population rate and the development of technology and industry. Metals transported into water by anthropogenic activities, natural events and dead organisms settle over time and accumulate in sediment and pass back into the water environment (Ercişli, 2016). Aquatic organisms such as sediment, water and fish are used in the determination of metal pollution in aquatic ecosystems and it has been reported that metal accumulation and effects are not the same in every fish and vary according to the type of metal, the concentration of accumulation, the duration of the effect, the age, reproduction period, nutrition and habitat of the organisms (Çetin et al. 2016). Fish take metals mainly through the gills. Fish gills are important tissues in metal accumulation due to their high storage properties (Rajar et al., 2024). Iron (Fe), nickel (Ni), zinc (Zn) and copper (Cu) are essential metals (Yunusa et al., 2023; Singh and Sharma, 2024). Essential metals are necessary for growth and development in living organisms. Deficiency or excess of essential metals may cause undesirable effects in living organisms (Singh and Sharma, 2024). Arsenic (As) is a highly toxic heavy metal that has no essential biological role in living organisms (Hughes et al., 2011) and exists in normal water. Nickel (Ni), one of the essential metals, swells the gill lamellae, leading to increased oxygen consumption, respiratory stroke volume and respiratory frequency (Pane et al. 2003). It has been determined that Ni has both a vital role in iron (Fe) metabolism and a role in the absorption of iron from the intestines (Latund-Dada et al. 2006). Fe is an important trace element in zinc (Zn) hemostasis (Shim and Harris 2003). Zn, which tends to accumulate in the gills, initially increases mucus secretion in the gills and then decreases mucus, leading to increased susceptibility to microbial infections (Schelkle et al. 2009). Exposure to high concentration of Fe has also been reported to reduce the concentration of copper (Cu) transporters and eventually lead to a reduction in Cu absorption (Chandrapalan and Kwong, 2020). The presence of metals in sediment poses a threat to aquatic organisms through accumulation and biomagnification (Misra et al., 2024).

In this study, the accumulation concentration of As, Cu, Fe, Ni and Zn in gill tissue, water and sediment samples of brown trout, rainbow trout and shabut collected from Atatürk Dam Lake, one of the important water resources of the Southeastern Anatolia Region, were investigated by inductively coupled plasma mass spectrometry (ICP-MS).

MATERIALS and METHODS

Working Area

Atatürk Dam is located between Adıyaman and Şanlıurfa provinces and is used for energy and irrigation purposes and is also very important for the fishery sector (Duman and Çelik 2001).

Fish Sampling

The study was conducted in June and July without sex determination, taking into account the reproductive period of the fish. The approximate locations where the fish samples were collected are shown on the map (Figure 1). The fish samples used in the study were selected to be at least 500 to 1500 grams (g) (60 in total). The lifeless fish samples were placed in polypropylene containers and brought to the laboratory. The standard, fork and total lengths of the collected fish samples were measured on a measuring board with an error of ±1 mm and their weights were measured on a Weightlab brand precision balance and gill tissue samples were taken from the fish. Four water samples (10 ml) and four sediment samples (10 g) were also collected from the places where the fish samples were collected.

Figure 1: Areas where fish samples were collected.

Inductively Coupled Plasma Mass Spectrometry Analysis

The tissue samples were treated with 8 ml of 65% nitric acid followed by 3 ml of 30% hydrogen peroxide and placed in a Teflon reactor. Thermal incineration in the microwave was performed gradually at 130 ºC for 10 minutes, 150 ºC for 10 minutes and 180 ºC for 10 minutes. After incineration, distilled water was added to the ash and filtered through filter paper. Heavy metal analyses in the filtrate were measured by inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer, Inc., Waltham, MA, USA) (Ütme and Temamoğulları 2021). The same procedure was applied to water and sediment samples. In the study, limit of detection (LOD) and limit of quantification (LOQ) values were determined as 0.05614 ppb and

0.1853 ppb for As; 0.04119 ppb and 0.136 ppb for Cu; 1.41 ppb and 4.653 ppb for Fe; 0.0188 ppb and 0.062304 ppb for Ni; 1.042 ppb and 3.4386 ppb for Zn, respectively. Correlation coefficient (R^2) values in the calibration equation were found as 0.9998 for Fe; 0.9999 for Ni; 0.9998 for Zn; 0.9999 for Cu; 0.9974 for As, respectively. Relative standard deviation (RSD) value in the calibration equation was as 3.7 for Fe; 9.8 for Ni; 4.1 for Zn; 4.7 for Cu; 8.7 for As, respectively. Recovery study was found as 99.87 for Fe; 102.64 for Ni; 99.35 for Zn; 100.82 for Cu; 100.06 for As, respectively.

Statistical Analysis

Differences between normally distributed groups were analyzed by one-way analysis of variance (ANOVA) and the significance of the differences was checked by post hoc Duncan test. This two test were performed to determine the relationship between the weight and length of the fish and the accumulated heavy metals in the gills. SPSS version 23 for Windows (IBM Corp., Armonk, NY, USA) was used for statistical analysis. $p<0.05$ was considered statistically significant. Results are presented as mean ± standard error of the mean (S.E.M.).

RESULTS

As a result of the study, it was observed that different concentration of heavy metal accumulation were found in the gill tissues of rainbow trout, brown trout and shabut fish (Table 1). It was determined that the difference in Fe and Ni in the gills between brown trout and shabut fish species was statistically insignificant ($p>0.05$), while the difference between rainbow fish and these species was statistically significant ($p<0.05$). Ni was found to accumulate less in rainbow trout than in other fish species. It was determined that Fe, Ni, Zn and Cu, except As, accumulated less in the gill samples of rainbow trout. It was determined that the concentration of As was similar between Rainbow trout and Shabut fish species; and the concentration of As accumulation in Brown trout was less and statistically significant ($p<0.05$). The difference in Cu and Zn accumulation in brown trout compared to other fish gills was statistically significant ($p<0.05$). In terms of the concentration of Cu accumulation in the gills, it was determined that Shabut > Brown trout > Rainbow trout, respectively, and the difference between all species was statistically significant ($p<0.05$). In terms of the concentration of Zn accumulation in the gills, it was determined that Brown trout > Shabut > Rainbow trout, respectively, and the difference was statistically significant. It was determined that As and Cu accumulated more in the gills of shabut than in the gills of other fish ($p<0.05$). In addition it was observed that as the concentration of Cu, Fe, Ni, Zn and As in the water and sediment environment increased, the amount of accumulation in the gill tissues of fish increased.

Table 1. Potentially toxic elements, ppb, mean ± SEM.

Groups	Rainbow Trout	Brown Trout	Shabut	P<0.05
Fe±SEM	3387.26^{a}±275.04	73046.18^{b}±14832.11	51711.22^{b}±3381.78	0.000
Ni±SEM	5.422^{a}±0.48	99.105^{b}±16.23	76.97^{b}±10.21	0.000
Zn±SEM	4036.33^{a}±172.18	112768.71^{c}±8601.24	23561.50^{b}±1117.33	0.000
As±SEM	172.51^{b}±17.57	72.61^{a}±5.32	232.66^{b}±51.63	0.003
Cu±SEM	296.57^{a}±15.91	502.76^{b}±25.76	706.72^{c}±36.71	0.000

The difference between the averages Decribed with different letters in the same row is significant ($p<0.05$).

In Table 2, it was observed that there was no statistically significant difference ($p>0.05$) in the concentration of As, Cu, Fe, Ni and Zn in the gill tissue samples of rainbow trout examined based on weight, while only Cu accumulation was statistically significant in terms of length ($p<0.05$). In addition, it was determined that the concentration of Fe in rainbow trout gills collected from Birecik region was higher than the other regions, and this difference was statistically significant ($p<0.05$). In Table 2, it is seen that there is no statistically significant difference ($p>0.05$) in the concentration of As, Cu, Fe, Ni and Zn in the gill tissue samples of brown trout analyzed according to weight, length and regions where the samples were collected.

In Table 2, it was determined that there was no statistically significant difference ($p>0.05$) in the concentration of Fe and Ni in the gill tissue samples of shabut fish according to weight and length, the difference in the concentration of Fe was not statistically significant in terms of length, the difference was statistically significant ($p<0.05$) in terms of weight (600-1110g); the difference in terms of metal accumulation between the regions where the samples were collected was not statistically significant ($p>0.05$).

Table 2. The concentration of potentially toxic elements in the gill tissues of fish caught from Atatürk Dam Lake. ppb. average ± SEM.

Species	Parameters		N	Fe	Ni	Zn	As	Cu	p<0.05
Rainbow trout (Oncorhynchus Mykiss)	**Weight(g)**	500-699	5	3977.38±785.52	5.92±1.31	4365.16±448.58	158.24±58.46	297.88±21.21	Fe:0.091
		700-799	5	4152.9±490.61	5.49±0.42	4054.44±381.57	192.70±22.92	338.60±37.24	Ni:0.937
		800-1100	5	2620.2±230.82	5.23±0.77	3888.3±271.38	184.38±8.58	283.96±33.15	Zn:0.731
		1101-1400	5	2798.56±242.79	5.03±1.33	3837.42±316.76	154.74±39.80	265.84±34.04	As:0.857
		Total	20	3387.26±275.04	5.42±0.48	4036.33±172.18	172.51±17.57	296.57±15.91	Cu:0.447
	Length(cm)	32-37	10	3634.1±412.02	5.59±0.68	3987.74±260.54	178.45±30.05	332.85±18.85	Fe:0.384 Ni:0.728 Zn:0.786 As:0.745 Cu:0.018
		38-43	10	3140.42±368.91	5.24±0.7	4084.92±238.26	166.58± 19.82	260.29±20.52	
		Total	20	3387.26±275.04	5.42±0.48	4036.33±172.18	172.515±17.57	296.57±15.91	
	Area	Birecik	10	3940.79±471.13	5.58±0.65	4242.57±278.31	191.01±24.33	309.19±26.16	Fe:0.040 Ni:0.743 Zn:0.241 As:0.305 Cu:0.443
		Bozova	10	2833.73±171.32	5.25±0.73	3830.09±195.61	154.02±25.22	283.95±18.69	
		Total	20	3387.26±275.04	5.42±0.48	4036.33±172.18	172.51±17.57	296.57±15.91	
Brown Trout *(Salmo Trutta)*	**Weight(g)**	600-799	10	52388.82±5214.93	94.03±22.56	121842.15±11699.29	73.29±5.18	473.69±28.48	Fe:0.170 Ni:0.764 Zn:0.304 As:0.903 Cu:0.270
		800-999	10	93703.54±28404.74	104.18±24.46	103695.28±12537.83	71.94±9.63	531.84±42.47	
		Total	20	73046.18±14832.11	99.1±16.23	112768.71±8601.24	72.61±5.32	502.76±25.76	
	Length(cm)	27-32	10	53537.38±5358.46	92.42±24.58	112480.13±10866.9	66.55±5.16	485.53±28.07	Fe:0.196 Ni:0.692 Zn:0.974 As:0.266 Cu:0.518
		33-38	10	92554.98±28668.07	105.79±22.32	113057.3±13937.63	78.68±9.21	520.00±44.15	
		Total	20	73046.18±14832.11	99.1±16.23	112768.71±8601.24	72.61±5.32	502.76±25.76	
	Area	Birecik	10	46982.52±5054.56	87.7±23.18	116236.54±13361.42	73.79±5.21	458.57±29.43	Fe:0.078 Ni:0.497 Zn:0.698 As:0.832 Cu:0.086
		Bozova	10	99109.84±27428.91	110.51±23.38	109300.89±11452.75	71.44±9.60	546.96±38.77	
		Total	20	73046.18±14832.11	99.1±16.23	112768.71±8601.24	72.61±5.32	502.76±25.76	

Table 2-Continuation. The concentration of potentially toxic elements in the gill tissues of fish caught from Atatürk Dam Lake. ppb. average ± SEM.

Shabut ***(Tor Grypus)***	**Weight(g)**	600-1110		44862.55±4757	66.36±15.49	23556.33±1674.27	294.77±99.53	651.67±37.09	Fe:0.039 Ni:0.311 Zn:0.996 As:0.239 Cu:0.138
		1115-1625	10	58559.89±3903.19	87.58±13.22	23566.68±1570.98	170.55±22.21	761.78±60.35	
		Total	10	51711.22±3381.78	76.97±10.20	23561.50±1117.33	232.66±51.63	706.72±36.71	
			20						
	Length(cm)	40-52	10	48905.92±3737.61	75.95±13.99	22988.66±1212.54	289.50±93.38	661.42±35.18	Fe:0.422 Ni:0.924 Zn:0.621 As:0.283 Cu:0.227
		53-65	10	54516.52±5706.89	77.99±15.61	24134.35±1930.80	175.82±42.66	752.03±63.23	
		Total	20	51711.22±3381.78	76.97±10.20	23561.50±1117.33	232.66±51.63	706.72±36.71	
	Area	Birecik	10	51077.48±6655.34	67.28±15.13	23601.68±2012.22	261.25±102.78	708.99±73.39	Fe:0.857 Ni:0.356 Zn:0.972 As:0.594 Cu:0.953
		Bozova	10	52344.96±1975.94	86.66±13.78	23521.33±1105.34	204.07±22.60	704.46±17.43	
		Total	20	51711.22±3381.78	76.97±10.20	23561.50±1117.33	232.66±51.63	706.72±36.71	

N: Number of Group Samples.

The concentration of As, Cu, Fe, Ni, Ni and Zn in water and sediment samples taken from the places where fish samples were collected during the study are given in Table 3. It was determined that the concentration of Fe and Cu were generally high in the water samples and especially Bozova Ni (1st and 2nd region) and Zn (2nd region) metals were below the detection limits. According to the reference values of the surface water quality regulation of the Republic of Turkey, it was determined that the concentration of Cu (1, 2, 3 and 4 regions) and Fe (1 and 3 regions) were above the reference values, while the concentration of As, Ni and Zn were below the reference values. In our study, it was determined that the concentration of As, Cu, Ni and Zn in the water samples were below the reference limits determined by the World Health Organization (2022) and the European Commission (1998), while the concentration of Fe in the collected water samples was above WHO reference values (Table 3).

Table 3. The Fractions of As, Cu, Fe, Ni and Zn in water samples and recommended reference values(ppb).

Area	As	Cu	Fe	Ni	Zn
1	9.9	12.6	145.7	<0.000	13.8
2	9.3	19.8	11.4	<0.000	<0.000
3	8.6	9.5	144.6	0.4	39.7
4	9.3	17.5	34.4	0.2	5.6
Maximum allowable environmental quality standard (Yer üstü su kalitesi yönetmeliği, 2016)	53	3.1	101	34	231
WHO (2022)	10	2000	10	70	-
Directive of the Council of the European Union (1998)	10	2000	10	20	-

1 and **2**. Bozova district, **3** and **4**. Birecik district.

In addition, according to the heavy metal limits determined in sediment samples according to MacDonalds (2000), it was determined that Cu, Fe, Ni and Zn analyzed in our study were above the threshold effect value, but As was below the threshold reference value in all regions; Cu, Fe, Ni (1st, 2nd, 3rd region) and Zn (1st and 2nd region) accumulation were above the possible effect reference value, Ni (4th region), Zn (3rd and 4th region) and As in all regions were below the possible effect reference value. According to the serious and toxic effect reference values, it was determined that As, Ni and Zn were below these values in all regions, while Cu and Fe were above the serious and toxic effect reference values (Table 4).

Table 4. The Fractions of As, Cu, Fe, Ni and Zn in sediment samples and the recommended reference values (ppb).

Area	As	Cu	Fe	Ni	Zn
1	4941.78	2909064.10	4511931.96	41184.14	336847.20
2	5942.07	1712703.17	9930015.32	56969.78	358343.76
3	4924.13	2161277.37	4478824.61	36510.56	286369.51
4	4923.67	2784446.15	4213026.84	33961.20	307047.65
The Threshold Effect Value (Macdonald et al., 2000)	9790	31600	35800	22700	121000
Possible Impact Value (Macdonald et al., 2000)	17000	197000	913000	36000	315000
The Serious Impact Value (Macdonald et al., 2000)	33000	110000	250000	75000	820000
Toxic Effect Value (Macdonald et al., 2000)	17000	86000	170000	61000	540000

Threshold impact value: Below this value, it rarely causes an adverse effect; Potential effect concentration: Above this value, it will cause an adverse effect; Serious impact quantity: Sediment is contaminated with heavy metals and above this value causes a serious adverse effect on organisms living in the sediment; Toxic impact quantity: When the sediment contains high concentration of heavy metals and causes toxic effects on the organisms living in this sediment (MacDonald et al., 2000).

DISCUSSION

Water, sediment, and fish are mainly used in the risk assessment of water pollution (Esmaeilzadeh et al., 2023; Singh et al., 2024). In our country and the world, many studies have been carried out using water, sediment, and fish gills to evaluate the accumulation of metals that have an important role in water pollution (Oymak et al., 2009; Tashi et al., 2022).

In our research, it was determined that Cu accumulation was higher in rainbow trout gills (32-37 cm) and Fe accumulation was statistically significant ($p<0.05$) in the gills of 600 - 1110 gram shabut in terms of weight. It was determined that Ni in rainbow trout gills and As in brown trout gills accumulated less than the other two fish species ($p<0.05$). It was determined that As and Cu accumulation in shabut fish was higher than in other fish species ($p<0.05$). In general, it was determined that rainbow trout showed less accumulation of metals except As in the gills of Shabbut and brown trout.

Oymak et al. (2009) determined Cu 1230±350 ppb, Fe 88850±29610 ppb, Ni 350±140 ppb, and Zn 13350±5120 ppb in 12 gill samples of shabut collected from Atatürk Dam by ICP-OES (inductively coupled plasma-optical emission spectrometry). In this study, which we conducted with ICP-MS device in Atatürk Dam in 2023, it was determined that the concentration of Cu, Fe, and Ni were less in different gill samples of shabut fish, while the concentration of Zn increased. This difference in the concentration of Cu, Fe, Ni and Zn in the studies conducted in the same fish species can be explained by the difference in time and analyzers. Tashi et al. (2022) reported that Fe, Ni, and Zn contents (ppb) were 177100±36170, 59±14 and 11720±4700, respectively, and there was a positive correlation between length and Fe accumulation in 9 trout gills in Punatsang Chhu river in Bhutan using ICP-OES device. In our study, there was no correlation between Fe accumulation and length in rainbow trout gills. In addition, Fe and Zn were determined less in our study results than Tashi et al. (2022). In our study, metal accumulations in gill samples were found to be Zn > Fe > Cu > As > Ni in rainbow trout, Zn > Fe > Cu > Ni > As in brown trout and Fe > Zn > Cu > As > Ni in Shabut fish, respectively. It is thought that this may be due to the concentration and duration of metal exposure of the samples used in the analysis, as well as the difference and sensitivity of the devices used.

Many researchers have stated that there are significant differences in heavy metal accumulation concentration between different species (Akgün 2007; Özvar 2020). They stated that the difference in metal accumulation in the gills may be due to the complexation of metals with mucus, which cannot be removed between the coverslips during the preparation of gill tissues for analysis, and the difference in the methods used (Yılmaz 2009). It has been reported that the reason for the difference in metal accumulations in fish may be due to differences in species, swimming behaviors, habitats, metabolic activity, feeding habits, age and size, and the methods and devices used in the analysis (Özvar 2020). Similarly, it is thought that the reason for the different the concentration of As, Cu, Fe, Ni and Zn in the gill tissues of different fish species may be due to the different methods and methods used in the analysis. In addition, Fe concentration in the gills of rainbow trout collected in Bilecik region were found to be high in our study. The heavy metal pollution detected in fish was thought to be due to the fact that Birecik region is more exposed to pollutants such as industrial and urban wastes.

Many studies have been conducted to determine heavy metal accumulation in Atatürk Dam water and sediment samples (Karadede and Ünlü 2000; Alhas et al., 2009; Ural et al. 2011; Uçkun et al 2017; Bayhan, 2021; Uçkun and Uçkun 2021). In our study, it was determined that Cu and Fe accumulated in sediment samples taken from the regions where fish gill samples were collected, was above the serious and toxic effect threshold values. In addition, it was determined that the concentration of Cu and Fe in the water samples taken from the regions where the sediment samples were taken, was high and Fe was above the limits set by WHO. Moreover, Cu (in all regions) and Fe (in regions 1 and 3) in the water samples were above the reference values according to TS regulation, but below the reference values of EU.

Karadede and Ünlü (2000) determined Fe > Zn > Cu > Ni concentration in Bozova sediment samples, Fe > Ni > Zn > Cu concentration in Akpınar sediment samples, Fe > Ni > Zn > Cu concentration in Bozova sediment samples, and Zn > Fe > Cu > Ni concentration in Akpınar sediment samples, respectively; Zn > Fe > Cu > Ni in Bozova water samples, Zn > Cu > Ni in Akpınar water samples, but they could not detect

Fe. They stated that Fe was the most abundant in sediment samples and Zn was more abundant in water samples. Alhas et al. (2009) stated that the concentration of Ni > Fe > Zn > Cu in Bagpinar (Adıyaman) and Akpinar (Adıyaman) sediment samples of Ataturk Dam were determined by ICP-OES device in Bagpinar (Adıyaman) and Cu > Ni > Fe > Zn in Akpinar (Adıyaman) and Cu and Ni could not be determined in water samples and Zn > Cu concentration were determined. Ural et al. (2011) reported that Fe was in higher concentrations in sediment (Fe > Ni > Zn > Cu) and water (Fe > Zn > Cu) samples collected from Atatürk Dam, but they could not detect Ni in water samples. Uçkun et al. (2017) determined the concentration of metals in sediment samples collected from Atatürk Dam by ICP-MS device as Zn > Fe > Cu > N i > As and Fe > Zn > Cu > Ni > As in water samples, respectively. In our study, Fe > Cu > Zn > Ni > As in sediment samples and Fe > Cu > Zn > As > Ni in water samples. Uçkun et al. (2017) also stated that heavy metals accumulated more in sediment samples than in water. In our study, it was observed that more metals accumulated in sediment than in water. Uçkun and Uçkun (2021) determined the concentration of Ni > Cu in sediment samples and Cu > Ni in water samples, respectively, with the ICP-MS device collected in Atatürk Dam lake. In a study conducted in Atatürk Dam waters, it was determined that the concentration of Zn and Ni was below 5 ppb and Fe was 200 ppb (Bayhan 2021). In our study, it was observed that the concentration of Zn (Region 2) and Ni in the water samples was compatible with the study of Bayhan (2021), but the concentration of Fe was higher than our study. In this study, more Fe was found in both water and sediment compared to other metals. When compared with Table 4, it was determined that the concentrations of Cu and Fe in sediment samples were above the serious and toxic effect values. It has been stated that heavy metal accumulation above the toxic effect value will have a negative effect on organisms living in the sediment (Mac Donalds. 2000).

Çağlan Kaya (2021) found the following average values for Cu and Zn in the collected water samples, respectively: 0.93 μg/L for Cu and 2.99 μg/L for Zn in Lake Beyşehir; 0.09 μg/L for Cu and 6.91 μg/L for Zn in Lake Eğirdir; 0.02 μg/L for Cu and 1.24 μg/L for Zn in Suğla Lake; 0.09 μg/L for Cu and 10.22 μg/L for Zn in Karataş Lake; 0.23 μg/L for Cu and 2.71 μg/L for Zn in Kovada Lake; 6.63 μg/L for Zn in Gölhisar Lake; 8.75 μg/L for Zn in Çivril Lake. In our study, the average values were 14.85 μg/L for Cu and 14.77 μg/L for Zn. In the same study Cu and Zn in collected sediment samples, respectively: 17.42 mg/kg for Cu and 53.62 mg/kg for Zn in Lake Beyşehir; 12.65 μg/L for Cu and 28.96 μg/L for Zn in Lake Eğirdir; 19.79 mg/kg for Cu and 50.06 mg/kg for Zn in Lake Çivril; 16. 30 mg/kg for Cu and 53.974 mg/kg for Zn; 25.86 mg/kg for Cu and 41.61 mg/kg for Zn in Karataş Lake; 50.90 mg/kg for Cu and 73.57 mg/kg for Zn in Kovada Lake; 23.42 mg/kg for Cu and 41.27 mg/kg for Zn in Gölhisar Lake. In our study, the average values were 23.91 mg/kg for Cu and 32.21 mg/kg for Zn. It is thought that the difference in the amount of accumulation detected may be due to regional differences.

Şengül (2024) found that Cu metal accumulated the most in the water of Gölova Dam Lake. In this study in Atatürk Dam Lake, it was observed that the accumulation level of Cu metal was high.

CONCLUSION

Although the Turkish Food Codex does not set a specific maximum limit value for the metals included in the study in fish meat, general food safety principles require that the concentration of metal accumulation should be at a concentration that does not harm human health. By keeping fish consumption at an appropriate concentration with a balanced and varied diet, it can be prevented from harming human health with substances such as Cu, Fe, Ni, Zn and As for which limit levels have not been determined yet. Atatürk Dam Lake, located on the Euphrates River, is home to various aquatic creatures and meets the fish and water needs of the people of the region. Monitoring of dam lake pollution is extremely important for environmental and public health. Considering all anthropogenic activities, the risk of metal accumulations in Atatürk Dam increasing in the future is quite high. Repetition of this study in Ataturk Reservoir at regular intervals is important for aquatic balance and human health.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: FT and OA contributed to the project idea, design and execution of the study. FT, OA and ZG contributed to the acquisition of data. FT and OA analysed the data. FT, OA and ZG drafted and wrote the manuscript. FT, OA and ZG reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was carried out at Harran University Reserch Animals Application Center. This research was approved by The Ethics Committee of the Faculty of Veterinary Medicine, Harran University (HRUHADYEK, Ref No: 229988, Tarih: 07/2023)

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RESEARCH ARTICLE

Immunomodulatory Effects of Inactive Parapoxvirus Ovis Administration to Pregnant Heifers on Colostrum and Calf Health

Halime KARA[1]*, Mehmet ŞAHAL[2]

[1]Laboratory and Veterinary Health Program, Health Vocational School, Ankara Yıldırım Beyazıt University, Ankara, Türkiye
[2]Department of Internal Medical, Faculty of Veterinary Medicine, Ankara University, Ankara, Türkiye

ABSTRACT

The aim of this study was to evaluate the effect of inactive Parapoxvirus ovis (IPPVO) immunomodulation protocol administration to pregnant heifers in the third trimester of pregnancy on colostrum quality, passive transfer success and calf health. Animal material of the study included 40 Holstein breed pregnant heifers and their calves, 20 of them was IPPVO group and rest 20 was control group. Totally 3 doses of 2 ml IPPVO (Zylexis, Zoetis, USA) was administered intramuscularly to study group in 25, 23 and 21 days before estimated birth date. Also control group was injected the same amount of isotonic NaCI %0.9 with IPPVO in the same days. Colostrum samples from heifers after birth and blood samples from calves 24-48 hours after birth were taken and sera were extracted. Immunoglobulin G (Ig G) concentrations of colostrum and serum samples were measured by using enzyme linked immunoassay (ELISA) method. Calves' health status were observed during first 30 days. There was no adverse effect was detected in heifers administered IPPVO during study procedure. IgG concentrations of heifers that received IPPVO was 59.64 mg/ml; that of control group was 59.62 mg/ml and the difference between groups was statistically insignificant ($p>0.05$). Average serum IgG concentration of calves born from heifers that received IPPVO was 32.6 mg/ml; that of control group was 28.7 mg/ml. When the yielded results of daily health status controls of two claves groups evaluated, no statistically significant difference was detected in terms of developing any disease and calf deaths ($p>0.05$).

Keywords: Colostrogenesis, Holstein Pregnant Heifer, IgG, Immunomodulation, Inactive Parapoxvirus Ovis

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Gebe Düvelerde İnaktif Parapoxvirus Ovis Uygulamasının Kolostrum ve Buzağı Sağlığı Üzerine İmmunmodulatorik Etkileri

ÖZ

Sunulan bu çalışmanın amacı sığırlarda gebeliğin son döneminde uygulanan inaktif Parapoxvirus Ovis (İPPVO) immunmodulasyon protokolünün kolostrum kalitesi, pasif transfer başarısı ve buzağı sağlığı üzerine etkilerinin araştırılmasıdır. Çalışmanın hayvan materyalini 20 kontrol ve 20 İPPVO grubu olmak üzere 40 adet Holstein ırkı gebe düve ve buzağıları oluşturdu. İPPVO grubuna muhtemel doğum tarihlerine 25, 23 ve 21 gün kala, toplamda 3 doz, 2 ml lik İPPVO preparatı, intramusküler (Zylexis, Zoetis, USA), kontrol grubuna ise aynı planda ve hacimde izotonik NaCI %0.9 enjeksiyonu yapılmıştır. Doğumla birlikte annelerden kolostrum örnekleri, buzağılardan ise doğumdan 24 – 48 saat sonra kan alınarak serumları çıkartılmıştır. Kolostrum ve serum örneklerinde ELISA yöntemi kullanılarak IgG konsantrasyonları ölçülmüştür. Buzağılar ilk 30 günlük dönemde sağlık yönünden takip edilmiştir. Çalışma kapsamında İPPVO preperatı kullanılan düvelerde herhangi bir yan etki gözlenmemiştir. İPPVO uygulanan düvelerin kolostrum IgG konsantrasyonları 59.64 mg/ml, kontrol grubunda ise 59.62 mg/ml olarak ölçülmüş ve aradaki fark istatistik yönden anlamsız bulunmuştur ($p>0.05$). İPPVO uygulanan düvelerden doğan buzağıların ortalama kan serumu IgG konsantrasyonu 32.6 mg/ml, kontrol grubunda 28.7 mg/ml olarak ölçülmüştür. Çalışma kapsamında iki grup buzağıları arasında yapılan günlük sağlık kontrolleri sonucunda toplanılan veriler değerlendirildiğinde hastalığa yakalanma ve buzağı ölümleri yönünden de anlamlı bir farklılık tespit edilmemiştir ($p>0.05$).

Anahtar Kelimeler: Kolostrogenezis, Gebe Holstein Düve, IgG, İmmunmodülasyon, İnaktif Parapoxvirus Ovis

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ORCID ID; *HK: 0000-0001-8202-5882, MŞ: 0000-0003-3189-5778*

*Corresponding author e-mail: hkara@aybu.edu.tr

INTRODUCTION

Passive transfer (PT) is a process based on absorption and transfer to systemic circulation of immune components with nutritional substances in colostrum of cow that in puerperium through digestive system of calf (Zarcula 2008; Aydoğdu et al 2019). Calves with passive transfer deficiency (PTD) have 1,6 fold increased risk of developing any disease and their average daily live weight gain 0,06 kg less than normal calves until weaning (Kara and Ceylan 2021). While death rate of calves having less than or equal to 2 mg/ml blood IgG concentrations is over than 50%, mortality of claves having more than 18mg/ml blood IgG concentrations is less than 5% (Besser and Gay 1994). Three Q formula (Quantity, Quality, Quickly) is important for succeeding passive transfer (Jaster 2005). Also, PT success can be affected by administration method of colostrum, presence of cow, metabolic diseases of cow, various physiologic behaviors of calves and cows and calving season (Gökçe and Erdoğan 2013).

Because it is known that IgG concentrations in colostrum and calf blood is correlated, only adequate quality of colostrum use is required (Quigley 2007). PTD risk is 15,422 fold increased in calves fed with poor quality colostrum (Kara and Ceylan 2021). Colostrum quality can be affected by age, breed, vaccination status, (dry period) nutrition, health status, body condition score of the cow, the duration of dry period, calving season, milk leakage from breast in prepartum period, the time between calving and colostrum milking (Kaygısız and Köse 2007; Godden 2008; Gulliksen et al 2008; Cortese 2009; Conneely et al 2013; Phipps et al 2017; Kara et al 2020; Kara and Ceylan 2021; Kurtdede et al 2022). It is known that IgG concentrations in colostrum is directly related to that of mother's blood serum, immunomodulation and immunosuppression applications during colostrogenesis affect IgG concentrations in colostrum. Long acting corticosteroid use in the period of colostrogenesis reduces the IgG concentrations in colostrum (Gökçe ve Erdoğan 2013). Some of the immonumodulatory applications to mother in dry period in order to increase the colostrum quality are levamizol, natural zeolite, Saccharomyces cerevisiae, Corynebacterium cutis, Propionobacter acnes, mannan oligosaccharide, clinoptilolite, vitamin E and some minerals as selenium (Se) (Heinrichs et al 2003; Şentürk er al 2003; Zarcula et al 2008; Waldner and Rosengren 2009; Hall et al 2014; Çalık 2016).

Immunomodulatory term is used for substances that have stimulatory, suppressive and regulatory effects on the immune system (Kart et al 2010). Applications that immunostimulants used for is named as immunostimulation (Thacker 2010). Immunomodulation is classified in two groups as "specific" that is special for a certain pathogen and "non-specific" that is not directed against a specific pathogen (Galeotti 1998; Dhama et al 2015). While vaccines prepared for a certain agent constitute specific immunomodulators, various microorganisms or their metabolites, animal and herbal extracts, complex carbohydrates, nutritional factors, cytokines and various chemicals are some of non-specific immunomodulators (Galeotti 1998; Pirofski and Casadevall 2006; Yanar and Aktaş 2021; Atlı and Şimşek 2022). Immunomodulation is applied for generating immunity against infective agents, maintaining that the immunity is faster, more effective and longer lasting; alleviating the stress with immunosuppressive effects (Dhama et al 2015). Non-specific immunomodulators that are applied in veterinary clinical practice are used especially for supporting immune system additional to therapy, augmentation of effect of vaccines or protection and treatment of cancer cases (Kart et al 2010; Mohamed et al 2013). The most used non-specific immunomodulators applied in veterinary practice are vitamin C, vitamin D, vitamin E, vitamin A and its precursor βeta carotene, various amino acids, selenium, zinc, beta glucan, levamizol, inactive parapoxvirüs ovis (IPPVO), lysate of Corynebacterium cutis and Propionibacterium acnes and echinacea (Kim et al 2007; Cao et al 2015; Aydın and Aktaş 2021).

PPVO, in other words Orf virus (OV, ORFV), is the pathogen of a zoonotic disease named as "ecthyma disease of sheep" that is so contagious and characterized of crusted papulles especially in mucous membranes in goats and sheep (Nandi et al 2007). Results of in vivo and in vitro studies reveal that PPVO proteins induce immunomodulatory activity when used as combination with inactive vaccine virus particules (Fleming and Mercer 2007). As a strong immunomodulatory, PPVO activates most of the cells of the natural immune system via mediating rapidly the responses of humoral and cellular immunity (Orta et al 2020). By way of these activations, secretion of various chemokines and cytokines are induced. Whitin in immunoreaction, neutrophyles, natural killer (NK) cells and dentritic cells (DC) migrate to infection area (Wang and Luo 2018). IPPVO preparates derived from chemically inactivated PPVO are licensed as paraimmunity activator and widely used as immunomodulatory in veterinary field (Fachinger et al 2000; Adams and Horohov 2013). It was reported that the immunomodulatory effect was observed in the controls performed 4 days after IPPO injection in cattle (Erbasan and Mamak 2023).

The aim of this study was to evaluate the effect of inactive Parapoxvirus ovis (IPPVO) immunomodulation protocol administration to pregnant heifers in the third trimester of pregnancy on colostrum quality, passive transfer success and calf health.

MATERIALS and METHODS

Ethical approval

The study was conducted upon the approval of Ankara University Local Ethics Committee of animal experiments with approval number of 2019-5-44.

Animal Material

The animal material of the study consisted of 40 Holstein heifers and their offspring reared in a private farm in Ankara. In order to eliminate the effects of breed, age and nutrition on colostrum quality, heifers at the same age, breed, fed in the paddock with the same ration included in the study. Heifers were applied immunomodulation three month period including February-April dates. Animals were selected as control and study group respectively. Heifers detected any disease (like lameness) during the pre-study examination excluded from the study. Animals having body condition score 3.5±0.5 included in the study. Newborn calves were fed with amount of 12% of their live weight colostrum via bottle in the first 4 hours.

Study plan

Artificial insemination records and estimated birth days of animals which included in the study were determined and noted. Pregnancy period was accepted as 280 days. Totally 3 doses of 2 ml Zylexis (Zoetis, USA) that includes IPPVO D1701 strain were administered intramuscularly to heifers in the study group in 25, 23 and 21 days before estimated birth date. Also, heifers in the control group was injected intramuscularly the same amount of isotonic NaCI %0,9 with IPPVO as placebo in 25, 23 and 21 days before estimated birth date. Heifers were milked with portable single automatic milking machines within the first 2 hours following calving. A sample of 50 ml colostrum was taken into tubes before feeding and stored in a -20 °C freezer. Blood samples from calves were taken between 24-48 hours of age (Figure 1). Colostrum and blood sera were stored at -20 °C freezer.

until IgG analysis. IgG analysis was conducted with commercial enzyme linked immunoassay (ELISA) kits (Bovine Immunglobulin ELISA kit/Bio-X, Belgium-Lot No: IG19B04) according to the company's directions.

Statistical analysis

The data were summarized in tables and expressed as descriptive statistics; scatter plots were used. Independent samples t test was used for comparing blood IgG concentrations of IPPVO and control groups and Mann-Whitney U test was used for comparing morbidity (diarrhea, respiratory system infections, omphalitis and joint infections) rates of calves and colostral IgG concentrations between groups. While Pearson correlation analysis was being used for determining the relation between evaluated parameters, Chi-square test was used for comparison of groups in terms of death and morbidity rates of calves. All mentioned statistical analysis was conducted by using IBM SPSS 25 package program and significance level was accepted as $p<0,05$.

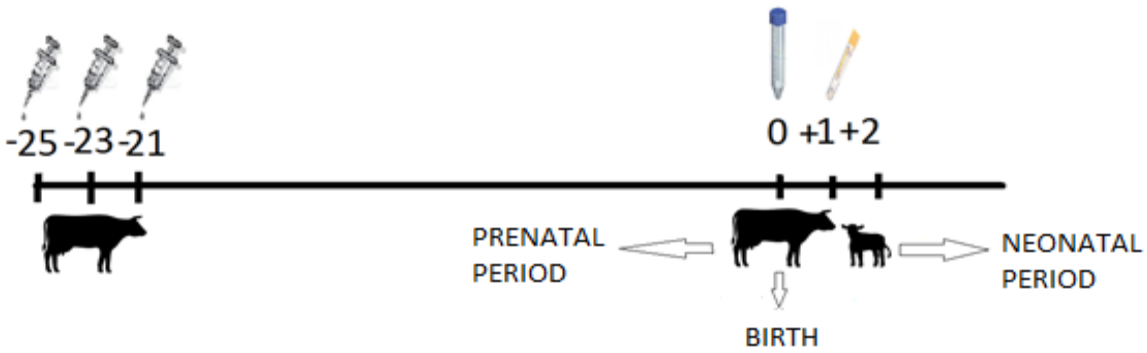

Figure 1: The days when the treatment was applied to the heifers and the days when blood and milk samples were collected from the heifers and calves.

RESULTS

There was no adverse effect reported in pregnant heifers that applied IPPVO commercial preparate (Zylexis, Zoetis / USA) via intramuscular injection 25, 23 and 21 days before birth.

Within the scope of the study, colostrum samples were taken from a total of 40 heifers, 20 in the control and 20 in the study group (IPPVO). While mean colostral IgG concentration of control group was measured as 59.62 mg/ml, it was found as 59.64 mg/ml in study group (Table 1). There was no statistically significant difference between groups ($p>0.05$).

Although each group contained 20 heifers, as a result of a heifer's twin birth, 21 calves was attained from IPPVO group. Within the scope of the study, blood samples were taken from newborn calves 24-48 hours after birth and blood samples of calves died within

first 24 hours after birth excluded from the study. One calf from control group and 4 calves from IPPVOP group died within first 24 hours after birth. Mean blood IgG concentration of calves in control group (n:19) was 29,9 mg/ml; that of calves in IPPVO group (n:17) was 32.6 mg/ml (Table2) and). the difference was statistically insignificant (p>0.05
Totally 41 calves were born, 20 of them was in the control group and 21 of them was in IPPVO group. In the first 30 days period, 7 of 41 calves died (17.1%); 3 of them (15%) were from control group, 4 of them were (19%) from IPPVO group. While all of deaths was occurred within firs 24 hours in IPPVO group, 1calf died within first 24 hours and 2 calves died 30 days period after 24 hours in control group (Table 3). When calf deaths evaluated in the farm, there was no statistically significant difference defined between groups (p>0.05)

Table 1. Colostral IgG concentrations (mg/ml) of heifers by groups

	Group	N	Mean	Median	SD	SE	p
Colostrum IgG (mg/ml)	Control	20	59,62	42,7	35	7,84	**0,947**
	IPPVO	20	59,64	51,7	34,1	7,63	

*N: Animal count in the group, SD: standard deviation, SE: standart error.

Table 2. Blood IgG concentarions (mg/ml) of calves 24-48 hours after birth

Independent Sample T-Test

	Group	N	Mean	Median	SD	SE	p
Blood IgG (mg/ml)	Control	19	29,9	28,7	12,1	2,77	**0,486**
	IPPVO	17	32,6	33,3	10,8	2,63	

*N: Animal count in the group, SD: standard deviation, SE: standart error.

Table 3. Number of dead and alive calves in the first 24 hours and between 1 and 30 days.

	Groups			
Deaths	**Control 20 calves**	**IPPVO 21 calves**	**Total**	**p**
First 24 hours	1 (%5)	4 (%19)	5 (%12,2)	
1 – 30 days	2 (%10)	0 (%0)	2 (%4,9)	**0,192**
Total	3 (%15)	4 (%19)	7 (%17,1)	
Alive	17 (%85)	17 (%81)	34 (%82,9)	

When calves that can be followed after receiving passive transfer compared, no statistically significant difference was detected between groups (p>0.05) (Table 4).
Within the scope of study, daily health checks were carried out on newborn calves for 30 days. Calves that died in the first 24 hours were excluded from evaluation during health checks, as their passive transfer success could not be evaluated. After evaluation of calves that had treatment at least for one of the following diseases: diarrhea, respiratory infections, fever, omphalitis and arthritis in first 30 days, it was found out that 11 of 17 calves (57.9%) in control group and 11 of 19 calves (64.7) in IPPVO group and totally 22 of the 36 calves (61.1%) had at least one of the mentioned diseases signs (Table 5). When evaluated in terms of the occurrence of at least one of the diseases, the statistical difference between the groups was found to be insignificant (p>0.05).
Nine calves (47.4%) from control group and 6 (35.3%) calves from IPPVO group and totally 15 calves (41.6%) had diarrhea in the first 30 days period (Table 5). No statistically significant difference was detected between groups in terms of newborn calf diarrhea (p>0.05).

Table 4. Comparison of alive and dead calves in terms of passive transfer success

	Groups			
Passive Transfer	**Control 19 calves**	**IPPVO 17 calves**	**Total**	**p**
Died after 24 hours	2 (%10)	0 (%0)	2 (%4,9)	**0,487**
Alive	17 (%89,5)	17 (%100)	34 (%94,4)	

Table 5. Disease occurrence status in calves in the first 30-day period

	Groups			p
Disease types	***Control***	***Ippvo***	***Total***	
Diarrhea	*9(47,4%)*	*6(35,3%)*	*15(41,6%)*	***0,192***
Respiratory disease	*2(10,5%)*	*5(29,4%)*	*7(19,4%)*	***0,153***
Occurrence status of at least one disease	*11(57,9%)*	*11(64,2%)*	*22(61,1%)*	***0,144***

Additionally, 2 calves (10.5%) from control group and 5 calves (29.4%) from IPPVO group and totally 7 calves (19.4%) represented respiratory infection signs in the first 30 days period (Table 5). Also, any statistically significant difference wasn't found between groups (p>0.05).

When the effect of prolonging the pregnancy period on colostrum quality both in the control and IPPVO group was evaluated in the work plan prepared with reference to 280 days in heifers; it was determined that the relationship between these continuous variables showed a negative trend (r=-0.115) (r=-0.254), but the correlation between both values was not statistically significant (p=0.613) (p=0.267) (Figure 2).

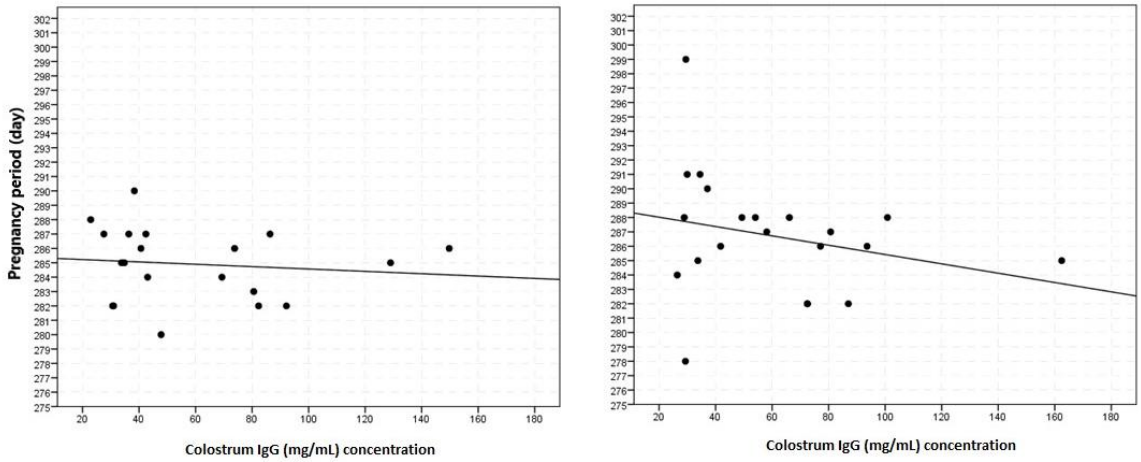

Figure 2. Evaluation of pregnancy period and colostrum IgG (mg/ml) concentration in control group (left) and IPPVO group (right) heifers.

DISCUSSION

Although the success of PT and the importance of colostrum are well known, the prevalence of PTD is still quite high (Gökçe and Erdoğan 2013; Güzelbekteş and Aydoğdu 2024). When the factors affecting the success of PT are considered, among many variables, the amount, quality, and timing of colostrum given to the calf (3 Q rule) stand out (Zarcula et al 2008).

Colostrum quality has a diverse place among all these variables. Also; it can be affected by variables such as the cow's age, breed, vaccination applications in dry period, nutrition, season, health status, dry period, and the time between colostrum milking and birth (Gökçe and Erdoğan 2013). It was reported that colostral IgG concentration of Holstein cows varies between 99 mg/ml and 186 mg/ml and average of 76 mg/ml (Swan et al 2007). According to the results of different studies, it has been revealed that the colostrum average IgG concentrations of first-born Holstein heifers are between 69.66 mg/ml and 119 mg/ml (Saucedo-Quintero et al 2004; Kehoe et al 2011; Aydoğdu and Güzelbekteş 2018; Kara and Ceylan 2021). In the presented study, the average colostrum IgG concentrations were measured as

59.62 mg/ml in the control group and 59.64 mg/ml in the IPPVO group. Although the average IgG concentration measured for both groups was above the 50 mg/ml, which is the threshold value of quality colostrum, it was found to be low compared to other studies on first-born Holstein heifers (Gökçe and Erdoğan 2013). For this reason, it would be beneficial to carry out additional practices to increase the overall colostrum quality in the farm where the study was conducted.

It is known that there is a linear relationship between colostrum quality, that is, the IgG concentration in its content, and the IgG level in the cow's blood serum. Because of this, studies have been carried out for many years to increase the colostrum quality and therefore the success of PT by targeting the colostrogenesis period and strengthening the immune systems of cows (Gökçe and Erdoğan 2013). According to results of the studies conducted by Şentürk et al. (2003) on pregnant heifers and Krakowski et al. (1999) on pregnant mares revealed that levamisole protocols targeting colostrogenesis increased colostrum quality and PT success (Krakowski et al 1999; Şentürk and Polat 2003).

In two different studies, it was reported that IgG levels in colostrum and calf blood serum increased with vaccinations against calf diarrhea at the end of pregnancy (Güngör and Baştan 2004; Sancak and Gül 2021). Çalık (2016) applied commercially available Corynebacterium cutis lysate to pregnant heifers and Turna Yılmaz et al. (2011) targeted colostrogenesis in pregnant sheep, and they reported that colostrum quality and PT success increased (Turna Yılmaz et al 2011; Çalık 2016). In this study, a commercially available IPPVO preparation was administered for 3 doses at the end of pregnancy, similarly targeting colostrogenesis, but it was determined that the IgG concentrations in colostrum and calf blood serum were not different from the control group. When other immunomodulation studies targeting colostrogenesis in order to increase colostrum quality and therefore PT success in calves are examined; It has been interpreted that the reason why the findings of this study differ from other immunomodulators that significantly increase colostrum quality may be related to the mechanism of action of the applied immune stimulant and the immune stimulation protocols.

In a research passive transfer success and calf health in different dairy farms were evaluated and it was reported that the average blood serum IgG level of calves were 19±10 mg/ml (Johnson et al 2017). The rate of calves suffering from diarrhea in the period until weaning varied between 24.1% and 74.4% among farms with the average recorded as 48.2%. In the same period, the rates of respiratory system diseases in calves were found to be between 20.4% and 77.8%, with the average as 45.9%. It has been determined that the rate of calves suffering from diarrhea is especially widespread in the first 2 weeks, while respiratory system diseases appear in the 4–9-week period. In the same study, calf mortality rates were recorded as 3.1% in the first month.

Lombard et al. (2020), in their evaluation of 2360 heifer calves on 103 farms; They reported PTY as 12%, the rate of having at least one disease as 34.3, and mortality as 3.2% (Lombard et al 2020). Urie et al. (2018), in their study involving 13 states and 104 enterprises across the USA, reported calf deaths as 5% until the weaning period in 2545 calves. They reported that at least one morbidity case was detected in 33.8% of 2545 calves, diarrhea was detected in 17.2% of the calves, and pneumonia was detected in 27% (Urie et al 2018). There is no large population study examining calf deaths in Turkey. Şahal et al. (2018) state that neonatal calf death rates in Turkey are estimated to be around 15%, that calf death rates in European countries are around 10 - 15%, and that this rate can be reduced to 5% in farms with good manegement (Şahal et al 2018). Akyüz et al. (2017) report that calf deaths are 10% in state farms (General Directorate of Agricultural Enterprises) and more than 50% in commercial farms (Akyüz et al 2017). Yüceer and Özbeyaz (2008) reported that 81 calves from 90 cows were born alive, and 5 calves (6.17%) died due to diarrhea and respiratory system infections in the first month of life (Yüceer and Özbeyaz 2010). In the evaluation made by subtracting the deaths occurring in the first 24 hours within the scope of this study, the calf mortality rate was recorded as 10% (2/19) in the control group and (0) in the IPPVO group. When all calves included in the study were evaluated, the mortality rate in the farm was found to be 4.9% (2/36). The results obtained in the study in terms of calf deaths were found to be well below the country average and were found to be compatible with good management averages. It was understood that the calf mortality rate in the farm was below 5% and that it met the targeted success criteria. Based on colostrum IgG averages, PT success was found to be compatible with literature information (Şahal et al 2018). When the diseases that occurred in the calves included in the study during the research were evaluated; In the control group, 47.4% had diarrhea, 10% had respiratory system infection, and 57% had at least one disease of different organ system. In the IPPVO group, diarrhea was observed in 35%, respiratory system infection was observed in 29.4%, and at least one disease was observed in 64.7%. Considering the entire population together, during the 30-day monitoring period, 41.6% of the 36 calves had diarrhea, 19.4% had respiratory system infection, and 61.1% had at least one disease. When the results obtained are evaluated; It was understood that the cases of diarrhea in calves were compatible with the literature information and the rate of respiratory system infection was lower than some of the literature data. This is because; It has been understood that diarrhea is more intense in the first month of life in calves, and respiratory system

infection is more common in calves, according to the literature, especially in the second month and during the weaning period. When the case of at least 1 disease in calves was evaluated, it was determined that the disease rate increased due to other diseases, especially omphalitis and trauma, but the results obtained were compatible with the literature information (Yüceer and Özbeyaz 2010; Sancak and Gülhan 2021; Şimşek and Akkan 2021).

In previous studies conducted in the same region, it was reported that the average gestation period for Holstein heifers ranged between 277.6 - 281.5 days (Koçak et al 2007; Koçak et al 2008; Şahin and Ulutaş 2010). In the presented study, it was determined that the pregnancy of 1 heifer lasted less than 280 days, the pregnancy of 1 heifer lasted 280 days, and the pregnancy of 38 heifers lasted longer than 280 days. The average gestation period in the IPPVO group was recorded as 286.85 days, and in the control group the average pregnancy period was 284.9 days. Hurley and Theil (2011) mention that IgG has a half-life (Hurley and Theil 2011). Although the half-life of IgG varies between 1-3 weeks, the half-life of IgG1, which is the predominant IgG subtype in colostrum, is reported to be shorter than IgG2 (Cervenak and Kacskovics 2009). Husband et al. (1972) reported that the average half-life of IgGs was approximately 16 days (Husband et al 1972). It was thought that the prolongation in the birth dates of most heifers within the scope of the study could be effective in the formation of the targeted IgG concentrations in the trial group. Even though there was a negative trend between those two parameters, it was not statistically significant because of the small number of trial animals.

CONCLUSION

As a result, it was observed that the IPPVO protocol applied to heifers at the end of pregnancy did not have any side effects related to pregnancy, and the immunomodulation protocol applied did not have a positive effect on colostrum quality and blood passive transfer success of calves. In addition, it was determined that the applications did not have any effect on calf sickness rates and calf mortality. However, it was observed that prolonged gestation period had a negative effect on colostrum quality and this effect was not statistically significant in the studied population.

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Ethical approval: The study was conducted upon the approval of Ankara University Local Ethics Committee of animal experiments with approval number of 2019-5-44

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RESEARCH ARTICLE

Investigation of Novel Hematological Index Variations in Cats Naturally Infected with Feline Panleukopenia Virus

Ömer AYDIN[1]*

[1]*Department of Internal Medicine, Faculty of Veterinary Medicine, Atatürk University, Erzurum, Türkiye*

ABSTRACT

Feline panleukopenia is a viral infection that impacts cats of all age groups, with particularly high mortality rates observed around the age of 3 months. The disease spreads through the fecal-oral transmission route. This study aims to evaluate the levels of various inflammatory hematologic indices—specifically, the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), systemic inflammation response index (SIRI), and systemic immune-inflammatory index (SII)—in cats naturally exposed to Feline Panleukopenia Virus (FPV), and to assess the potential of these indices as markers of inflammation. Two groups were included in the study: a control group consisting of 20 healthy cats and an experimental group consisting of 40 cats showing symptoms of anorexia, vomiting, and/or diarrhea, diagnosed with FPV using a rapid test kit. All cats in both groups were between 0 and 3 months old and represented a variety of breeds and genders. No significant differences were found between the groups for the NLR and LMR values (with p-values of 0.054 and 0.627, respectively). However, the PLR was significantly higher in the FPV-infected group compared to the control group ($p<0.001$). In contrast, the SIRI ($p<0.001$) and SII ($p=0.002$) values were notably lower in the FPV-infected cats. In conclusion, this study revealed that there were significant differences in haematological indices between the two groups and PLR, SIRI and SII were important markers to reflect the inflammatory status in FPV infection.

Keywords: Feline panleukopenia virus, Lymphocyte to monocyte ratio, Neutrophil to lymphocyte ratio, Systemic immune-inflammation index, Systemic inflammatory response index

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Feline Panlökopeni Virüsüyle Doğal Enfekte Kedilerde Yeni Hematolojik İndeks Varyasyonlarının Araştırılması

ÖZ

Feline panlökopeni, tüm yaş gruplarındaki kedileri etkileyen viral bir enfeksiyondur ve özellikle 3 aylık civarında yüksek ölüm oranları görülür. Hastalık fekal-oral bulaşma yolu ile yayılır. Bu çalışmanın amacı, doğal olarak Feline Panlökopeni Virüsüne (FPV) maruz kalan kedilerde çeşitli inflamatuar hematolojik indekslerin (özellikle nötrofil-lenfosit oranı (NLR), trombosit-lenfosit oranı (PLR), lenfosit-monosit oranı (LMR), sistemik inflamasyon yanıt indeksi (SIRI) ve sistemik immün-inflamatuar indeks (SII) düzeylerini ve bu indekslerin inflamasyon belirteçleri olarak potansiyelini değerlendirmektir. Çalışma 20 sağlıklı kediden oluşan bir kontrol grubu ve anoreksi, kusma ve/veya ishal semptomları gösteren ve hızlı test kitiyle FPV tanısı koyulmuş olan 40 kediden oluşan bir deney grubu olarak iki gruptan oluştu. Her iki gruptaki kediler 0 ila 3 aylık yaşta, çeşitli ırk ve cinsiyetlerden oluştu. Gruplar arasında NLR ve LMR değerleri açısından anlamlı bir fark bulunmamıştır (p-değerleri sırasıyla 0,054 ve 0,627'dir). Ancak, PLR değeri FPV ile enfekte grupta kontrol grubuna kıyasla anlamlı derecede yüksekti ($p<0,001$). Buna karşılık, SIRI ($p<0,001$) ve SII ($p=0,002$) değerleri FPV ile enfekte kedilerde belirgin şekilde daha düşüktü. Sonuç olarak, bu çalışma iki grup arasında hematolojik indekslerde önemli farklılıklar olduğunu ve PLR, SIRI ve SII'nin FPV enfeksiyonunda inflamatuar durumu yansıtmada önemli belirteçler olduğunu ortaya koymuştur.

Anahtar kelimeler: Feline panlöpeni virüs, Lenfosit-monosit oranı, Nötrofil-lenfosit oranı, Sistemik immun inflamasyon indeksi, Sistemik inflamasyon yanıt indeksi

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ORCID ID; *ÖA: 0000-0001-9444-1904*

*Corresponding author e-mail: aydinomer@atauni.edu.tr

INTRODUCTION

Feline Panleukopenia Virus is a highly transmissible and often deadly viral disease in felines, capable of surviving in the environment for extended durations. As a member of the parvovirus family, FPV consists of non-segmented, single-stranded deoxyribonucleic acid, with its genome typically spanning approximately 5.000 nucleotides. These viruses are among the smallest, with a diameter ranging from 18 to 28 nm (Leppard et al. 2007). FPV can result in a significantly high mortality rate, particularly in young kittens (Wolfesberger et al. 2012). The primary causes of death in FPV are severe sepsis and endotoxemia, along with significant erythrocyte imbalance (Decaro et al. 2005; Mylonakis et al. 2016; Gulersoy and Naseri 2022). The areas with the highest viral replication are the intestinal crypts, lymphoid tissue, and bone marrow, where mitotic activity is most prominent (Tuzio 2021). Following an incubation period of 2 to 10 days, acute cases of Feline Panleukopenia may present clinical signs including lethargy, anorexia, fever, vomiting, and diarrhea. However, in peracute cases, cats may suddenly die without developing any characteristic symptoms. Additionally, the disease may present in relatively mild or clinically indeterminate forms (Battilani et al. 2011). As the virus spreads throughout the body, affecting all tissues, including lymphoid tissues, a viremia ensues, potentially resulting in immunosuppression (Parrish 1995; Truyen and Parrish 2000). Therefore, cats with FPV often exhibit abnormal leukogram findings. The most common hematological finding in FPV is leukopenia, characterized by a decrease in white blood cell (WBC) count, typically presenting with neutropenia and lymphopenia (Shelton et al. 1990). Several mechanisms contribute to the development of neutropenia, including bone marrow suppression caused by the virus, increased utilization of neutrophils in tissues due to the inflammatory response, and neutrophil sequestration resulting from endotoxemia associated with FPV (Weiss and Wardrop 2010). Prognostic markers have attracted considerable attention in both veterinary and human medicine. Recently, the reliability of hematologic ratios has been explored as easily accessible and cost-effective prognostic and diagnostic tools for various inflammatory conditions. LMR and NLR are critical biomarkers as they reflect the balance of the innate and acquired immune systems. These markers are also involved in the pathogenesis of inflammation disorders (Zahorec 2001; Shumilah et al. 2021). In human medicine, inflammatory markers have a broad research scope across various disease conditions (Zahorec 2001; Pacheco-Barcia et al. 2020; Shumilah et al. 2021; Ertan et al. 2022; Kamiya et al. 2022). SII is derived from a formula that utilizes the platelet, lymphocyte, and neutrophil counts determined through a complete blood count. It is presented as an important indicator of systemic inflammatory conditions (Yazlık et al. 2022). In human medicine, it has been used as an indicator of inflammatory status in tumor-related diseases (Sun et al. 2019). Although there have been several studies investigating various hematologic indices in cats with inflammatory, autoimmune, and other diseases, no study has been found that investigates the values of LMR, SIRI, and SII in cats with FPV. The hypothesis of this study is to explore the extent to which the levels of hematologic indices may change in the case of an inflammatory disease such as FPV.

Thus, the aim of this study is to investigate the biomarkers such as NLR, LMR, PLR, SIRI, and SII, which are frequently used in human medicine but relatively new in veterinary practice, in cats infected with FPV and to determine their blood levels.

MATERIALS and METHODS

Animal

The study was authorized by the Local Ethics Committee of Atatürk University (Decision Number: 2024/13). The animals included in the study, both experimental (40 cat) and control groups (20 cat), were cats aged 0-3 months from different breeds and genders. The disease in the naturally infected animals with FPV was diagnosed through clinical, hematological, and rapid diagnostic kit tests (Asan Easy Test®, Korea), while the healthy control group consisted of clinically and hematologically healthy cats brought in for routine vaccination.

Inclusion/Exclusion Criteria for FPV

The study included cats displaying clinical signs of FPV, such as depression, anorexia, diarrhea, vomiting, and dehydration. According to the anamnesis, the affected kitten's illness was reported to have occurred within a 3-5 day period. Kittens that had received treatment at another private veterinary clinic or institution were excluded from the study. Immunochromatographic tests, such as GenBody FeLV Ag/FIV Ab Combo (Korea) and Asan Easy Test® (Korea), were used to detect and eliminate infections like feline leukemia virus, feline immunodeficiency virus, and feline coronavirus, which are commonly observed in kitten populations. Kittens infected with these viruses were excluded from the study. Additionally, cats diagnosed with ascaridiosis were excluded. Cats vaccinated with a polyvalent vaccine (including a panleukopenia strain) within the last 3 weeks were also excluded from the study.

FPV Rapid Test Kit Procedure

Before the test, all specimens were allowed to reach room temperature, approximately. The test kit was removed from its protective casing and placed on a

flat surface. Fecal samples were collected from four different regions or directly from the feline colon. The collected fecal sample was placed into the assay solution in a tube, and the sample was mixed with the swab until it dissolved. 3–4 drops (approximately 100 μL) of the solution were added to the sample well. The test results were read within 10 minutes.

Feline Coravirus Ab Test Kit Procedure

All specimens were allowed to reach room temperature. Subsequently, the test kit was removed from its protective casing and placed on a flat surface. Using the capillary tube provided in the kit, 10 μL of whole blood (or 5 μL of serum or plasma) was transferred into the assay solution tube. The specimen was gently mixed with the diluent buffer by stirring with the capillary tube. Afterward, the mixture was transferred from the tube using the disposable dropper provided in the kit, and three drops of the solution were added to the test device. The test results were interpreted within 10 minutes.

FeLV Ag/FIV Ab Test Kit Procedure

All samples were placed in the test devices and allowed to come to room temperature before testing. The test kit was removed from its protective casing and placed on a flat surface. Using the provided capillary tube, 20 uL of whole blood was collected and 1 drop of blood was added to the specimen well. Then 3 drops of assay solution were added vertically to the sample well. So the test results were interpreted within 10-15 minutes.

Collection and Analysis of Blood Samples

For haematological analysis of each animal in both control and experimental groups, 1.5 mL blood samples were taken from the cephalic vein (*vena cephalica antebrachii*) and transferred into tubes containing EDTA (Hema-Tube EDTA K3, Turkey). These blood samples were analysed very quickly using an Abacus junior Vet 5 (Hungary) haemogram device.

Hematological Analyses

Hematological indices, including LMR, PLR, NLR, SII, and SIRI, were determined and presented using the absolute values obtained from the hemogram analyzer, as described below:

NLR: Neutrophil cont/Lmphocyte count

PLR: Platelet count/Lymphocyte count

LMR: Lymphocyte count/Monocyte count

SIRI: Monocyte count x Neutrophil count/Lymphocyte count

SII: Neutrophil count x Platelet count/Lymphocyte count (Hrubaru et al. 2022).

Statistical Analyses

In a study conducted on cats with panleukopenia, a power analysis of the NLR hematological index values was performed (effect size=1.12; α=0.05, and power=95%). The results indicated that at least 18 animals per group would be required for the study to achieve statistically meaningful results (Yanar 2024). SPSS software version 27.0.1 was utilized for the data analysis. The normality criteria of the data were determined by Shapiro-Wilk normality test. Group comparisons of the normally distributed data were made by Independent samples t-test and group comparisons of the non-normally distributed data were made by Mann-Whitney U test. The significance criterion for group comparison was accepted as $p<0.05$.

RESULTS

Clinical Presentation

It has been determined that the respiration rate, pulse rate, and rectal temperature in cats with FPV are significantly higher compared to the control group cats ($p<0.001$) (Table-1). Additionally, clinical signs such as anorexia, lethargy, vomiting and/or diarrhea in some cats, and abdominal pain on palpation have also been observed in cats with FPV.

Table 1. Clinical parameter indicators between groups

	Groups		
Parameters	**Control (n=20)**	**FPV (n=40)**	**P value**
	$\bar{x}\pm$sd	**$\bar{x}\pm$sd**	
HR (Heartbeats/minute)	134.30±9.14	152.47±8.90	<0.001
RR (Respirations/minute)	31.90±5.87	57.37±5.39	<0.001
	Median value (Q1-Q3)	**Median value (Q1-Q3)**	**P value**
RT (°C)	38.5 (38.2-38.8)	39.7 (38.8-39.9)	<0.001

RT: Rectal temperature; RR: Respiration rate; HR: Heart rate

Breed and Sex Characteristics in Cats with FPV

It has been determined that 23 of the cats with FPV are female (%58), while the remaining 17 (%42) are male. Regarding breed, 12 of the FPV-positive cats are mixed breed (%30), 17 are of the British breed (%43), 8 are of the Scottish breed (%20), and 3 are Iranian breed crossbred (%7). Additionally, it was found that 17 of the infected cats are between 0-1 months old (%43), while 23 are between 2-3 months old (%57).

Haematological Findings

The data for hematological indices are presented in Table-2 and Figure-1 for both the control and FPV groups. Hematologically, WBC, NEU count, MON count, LYM count, SIRI, and SII values were significantly lower in the FPV group compared to the control group, while the PLR value was higher (all values $p<0.001$, except for SII, which was $p=0.002$). No significant differences were observed between the FPV and control groups in terms of PLT, NLR, and LMR values (The p-values are as follows: $p=0.063$, $p=0.054$, and $p=0.627$).

Table 2. A comparison of the hematological results between the FPV-infected cats and the control group

	Groups		
Parameters	**Control (n=20)**	**FPV (n=40)**	**P value**
	$\bar{x}\pm sd$	**$\bar{x}\pm sd$**	
WBC ($\times10^3/\mu L$)	10.63±2.27	2.77±1.32	<0.001
MON ($\times10^3/\mu L$)	0.36±0.12	0.12±0.08	<0.001
	Median value (Q1-Q3)	**Median value (Q1-Q3)**	**P value**
LYM ($\times10^3/\mu L$)	3.36 (2.57-4.87)	1.10 (0.61-1.45)	<0.001
NEU ($\times10^3/\mu L$)	5.47 (4.35-7.75)	1.32 (0.41-2.34)	<0.001
PLT ($\times10^3/\mu L$)	239.50 (199.00-324.25)	199.00 (109.25-292.00)	p=0.063
NLR	1.50 (1.02-3.10)	1.03 (0.27-1.96)	p=0.054
LMR	10.25 (6.48-14.39)	11.73 (5.65-19.58)	p=0.627
PLR	75.78 (45.51-101.98)	174. 43 (98.05-294.02)	<0.001
SIRI	0.65 (0.26-1.05)	0.10 (0.01-0.31)	<0.001
SII	360.03 (242.05-670.25)	190.88 (44.62-428.34)	p=0.002

LYM: Lymphocyte; LMR: Lymphocyte count to monocyte count ratio; MON: Monocyte; NEU: Neutrophil; NLR: Neutrophil count to lymphocyte count ratio; PLR: Platelet count to lymphocyte count ratio; PLT: Platelet; SII: Systemic immune inflammatory index; SIRI: Systemic inflammation response index; WBC: White blood cell. P<0.05 is considered statistically significant

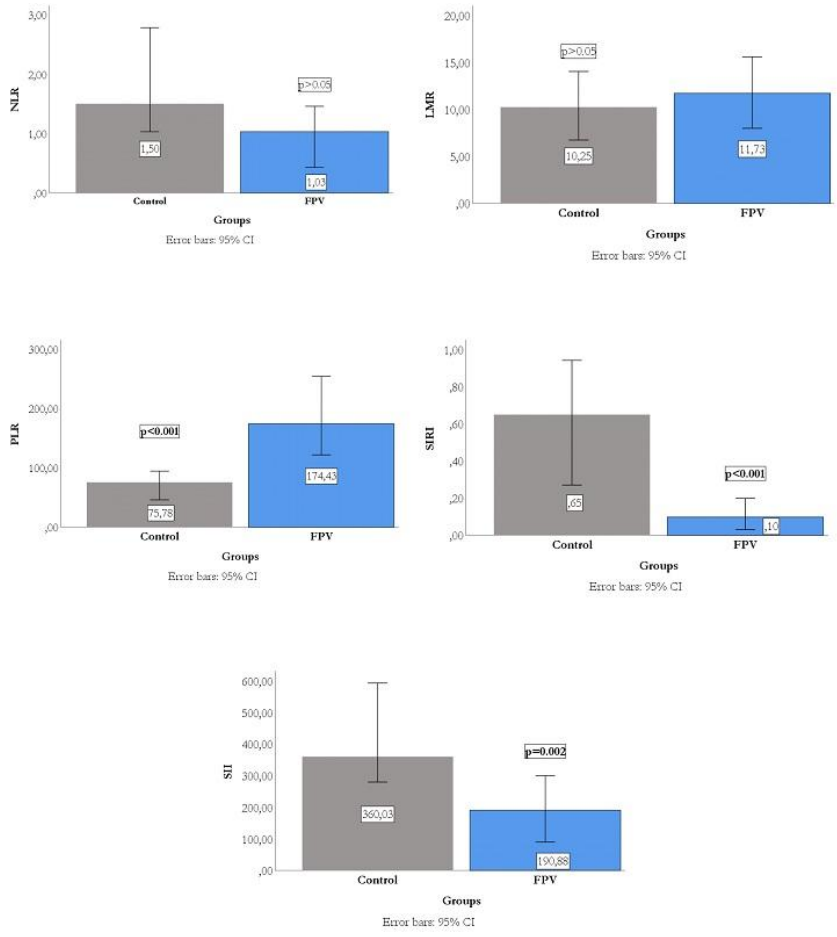

Figure 1: A comparison of the levels of hematological indices, including PLR, SIRI, SII, NLR, and LMR between the control and FPV groups

DISCUSSION

The primary objective of this study was to assess the levels of inflammatory blood parameters, including LMR, NLR, PLR, SII, and SIRI, in animals naturally infected with FPV. Although it has been reported that feline panleukopenia can be observed in all age groups (Neuerer et al. 2008), it has been shown that it is most commonly seen in cats around 3 months of age (Wolfesberger et al. 2012). In this study, the investigation of FPV infection in cats aged 0-3 months supports these findings.

It has been reported that in cases of acute FPV infection, vomiting, anorexia, diarrhea, and fever are the most common clinical signs. However, it has also been indicated that as the disease progresses, hemorrhagic diarrhea may occur clinically (Awad et al. 2019). A study on FPV reported that 34.2% of cats infected with FPV did not exhibit leukopenia, and in some infected cats, neither gastrointestinal signs nor leukopenia developed. This situation has been attributed to the fact that the sample collection may have occurred during the peracute phase of the infection (Kruse et al. 2010). In this study, similar to the aforementioned findings, most of the cats exhibited symptoms such as anorexia, vomiting, and/or diarrhea. However, none of these cases showed bloody diarrhea. This can be explained by the fact that the FPV-infected cats in this study were in the acute form, as referenced in the previous information. It has been reported that in the early stages of FPV infection, hyperthermia develops due to intense viremia (Riya et al. 2020), while hypothermia develops as the disease progresses (Porporato et al. 2018). Additionally, an increase in rectal temperature, respiratory rate, and heart rate in FPV-infected cats has been linked to sepsis (Gulersoy and Naseri 2022). In this study, it was found that rectal temperature, respiratory rate, and heart rate were significantly higher in the FPV group compared to the control group ($p<0.001$ for each value). This condition is likely attributed to sepsis, as indicated by the decrease in leukocyte indices in the FPV group (Gulersoy and Naseri 2022). Furthermore, the higher rectal temperature in the FPV group compared to the control group may be explained by the acute phase of the disease (Riya et al. 2020). It has been reported that in canine parvoviral enteritis, the intestinal crypts are more susceptible to destruction, and that the local intestinal immune defense in cats is more effective than in dogs, which is why hemorrhagic diarrhea does not develop in cats (Kruse et al. 2010). Therefore, the formation of diarrhea in FPV-infected cats, with no cases of hemorrhagic diarrhea, is likely due to this situation.

It has been stated that due to the myelosuppressive properties of FPV on the bone marrow, neutropenia and lymphopenia are observed in many cases (Gülersoy et al. 2023). The causes of neutropenia can be listed as bone marrow damage caused by the virus, increased usage in tissues, disruption in neutrophil production, and sequestration of neutrophils due to the effects of endotoxemia (Weiss and Wardrop 2010). Lymphopenia occurs due to the thymus and lymph node atrophy caused by the cytopenic property of the parvovirus, as well as the myelosuppressive syndrome that results from the infection (Sykes 2010). Monocytopenia is rarely observed in FPV, and it has been suggested that it may result from the uptake of the viral agent by monocytes and the subsequent destruction of these monocytes (Manikantaswamy et al. 2022). Monocytopenia has been indicated as a marker of poor prognosis and is associated with myelotoxicity (Kruse et al. 2010). Additionally, it has been stated that the severity of leukopenia is parallel to the intensity of clinical signs (Goddard et al. 2008). In addition to leukopenia, another significant hematological finding is thrombocytopenia, which occurs due to the increased consumption resulting from widespread intravascular coagulation or the destruction of megakaryocytes (Ghermai and Kraft 1987). On the other hand, it has been reported that thrombopoiesis can be stimulated due to the increased cytokine response in inflammatory conditions, which may lead to an increase in platelet count (Stokol 2010).

In the current study, it is believed that the abnormal hemogram findings in the FPV group, compared to the control group, are related to the immunosuppressive effect of the virus on the bone marrow. However, although thrombocytopenia was observed in some FPV-infected cats, no significant changes were observed between the groups. This may be related to the absence of consumptive coagulopathy or, as previously mentioned, the lack of DIC in many cats.

Hematological indices such as NLR, LMR, PLR, SII, and SIRI are calculated from hemogram data obtained through complete blood count analysis and are regarded as reliable indicators of systemic inflammation (Xia et al. 2023). In veterinary medicine, the NLR value has been reported to be elevated in cats with inflammatory diseases (Fries et al. 2022), SIRS, and sepsis (Gori et al. 2021). However, in a study on panleukopenic cats, it was found to be at lower levels in FPV-infected cats compared to the control group (Yanar 2024). A study on dogs with SIRS reported that NLR values were lower in septic dogs compared to non-septic SIRS dogs. The same study indicated that there could be different leukocyte responses in septic dogs, and this condition may be influenced by the effect of severe neutropenia (Pierini et al. 2019). In the current study, it is interesting that no significant difference was found in the NLR ratio between the FPV and control groups, although it was numerically lower in the FPV group. This may be due to a fourfold decrease in neutrophil count in the FPV group, along with a decrease in

lymphocyte values. Therefore, it is hypothesized that a situation similar to the one observed in the study by Pierini et al. (2019) may have occurred here as well. For these reasons, it can be concluded that NLR in this context may not provide a meaningful result in determining the inflammatory status in FPV disease. LMR is considered a novel indicator of systemic inflammatory status (Tsouloufi et al. 2021). In veterinary medicine, low LMR levels have been indicated to be linked to a poor prognosis (Davies et al. 2018). A study conducted on cats with different disease conditions found that LMR values were lower in comparison to the control group (Tsouloufi et al. 2021). A literature review on feline panleukopenia did not reveal any studies on LMR. However, in a study on cats infected with *Cystoisospora spp.* it was reported that infected cats exhibited an increase in MLR levels compared to the control group (indirectly indicating a decrease in LMR level). This condition has been suggested to result from monocyte activation and an increase in their numbers as a response to the inflammatory condition (Tuna and Kirkulak 2023). In the current study, no significant change was observed between the control and FPV group for the LMR value. It is hypothesized that this lack of change in LMR ratio is primarily due to the cytopenic nature of feline panleukopenia, which results in a decrease in both lymphocyte and monocyte levels.

In dogs with chronic enteropathy, it has been reported that PLR levels decrease with the recovery phase of the disease (Cristóbal et al. 2022). In a study on canine leishmaniosis, it was reported that there was no difference in PLR level for both experimental and control groups. This condition may have arisen due to thrombocytopenia in some dogs and lymphopenia in others, affecting their blood profiles (Durán-Galea et al. 2024a). In a study conducted on dogs with leptospirosis, it was reported that the SII level was higher in the group that did not survive compared to the group that survived. This condition has been attributed to the increased platelet activation and function (Durán-Galea et al. 2024b). In this study, it was found that the PLR level was elevated in the FPV group, whereas no significant difference was observed for the PLT value. The possible reason for this could be insufficient platelet activation in FPV (Durán-Galea et al. 2024b) or the lack of consumption coagulopathy (Ghermai and Kraft 1987). In this study, it can be said that the significant increase in PLR levels in the FPV group was due to the decrease in lymphocyte counts.

In studies conducted in human medicine on various diseases such as cervical cancer, COVID-19, and cardiovascular diseases, it has been reported that elevated levels of SIRI and SII reflect an unfavorable prognostic outcome (Huang et al. 2019; Xia et al. 2022; Xia et al. 2023). In a study conducted on dogs with leptospirosis, it was reported that the SII level was higher in the group that did not survive compared to the group that survived. It has been stated that this condition is characterized by an increase in neutrophils due to infection, accompanied by a decrease in platelet and lymphocyte counts (Durán-Galea et al. 2024b). Additionally, in dogs, it has been shown that the SII value decreases in cases of chronic inflammatory enteropathies (Cristóbal et al. 2022). In the current study, it was revealed that the FPV group value decreased for the SII value compared to the control group value. The reasons for these differences are hypothesized to include variations in immune response between species, as well as differences in the activation of the cellular or humoral immune system in different diseases, all of which could potentially influence the value of this parameter (Lou et al. 2005; Novak et al. 2023; Durán-Galea et al. 2024b). The literature review reveals that the SIRI is more widely utilized in human medicine. However, in veterinary medicine, recent studies have identified a few investigations conducted on different animal species. It has been reported in both human and animal studies that higher SIRI values are observed in disease conditions, and this has been attributed to inflammatory conditions (Xia et al. 2023; Aydın and Apaydın Yıldırım 2024; Erdogan et al. 2025). In this study, however, it was found that the SIRI value was significantly lower in the FPV-infected group compared to the control group. It is known that due to the characteristic feature of the FPV virus, it progresses with leukopenia, characterized by both neutropenia and lymphopenia, and therefore exhibits a cytopenic nature (Gülersoy et al. 2023). It is known that the SIRI value is determined by the NLR × monocyte count. In this study, a more significant reduction in neutrophil levels was noted in the FPV group when compared to the levels of lymphocytes and monocytes. Furthermore, it was noted that all three values (neutrophil, lymphocyte, and monocyte) were significantly lower in the FPV group. The SIRI value in this study is considered to be an important indicator not only for increases in inflammatory or infectious conditions but also for decreases, which could reflect bone marrow suppression or cytopenia.

This study has some limitations. First, the pathogen isolation was not performed using a highly accurate diagnostic method, such as PCR or RT-PCR (Awad et al. 2018). However, considering that current rapid diagnostic kits have a sensitivity of over 90% and a specificity close to 100% (Raheena et al. 2017), it can be seen that rapid diagnostic kits also possess high sensitivity. Secondly, blood samples were collected from the animals only once to determine the levels of the hematological indices. It is believed that obtaining multiple samples, or even using different treatment approaches, could help establish the cutoff values for these indices, which may be useful for determining the prognosis of the disease. Lastly, a representative sample of the animals' breeds was not achieved, and the fact that some animals received only one vaccination while others received no vaccination at all

may affect these parameters due to differences in immunity. Therefore, this situation is considered a limitation of the study.

CONCLUSION

It has been observed that hematological index parameters in cats with FPV yielded significant results, particularly the leukocyte index ratios such as PLR, SIRI, and SII values, which could be helpful in indicating the inflammatory status of the disease. To better understand this topic, it is considered essential to conduct large-scale studies to determine how changes in these hematological index values evolve with repeated measurements under different treatment protocols, and how these changes reflect the prognosis. This would help clarify the prognostic value of these hematological indices.

Conflict of interest: The author have no conflicts of interest to report.

Authors' Contributions: ÖA carried out the design of the article, collection of samples, data processing, analyses, writing and review of the manuscript.

Ethical approval: This study was carried out at Atatürk University Animal Hospital. This research was approved by Atatürk University Local Ethics Committee (Decision Number: 2024/13; Date: 25.12.2024)

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Explanation: This study has not been presented as abstract or full text in any congress or symposium.

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RESEARCH ARTICLE

Morphometric Analysis of the Skull in the Holstein Cow: A Computed Tomography Study

Nimet TURGUT[1]*

[1]*Department of Anatomy, Faculty of Veterinary Medicine, Selçuk University, Konya, Türkiye*

ABSTRACT

Most craniometric studies have been conducted on dry skulls. This study aims to identify the craniometric characteristics of the skull in Holstein cows using Computed Tomography (CT) imaging. Fourteen Holstein cow heads were utilized, scanned via CT, and images were processed with the DICOM Viewer software program. Seventeen craniometric measurements (13 extracranial, 4 intracranial) were obtained through the program's multiplanar reconstruction tool, and 14 indexes were calculated based on these morphometric data. In Holstein cow, total length was 519.4 ±21.7 mm, basal length was 472.1 ±22.2 mm, viscerocranium length was 288.4 ±17.4 mm. Further, the greatest frontal breadth was 225.4±8.5 mm, while the length of the cranial cavity1, length of the cranial cavity2, maximum width, and maximum height of the cranial cavity were 140.5 ±6.4, 116.8 ±4.3, 103.3 ±4.4 and 96.6 ±4.7 mm, respectively. Skull index was 43.4±1.3, facial index was 78.3±3.7, basal index was 47.8±1.8, foramen magnum index was 83.1 ±3.2, cranial cavity index1 was 73.6 ±4.6, and length-width index1 was found to be 136.3 ±8.1. This study provides initial reference data on the morphometric properties of the Holstein cow skull, derived through a reproducible measurement protocol. These findings offer valuable insights for veterinary anatomists, radiologists, clinicians, and researchers in terms of both the data and methodology presented. Craniometric data may assist in diagnosing head region pathologies, pre-surgical planning (such as trepanation, dehorning, and facial surgery), and in applications of regional anesthesia. Additionally, these findings have potential future applications in assessing skull morphology changes related to breed and gender, and in correlating skull dimensions with meat and milk production data.

Keywords; Bovine, Cephalometry, Craniology

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Holstein Sığırında Kafatasının Morfometrik Analizi: Bilgisayarlı Tomografi Çalışması

ÖZ

Craniometrik çalışmaların büyük çoğunluğu kuru kafatası üzerinde gerçekleştirilmiştir. Bu çalışmanın amacı, Holstein sığırında Bilgisayarlı tomografi (BT) görüntüler üzerinde kafatasının craniometrik özelliklerini belirlemektir. Bu çalışmada toplam 14 adet dişi Holstein sığır başı kullanıldı. Başlar BT ile tarandı ve görüntüler DICOM Viewer yazılım programına aktarıldı. Programın multiplanar reconstruction aracı kullanılarak toplam 17 kraniyometrik (13 ekstracranial-4 intracranial) ölçüm gerçekleştirildi ve bu morfometrik veriler kullanılarak 14 adet index hesaplandı. Holstein sığırında total uzunluk 519.4±21.7, basal uzunluk 472.1±22.2, viscerocranium uzunluğu 288.4±17.4, frontal genişlik 225.4±8.5 mm iken, cavum cranii uzunluğu1, cavum cranii uzunluğu2, cavum cranii'nin maksimum genişliği ve yüksekliği sırasıyla 140.5±6.4, 116.8±4.3, 103.3±4.4 ve 96.6±4.7mm idi. Skull index 43.4±1.3, facial index 78.3±3.7, basal index 47.8±1.8, foramen magnum index 83.1±3.2, cavum cranii index1 73.6±4.6 ve uzunluk-genişlik index1 136.3±8,1 olarak belirlendi. Holstein sığırında tekrarlanabilir bir ölçüm protokolü ile kafatasının morfometrik özelliklerine ait ilk referans niteliğinde veriler elde edildi. Araştırma sonuçları hem sunulan veriler yönüyle hem de metodoloji yönüyle veteriner anatomistler, radyologlar, klinisyenler ve diğer araştırmacılara fayda sağlayabilir. Kraniometrik bilgi, baş bölgesinde şekillenebilecek patolojilerin tanısında, cerrahi öncesi planlamada (trepanasyon, boynuz kesimi ve yüz bölgesi cerrahisi vb.), bölgesel anestezi uygulamalarında katkı saylayabilir. Ayrıca bulgular gelecekte ırk ve cinsiyete bağlı kafatası morfolojisinin gelişimsel değerlendirilmesinde, etçi ve sütçü ırklarda kafatası boyutları arasındaki ilişkinin tanımlanmasında kullanılabilir.

Anahtar kelimeler: Sığır, Kraniyoloji, Sefalometri

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ORCID ID; *NT: 0000-0001-6446-2958*

*Corresponding author e-mail: nturgut@Selçuk.edu.tr

INTRODUCTION

The morphological and morphometric characteristics of the skull reflect the influence of evolutionary modifications, as well as genetic and environmental factors on individuals (Getty 1975; Hanken 1993; Zelditch et al. 2004). Over about 250 years of craniometric studies, researchers have investigated the origins of domestic animals, explored intra- and inter-species similarities and differences, identified developmental anomalies and variations, and classified animals based on skull size and shape (Wilckens 1876; Grigson 1974; Bartosiewicz 1980a; Evans and Christensen 1979). Craniometry has been applied to determine the typology of skulls found in archaeological contexts (Onar et al. 2012), in the development of stereotactic devices for central nervous system examination (Saito et al. 2004), and in the field of veterinary forensic science (Toledo González et al. 2020). Additionally, endocranial volume estimations have been made for certain individuals within the orders *Carnivora* (Finarelli 2006) and *Artiodactyla* (Finarelli 2011; Balcarcel et al. 2021) using models based on specific external skull measurements.

Morphometric measurements were carried out on the dry skull in European bison (Krasińska et al. 2008; Szara et al. 2023), water buffalo (Özkan et al. 2019), wild cattle (Grigson 1978; Brudnıckı et al. 2012; Balcarcel et al. 2021), domestic cattle (Grigson 1974; Parés Casanova and Jordana i Vidal 2008; Özkan et al. 2019), Simmental and Holstein Cattle (Çakar et al. 2024), native Asian cattle (Hayashi et al. 1981; Hayashi et al. 1988), Korean and Indonesian cattle (Nishida et al. 1983), Zebu cattle (Grigson 1980; Bökönyi 1997), Hungarian grey (Bartosiewicz 2006; Kőrösi 2013), Niata cattle (Veitschegger et al. 2018), Kuri cattle (Gambo et al 2015; Gambo et al. 2019), hybrids cattle (Krasinska 1988) and on the head region in some live cattle (Cabezas Congo et al. 2019; Lomillos and Alonso 2020; Neves et al. 2021). As a result of the studies, craniometric characteristics were identified in various animal species, revealing dimensional variations between breeds and sexes. However, most craniometric research in cattle to date has utilized dry skull samples.

The skull presents a complex anatomical structure that houses the brain, sensory organs, and components of the respiratory and digestive systems. CT imaging is highly effective for assessing intricate structures like the head, diagnosing bone tissue pathologies, and evaluating conditions related to paranasal sinus diseases and skull trauma (Stieger-Vanegas and Hanna 2022; Turgut et al. 2023). Recently, veterinary-specific CT devices capable of visualizing multiple body regions in large animals such as equidae and ruminants in a standing position have been developed. These devices enable efficient morphometric analyses and clinical evaluations on the acquired images (Stewart et al. 2021; Brounts et al. 2022). However, the accurate evaluation of these images also requires comprehensive radiological and morphometric data specific to the region of interest in various animal species.

The Holstein cattle breed is globally prevalent and holds substantial economic value due to its milk and meat yield capabilities, along with its high adaptability to various environments. Males of this breed are typically slaughtered at around 14–16 weeks or 12–15 months of age to meet meat production demands, as they exhibit rapid growth, while some are reared as breeding bulls. Female Holsteins, which are slower to mature, are raised until about 7–9 years due to their significant milk yield, thus constituting the majority of the Holstein population (ESK 2024, FOA 2024). Skull structure in cattle varies based on breed and gender, with meat breeds like Holsteins exhibiting shorter, wider skulls and dairy breeds characterized by longer, narrower skulls (Sasimowski 1987). Skull shape in cattle is influenced by factors such as brain development, sinus formation, and cornual process development (Barone 1999; Nickel et al. 1986) and continues to evolve with age (Bartosiewicz 1980a, 1980b; Kőrösi 2013; Neves et al. 2021). Skull shape serves as a key criterion for breed classification, with skull indices providing important data for defining morphological types. Thus, morphometric data on the head and other body regions in Holstein cows remain relevant. While general morphological characteristics of cattle skulls have been extensively documented, morphometric data specific to Holstein cows are limited, with no available intracranial measurements. This study aims to define the craniometric features of the Holstein cow skull using CT imaging. The resulting data may support determinations of skull morphology related to breed and gender, aid in intra- and inter-species craniometric datasets, assist in diagnosing bone-related pathologies, inform surgical planning for trepanation and dehorning, enhance regional anesthesia applications, and support veterinary forensic sciences in the analysis of animal-related evidence in criminal cases involving animals.

MATERIAL and METHODS

Animals

The study utilized 14 healthy Holstein cow heads with blunted horns, averaging an age of 5.4 ±1.7 years (range 1.5–8.0), obtained from a slaughterhouse in Konya, Turkey. Following clinical assessments conducted by the slaughterhouse veterinarian, the heads of the slaughtered animals were selected randomly. The ages of the animals were verified by inspecting their ear tags as well as assessing the condition and wear of their permanent incisors and canines (Schummer et al. 1979; Barone 1997). All procedures were conducted under the approval of the

Ethics Committee of Selçuk University Faculty of Veterinary Medicine, Experimental Animal Production and Research Center, under decision number 2024/077.

CT Scans

The heads were scanned using an MSCT device (Siemens Dual Source, Somatom Definition Flash, Germany) positioned perpendicular to the hard palate, with settings of 140 kV, 475–500 mAs, a 512 × 512 matrix, and a slice thickness of 0.6 mm. Scanning occurred within 24 h post-slaughter to minimize postmortem alterations, with the heads stored in cold conditions between slaughter and scanning. Axial reformat data sets of 1 mm thickness for the 14 animals that met the study's criteria (free from head and bone-related diseases, asymmetric development, and anomalies) were archived in Digital Imaging and Communication in Medicine (DICOM) format.

Extracranial and Intracranial Linear Measurements

The RadiAnt DICOM Viewer (Medixant, Poland) software was utilized to acquire morphometric parameters. DICOM data sets for each animal were transferred into the software, and linear measurements were conducted on the images using the Multiplanar Reconstruction (MPR) tool. Each linear measurement was repeated three times at the bone window setting (window level 300 HU and window width 2800 HU), and the arithmetic mean of the results was calculated. Measurement values were recorded in millimeters (mm). Anatomical structures were named following classical anatomy references (Sisson 1975; Nickel et al. 1986) and the Nomina Anatomica Veterinaria (NAV 2017). All craniometric measurements were visualized using Adobe Photoshop CC 2015.5 (Version: 25.5.0, Adobe Systems, San Jose, CA, USA).

To characterize the general head morphometry of the animals in this study, 14 indices were derived using data from 17 linear measurements, including 13 extracranial and 4 intracranial parameters. In extracranial measurements, bone reference points were established according to Von den Driesch (1976) (Figure 1). Reference points for intracranial measurements—comprising height, width, and two lengths—were defined by the author. For this purpose, the sagittal plane was aligned to pass through the midmedian plane, the dorsal plane through the nasion, and the transverse plane through the base of the hypophyseal fossa. Six points where these planes intersected the cranial cavity served as bone reference points for three intracranial measurements (height, width, and length 2). In the measurement of length 1 (LCC1), the basion and the intersection of the dorsal plane with the ethmoidal crest (crista galli) on the sagittal image were used as reference points (Figure 2). A total of 31 parameters, comprising both extra- and intracranial measurements along with calculated indices, are presented in Table 1 and Figures 1 and 2.

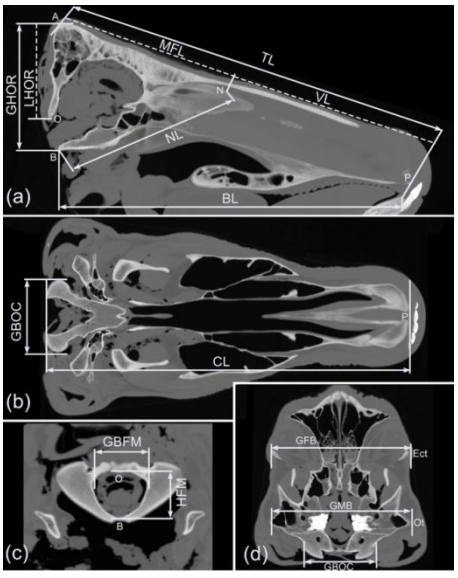

Figure 1. The extracranial measurements on CT image in Holstein cow. a) Sagittal CT section image. b-d) Dorsal CT section images. c) Transverse CT section image. A, Akrokranion; B, Basion; Ect, Ectorbitale; N, Nasion; O, Opisthion; Ot, Otion; P, Prosthion. See to section material and methods for details of abbreviations used in measurements.

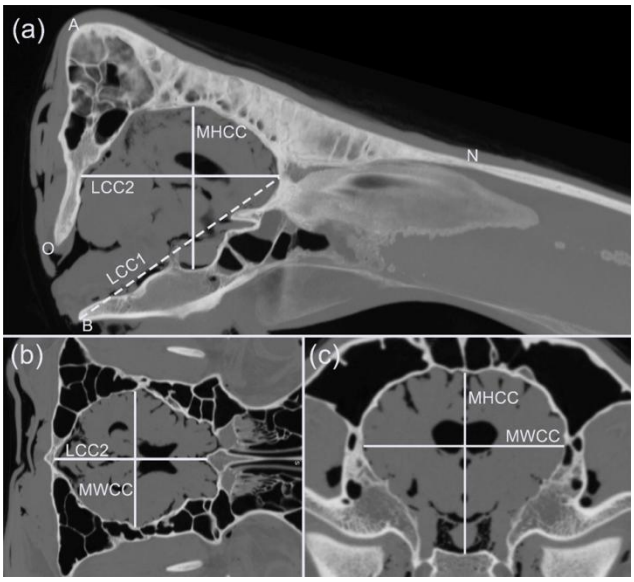

Figure 2: The intracranial measurements on CT image in Holstein cow. a) Sagittal CT section image. b) Dorsal CT section images. c) Transverse CT section image. A, Akrokranion; B, Basion; N, Nasion; O, Opisthion. See to section material and methods for details of abbreviations used in measurements.

Table 1. The craniometric measurements and the indices that are calculated

Parameters	Definition of measurements and indices	Description of measurements and indices	Figure
Extracranial measurements (mm)			
TL	Total length	Acrocranion-prosthion	Figure 1/a
CL	Condylobasal length	Aboral border of the occipital condyles-prosthion	Figure 1/b
BL	Basal length	Basion-prosthion	Figure 1/a
NL	Neurocranium length	Basion-nasion	Figure 1/a
MFL	Median frontal length	Acrocranion-nasion	Figure 1/a
GFB	Greatest frontal breadth	Ectorbitale-ectorbitale	Figure 1/d
VL	Viscerocranium length	Nasion-prosthion	Figure 1/a
GHOR	Greatest height of the occipital region	Basion-highest point of the intercornual ridge in the median plane	Figure 1/a
LHOR	Least height of the occipital region	Opisthion-highest point of the intercornual ridge in the median plane	Figure 1/a
GBOC	Greatest breadth of the occipital condyles		Figure 1/b,d
GMB	Greatest mastoid breadth	Otion-otion	Figure 1/d
GBFM	Greatest breadth of the foramen magnum		Figure 1/c
HFM	Height of the foramen magnum	Basion-opisthion	Figure 1/c
Intracranial measurements (mm)			
LCC1	Length 1: Lenght of the cranial cavity1	The most rostral point of ethmoidal crest to basion	Figure 2/a
LCC2	Length 2: Lenght of the cranial cavity2	The most rostral point of ethmoidal crest to the vermiform impression	Figure 2/a,b
MHCC	Maximum height of the cranial cavity	Hypophyseal fossa to internal lamina of frontal bone	Figure 2/a,c
MWCC	Maximum width of the cranial cavity	Greatest width distance between internal wall of parietal bones	Figure 2/b,c
Indices			
SI	Skull index	GFB/TL x 100	
FAI	Facial index	GFB/VL x 100	
FRI	Frontal index	GFB/MFL x 100	
BI	Basal index	GFB/BL x 100	
FMI	Foramen magnum index	HFM/GBFM x 100	
CCI1	Cranial cavity index 1	MWCC / LCC1 x 100	
CCI2	Cranial cavity index 2	MWCC / LCC2 x 100	
CCI3	Cranial cavity index 3	MHCC / LCC1 x 100	
CCI4	Cranial cavity index 4	MHCC / LCC2 x 100	
LLI1	Length- length index 1	MFL/VL*100	
LLI2	Length- length index 2	LCC1 /TL *100	
LLI3	Length- length index 3	LCC2 /TL*100	
LWI1	Length-width index 1	LCC1/MWCC*100	
LWI2	Length-width index 2	LCC2/MWCC*100	

Statistical Analysis

The SPSS software (version 29.0, Armonk, NY: IBM Corp. USA) was employed for statistical analysis. Descriptive statistics, including mean, standard deviation, minimum, and maximum values, were provided for both categorical and continuous variables. Pearson's correlation coefficient was used to assess relationships between two continuous variables, with p-values of $p<0.05$ and $p<0.01$ considered statistically significant. To evaluate the reliability of the three repeated measurements, 95% confidence intervals were calculated using the ıntraclass correlation coefficient (ICC), where an ICC above 0.75 is considered ideal. This coefficient is

acknowledged to range between 0 and +1 (Lee et al. 1989; McGraw and Wong 1996).

RESULTS

The statistical analysis in this study revealed a significant agreement among the three measurement sets ($p<0.05$). The 13 extracranial measurements, which were conducted for the first time on CT images of the bovine head, demonstrated high reproducibility (ICC: 0.879–0.996, mean: 0.965). Additionally, four intracranial measurements (LCC1, LCC2, MWCC, MHCC), defined by the author with novel reference points, also displayed strong reproducibility on bovine CT images (ICC: 0.887–0.949, mean: 0.920). Intracranial measurements, specifically, were effectively obtained from sagittal (height and two lengths), transverse (height and width), and dorsal (width and length) images at consistent levels on the MPR screen (Figure 2). These measurements clearly identified the cranial cavity's highest, widest, and longest points.

The study presents the results of 13 extracranial and 4 intracranial measurements conducted on multiplanar CT images of 14 Holstein cows, as shown in Table 2, along with the 14 calculated indices detailed in Table 3. Table 4 provides the relationships among the craniometric measurements and their associations with age, while Table 5 outlines the interrelationships among the indices. A cranial length of 519.4 ±21.7 mm and a maximum cranial width of 225.4 ±8.5 mm were observed in Holstein cows (Table 2).

A positive correlation was observed among the craniometric measurements exclusively between age and the VL value (p: 0.03, r: 0.582). Positive correlations were identified in the extracranial measurements, while intracranial and extracranial measurements exhibited correlations specifically between LCC1 and BL (p: 0.03, r: 0.574), LCC2 and GHOR (p: 0.01, r: 0.648) and LHOR (p: 0.01, r: 0.661), as well as MHCC and MFL (p: 0.003, r: 0.729) (Table 4). Analysis of the relationships among the indices revealed both positive and negative correlations, except for FMI. Notably, LWI1 showed a negative correlation with all indices except LWI2 (p: 0.007, r: 0.687) (Table 5).

Table 2. The morphometric data related to the craniometric measurements

Authors. Year	Specimen	Age	N-Sex	TL	CL	BL	NL	MFL	GFB	VL
Krasınska et al., 2008*	*Bison (European bison)*	5-27 y 5-22 y	152-F 154-M			447.0±12.6 471.8±14.3			274.6±12.2 318.9±15.3	
Özkan et al.,2019*	*Water buffalo (B. Bubalis L.)*	3-7 y	15 F	472.0±45.8(410.7-528.4)	482.3±45.9(420.8-541.8)	450.1±45.0(389.7-505.2)		213.9±16.4(183.7-239.1)	202.8±19.2(169.9-228.0)	273.5±37.4(229.2-322.6)
Grigson 1974*	*Cattle (Bos taurus L.)*	2 y ≤	18-F 17-M			414.8±31.8 429.4±30.1		210.8±24.4 218.2±20.4		
Grigson 1978*	*Cattle (Bos primigenius)*	2 y ≤	24-F 60-M			511.6±18.1 564.8±22.9		276.4±17.8 329.7±19.0		
Grigson 1980*	*Cattle (Bos indicus L.)*	2 y ≤	23-26 F,M			407.7±62.9		192.5±30.5		
Bartosıewıcz 1980*	*Cattle (Red Pied-Fleckvieh)*	0-2 m 1-7.7 y	21-F,M	263.4±27.3 484.9±27.2		236.7±27.1 437.8±28.1	132.3±18.1 215.5±10.0	145.2±13.0 223.1±14.4	131.1±15.6 211.7±20.8	124.9±16.2 261.9±23.1
Hayashi et al.,1981*	*Cattle (Sumatra)*	4 y ≤ 1-3 y	9-F 1-M	417.4±15.8		381.8±10.6		176.2±8.4	168.3±6.2	
Nıshıda et al.,1983*	*Cattle (Korean)*	4 y ≤ 2-3 y	8-F 8-M	474.2±9.8 484.9±26.2		423.6±15.0 433.5±19.9		199.9±10.9 212. ±15.6	209.3±8.2 231.1±9.2	278.0 269.0
Hayashi et al.,1988*	*Cattle (Banteng)*	4 y ≤	10-F 3-M	441.1±9.9 501.0±7.8		415.3±11.2 449.7±10.0		191.3±6.3 232.0±6.0	183.5±7.0 216.7±7.0	266.6 294.0
	Cattle (Bali)		10 F 8 M	391.3±13.7 424.9±19.6		383.4±11.5 405.1±21.8		160.0±9.8 184.9±10.3	178.0±5.0 200.1±9.3	242.0 253.7
	Cattle (Madura)		8-F	411.3±17.8		381.3±19.6		166.3±6.2	171.1±6.0	260.2
	Cattle (Aceh)		9-F	417.4±15.8		381.8±10.6		176. 2±8.4	168.3±6.2	251.7
	Cattle (Leyte)		6-F	405.7±21.4		365.0±22.2		169. 7±13.7	173.5±7.8	240.4
	Cattle (Korean)		8-F	474.3±9.8		423.6±15.0		199.9±10.9	209.3±8.2	278.0
Bartosiewicz 2006*	*Cattle (Hungarian grey)*	2-16 y	30-F 15-M	497.2±16.2 538.7±28.0		447.2±13.6 481.8-23.7		241.8±15.3 259.3±17.4	223.4±9.3 242.1±14.6	
Parés Casanova & Jordana i Vidal 2008*	*Cattle (Bos taurus L.)*	2.5 y <	502-F 76-M	533.0±37.1 579.1±49.2					235.3±14.2 272.5±16.8	380.1±38.1 408.9±42.7
Körösı 2013*	*Cattle (Hungarian grey)*	2-16 y	46-F(cow) 25-M(ox) 5-M(bull)	495.1 (454.1-555.9) 542.8 (462.1-579.5) 516.9 (462.2-541.0)	477.7 (446.5-524.5) 516.7 (451.5-547.5) 495.0 (455.8-515.2)	447.2 (415-490.0) 486.5 (459.6-518.8) 466.6 (421.0-483.5)	243.1 (218.6-270.0) 260.0 (240.0-280.0) 257.5 (240.0-270.0)	235.9 (203.4-271.2) 259.1 (216.7-280.5) 247.0 (226.0-268.5)	222.4 (202.5-247.2) 244.9 (213.7-267.4) 259.3 (222.4-274.5)	260.1 (206.2-300.1) 290.2 (248.4-311.6) 273.3 (242.3-290.8)
Cabezas Congo et al., 2019**	*Cattle (Criollo)*	Adult	198-F 19-M	456.2±29.2 446.3±11.92				281.8±27.6 291.8±34.6	206.3±42.9 183.2±20.3	168.4±15.3 197.4±39.9
Gambo et al.,2019*	*Cattle (Kuri cattle)*	9 m-10 y	15-F 15-M	498.7±47.5 503.8±67.5	466.2±32.6 470.7±41.6	468.3±32.3 478.7±46.1		225.3±21.7 236.3±31.0	205.3±20.4 221.0±25.2	260.1±25.7 257.5±29.2
Özkan et al.,2019*	*Cattle (Bos taurus L.)*	3-7 y	20-F	592.5±15.9(499.7-558.1)	519.6±15.9(485.7-543.4)	486.2±15.8(455.0-512.9)		233.5±12.1(214.1-255.9)	228.9±10.2(207.2-243.5)	298.0±15.0(271.2-326.8)
Lomillos and Alonso 2020**	*Cattle (Lidia)*	4-6 y	80-F 184-M	471.0±31.0 491.0±34.0					210±29.0 248±19.0	
Neves et al., 2021**	*Cattle (Jersey)*	1-11 m 16-24 m 25-58 m	18-F 17-F 13-F	308.3±54.8 432.9±17.6 441.5±13.4				181.1±24.0 238.2±20.1 226.9±9.6	262.2±39.8 338.2±17.8 340.0±15.3	127.2±34.6 194.7±12.8 214.6±12.0
Çakar et al., 2024***	*Cattle (Holstein)* *Cattle (Simmental)*	12-14 m	25M 29M	490.6±28.1 485.9±24.3	475.6±25.1 467.4±19.3	445.2±24.4 435.1±20.3		223.2±15.9 226.5±13.8	213.9±15.1 218.0±15.4	267.6±17.6 263.0±17.3
The present study****	*Cattle (Holstein cow)*	1.5-8 y	14-F	519.4±21.7(467.9-544.0)	506.9±21.5(453.8-541.0)	472.1±22.2(417.8-505.1)	235.3±8.5(219.9-244.5)	234.4±9.2(221.7-252.0)	225.4±8.5(209.7-236.9)	288.4±17.4(245.0-322.4)

Abbreviations: TL, Total length; CL, Condylobasal length; BL, Basal length; NL, Neurocranium length; MFL, Median frontal length; GFB, Greatest frontal breadth; VL, Viscerocranium length; F, Female; M, Male; y, years; m,months; * Skull study; ** Head study; *** Skull-surface scan; ****CT scan-cadavers study. Mean ± SD (minimum-maximum).

Table 2-Continuation. The morphometric data related to the craniometric measurements

Authors. Year	Specimen	Age	N-Sex	GHOR	LHOR	GBOC	GMB	GBFM	HFM	LCC1	LCC2	MWCC	MHCC
Özkan et al.,2019*	*Water buffalo (B. Bubalis L.)*	3-7 y	15 F	177.5±11.9 (155.0-191.7)	168.4±12.5 (140.6-184.5)	98.4±5.7 (89.2-107.8)	199.2±22.4 (167.7-226.8)	40.8±5.4 (29.5-56.2)	34.5±3.7 (29.0-45.1)				
Grigson 1974*	*Cattle (Bos taurus L.)*	2 y ≤	18-F 17-M	144.4±10.0 157.7±12.0		97.2±9.0 108.3±7.8							
Grigson 1978*	*Cattle (Bos primigenius)*	2 y ≤	24-F 60-M	190.7±13.3 222.5±12.0		116.8±6.0 137.0±6.3							
Grigson 1980*	*Cattle (Bos indicus L.)*	2 y ≤	23-26 F, M	118.0±17.4	114.4±26.3	87.5±15.0							
Hayashi et al.,1981*	*Cattle (Sumatra)*	4 y ≤ 1-3 y	9-F 1-M	116.9±10.4									
Nıshıda et al.,1983*	*Cattle (Korean)*	4 y ≤ 2-3 y	8-F 8-M	145.5±5.4 153.0±10.8									
Hayashi et al.,1988*	*Cattle (Banteng)*	4 y ≤	10-F 3-M	148.9±6.4 165.0±4.4									
	Cattle (Bali)		10 F 8 M	140.2±5.2 164.0±14.7									
	Cattle (Madura)		8-F	138.5±6.0									
	Cattle (Aceh)		9-F	116.9±10.4									
	Cattle (Leyte)		6-F	133.8±5.7									
	Cattle (Korean)		8-F	145.5±5.4									
Bartosiewicz 2006*	*Cattle (Hungarian grey)*	2-16 y	30-F 15-M		117.1±6.2 125.1±6.2		224.7±10.5 247.2±16.1						
Körösı 2013*	*Cattle (Hungarian grey)*	2-16 y	46-F(cow) 25-M(ox) 5-M(bull)	154.8 (114.0-171.2) 168.4 (149.3-200.5) 170.9 (146.5-188.6)	120.5 (104.7-156.7) 127.5 (115.8-159.7) 129.4 (114.3-151.2)	108.1 (92.4-129.0) 125.6 (110.3-198.6) 122.6 (117.9-129.6)	223.7 (202.3-258.6) 251.8 (218.8-275.0) 271.8 (227.3-288.6)	40.0 (30.7-51.6) 42.0 (27.7-64.3) 32.8 (26.5-35.6)	38.0 (31.0-43.2) 47.1 (35.7-45.7) 34.8 (32.7-36.3)				
Gambo et al.,2019*	*Cattle (Kuri cattle)*	9 m-10 y	15-F 15-M		101.3±6.7 104.3±12.3	99.9±8.4 108.7±8.5		38.5±3.8 36.3±3.4	39.7±3.2 39.5±3.2				
Özkan et al.,2019*	*Cattle (Bos taurus L.)*	3-7 y	20-F	170.3±7.5 (156.6-184.7)	131.3±7.3 (114.7-144.9)	113.3±5.7 (103.2-129.9)	231.5±11.3 (205.4-245.5)	42.7±3.0 (36.6-50.3)	38.9±2.2 (34.6-42.6)				
Çakar et al., 2024***	*Cattle (Holstein)* *Cattle (Simmental)*	12-14 m	25M 29M	158.6±9.3 157.6±12.5	122.2±9.6 122.1±10.8	109.7±7.3 112.8±7.5	210.9±16.9 212.2±15.3	40.1±4.5 40.4±5.8	39.4±3.2 38.9±4.3				
The present study****	*Cattle (Holstein cow)*	1.5-8 y	14-F	162.7±8.2 (146.8-173.6)	124.3±7.6 (112.2-134.6)	113.8±6.7 (105.0-125.0)	224.4±10.3 (199.4-236.8)	47.3±2.4 (45.1-52.4)	39.3±2.1 (35.5-43.3)	140.5±6.4 (130.6-152.4)	116.8±4.3 (110.0-124.9)	103.3±4.4 (97.2-110.9)	96.6±4.7 (87.4-104.6)

Abbreviations: GHOR, Greatest height of the occipital region; LHOR, Least height of the occipital region; GBOC, Greatest breadth of the occipital condyles; GMB, Greatest mastoid breadth; GBFM, Greatest breadth of the foramen magnum; HFM, Height of the foramen magnum; LCC1, Lenght of the cranial cavity1; LCC2, Lenght of the cranial cavity2; MWCC, Maximum width of the cranial cavity; MHCC, Maximum height of the cranial cavity; F, Female; M, Male; y, years; m,months; * Skull study; ** Head study; *** Skull-surface scan; ****CT scan-cadavers study. Mean ± SD (minimum-maximum).

Table 3. The morphometric data related to the indices

Authors. Year	Specimen	Age	N-Sex	SI	FAI	FRI	BI	FMI	CCI1	CCI2	CCI3	CCI4	LLI1	LLI2	LLI3	LWI1	LWI2
*Al-Sagair and Elmougy 2002**	*Camel (Malha)*	2-3 y 6-7 y	15-M 15-M	41.1±0.6 45.1±0.6	72.3±1.6 79.8±1.6												
*Yahaya et al., 2012**	*Camel (One-Humped)*	Adult	15-F 15-M					107.4±6.3 109.3±4.4									
*Yahaya et al., 2012**	*Camel (One-Humped)*	2-3 y	6-F 6-M	40.9±0.6 41.1±0.4	96.5±1.4 96.2±1.4			104.3± 1.6 102.8± 3.2									
*Zhu 2012**	*Tibetan Gazelle (Procapra Picticaudata)*	—	10-M	43.2±0.4	116.4±1.2												
*Yılmaz et al., 2020****	*Gazelles (Gazella subgutturosa)*	Adult	5-F 4-M	42.1±2.5 41.5±1.9				98.2±3.5 86.2±4.5									
*Choudhary and Singh 2015**	*Indian Blackbuck (Antelope cervicapra)*	Adult	6-F,M	45.9 ± 0.04 46.4 ± 0.04				98.7									
*Kataba 2015**	*Goat (Capra hircus)*	18 m ≤	15-F 15-M	54.4±3.4				88.5±5.6									
*Karimi et al., 2011**	*Sheep (Mehraban Sheep)*	Adult	8	53.6±3.3	85.4±1.9												
*Ömer and Alpak 2012**	*Sheep (Kıvırcık sheep)*	1 y	20-F 20-M	47.0±1.1 47.4±2.2	81.7±2.8 83.6±5.5												
*Gündemir et al., 2020**	*Sheep (Bardboka sheep)*	Adult	13-F 12-M	41.7±1.7 41.5±2.4				94.5±6.9 93.7±9.7									
*Özkan et al., 2019**	*Water buffalo (Bubalis bubalis L.)*	3-7 y	15-F	43.0±1.8 (39.6-46.0)	74.7±5.7 (66.0-84.7)	94.9±6.3 (83.7-106.7)	45.1±2.0 (42.1-48.3)	85.0±6.7 (78.2-104.6)					0.79±0.09 (0.67-0.96)				
*Parés Casanova and Jordana i Vidal 2008**	*Cattle (Bos taurus L.)*	2.5 y <	502-F 76-M	44.3±3.6 47.2±3.7													
*Cabezas Congo et al., 2019***	*Cattle (Criollo)*	Adult	198-F 19-M	43.1±7.0 36.5±3.6													
*Gambo et al.,2019**	*Cattle (Kuri cattle)*	9 m-10 y	15-F 15-M					103.7±11.3 109.2±10.9									
*Özkan et al., 2019**	*Cattle (Bos taurus L.)*	3-7 y	20-F	43.2±1.5 (39.8-46.0)	76.9±3.0 (70.5-81.5)	98.3±6.8 (89.1-113.9)	47.1±1.4 (44.3-50.5)	91.3±7.7 (79.9-108.6)					0.79±0.06 (0.66-0.87)				
*Neves et al., 2021***	*Cattle (Jersey)*	1-11 m 16-24 m 25-58 m	18-F 17-F 13-F	85.7±6.1 78.15±2.5 77.0±3.1													
*Lomillos and Alonso 2020***	*Cattle (Lidia)*	4-6 y	80-F 184-M	44.6±6.2 (26.8-58.1) 50.6±4.3 (27.3-61.6)													
*Çakar et al., 2024*****	*Cattle (Holstein)* *Cattle (Simmental)*	12-14 m	25M 29M	43.6±2.5 44.9±2.4	60.1±3.7 58.6±4.9	96.0±5.9 96.4±6.4	48.1±2.8 50.1±3.0	99.3±13.6 98.2±16.8					83.6±6.1 86.4±7.7				
*The present study******	*Cattle (Holstein cow)*	1.5-8 y	14-F	43.4±1.3 (41.9-46.0)	78.3±3.7 (72.3-86.1)	96.2±3.4 (90.4-102.5)	47.8±1.8 (44.4-50.5)	83.1±3.2 (78.2-89.4)	73.6±4.6 (68.0-84.9)	88.5±3.7 (84.1-94.1)	68.9±4.5 (62.5-76.2)	82.8±3.7 (78.8-90.2)	81.5±5.0 (71.2-92.0)	27.1±1.4 (24.8-29.0)	22.5±1.1 (21.1-24.5)	136.3±8.1 (117.8-147.0)	113.2±4.7 (106.2-118.9)

Abbreviations: SI, Skull index; FAI, Facial index; FRI, Frontal index; BI, Basal index; FMI, Foramen magnum index; CCI1, Cranial cavity index1; CCI2, Cranial cavity index2; CCI3, Cranial cavity index3; CCI4, Cranial cavity index4; LLI1, Length-length index1; LLI2, Length-length index 2; LLI3, Length-length index3; LWI1, Length-width index1; LWI2, Length-width index2. *Skull study; **Head study, ***3D model; ****Skull-surface scan, *****CT scan-cadavers study; F, Female; M, Male; y, years; m,months. Mean ± SD (minimum-maximum)

Table 4. The relationship between extra-intracranial measurements and age *(N=14)*.

	Age	TL	CL	BL	NL	VL	MFL	GMB	GBOC	GFB	GHOR	LHOR	LCC1	LCC2	MWCC	MHCC	GBFM
TL																	
CL		,884**															
BL		,903**	,971**														
NL		,562*	,613*	,690**													
VL	,582*	,886**	,920**	,929**													
MFL		,688**															
GMB		,663**	,573*				,553*										
GBOC								,614*									
GFB		,749**	,637*	,631*		,647*	,564*	,813**	,650*								
GHOR		,568*					,643*										
LHOR							,605*				,952**						
LCC1				,574*													
LCC2											,648*	,661*					
MWCC																	
MHCC							,729**										
GBFM																	
HFM									,670**	,572*							,747**

*, p< 0.05 ; **, p< 0.01

Abbreviations: TL, Total length; CL, Condylobasal length; BL, Basal length; NL, Neurocranium length; VL, Viscerocranium length; MFL, Median frontal length; GMB, Greatest mastoid breadth; GBOC, Greatest breadth of the occipital condyles; GFB, Greatest frontal breadth; GHOR, Greatest height of the occipital region; LHOR, Least height of the occipital region; LCC1, Lenght of the cranial cavity1; LCC2, Lenght of the cranial cavity2; MWCC, Maximum width of the cranial cavity; MHCC, Maximum height of the cranial cavity; GBFM, Greatest breadth of the foramen magnum; HFM, Height of the foramen magnum.

Table 5. The relationship between the indices *(N=14)*.

	FMI	SI	FAI	FRI	BI	CCI1	CCI2	CCI3	CCI4	LLI1	LLI2	LLI3	LWI1
FMI													
SI													
FAI		,782**											
FRI													
BI		,858**	,855**										
CCI1			,573*		,634*								
CCI2						,676**							
CCI3						,689**							
CCI4								,743**					
LLI1			,816**	-,654*	,541*	,613*		,571*					
LLI2								-,644*					
LLI3			,592*								,590*		
LWI1			-,601*		-,653*	-,997**	-,687**	-,705**		-,650*			
LWI2						-,676**	-1,000**						,687**

*, p< 0.05 ; **, p< 0.01

Abbreviations: FMI, Foramen magnum index; SI, Skull index; FAI, Facial index; FRI, Frontal index; BI, Basal index; CCI1, Cranial cavity index1; CCI2, Cranial cavity index2; CCI3, Cranial cavity index3; CCI4, Cranial cavity index4; LLI1, Length-length index1; LLI2, Length-length index 2; LLI3, Length-length index3; LWI1, Length-width index1; LWI2, Length-width index2.

DISCUSSION

This study provides pioneering descriptive data on the morphometric characteristics of the Holstein cow skull using CT imaging. Indices were calculated based on specific morphometric measurements of the skull, with mean±SD values of craniometric measurements and indices detailed in Tables 2 and 3 alongside comparisons to existing literature. The craniometric data for Holstein cows were compared with data from other members of the *Bovidae* family (Table 2), while indices were contrasted with those from the order *Artiodactyla* (Table 3). Despite variations in nomenclature used for certain linear measurements in the literature, the measurement reference points align well with those used in the current study.

A review of previous studies indicates that craniometric measurements are generally larger in males within species of the *Bovidea* family (Table 2). Similarly, index values tend to be higher in males across several *Artiodactyla* species, including camels (Al-Sagair and Elmougy 2002; Yahaya et al. 2012), sheep (Ömer and Alpak 2012), and cattle (Parés Casanova and Jordana i Vidal 2008; Gambo et al. 2019; Lomillos and Alonso 2020) (Table 3).

In this study, the CL and BL in Holstein cows were 506.9 ±21.5 mm and 472.1 ±22.2 mm, respectively. The CL in Holstein cows was found to be greater than in other breeds, except for domestic cattle (Özkan et al. 2019) and male Hungarian greys (Körösı 2013), while the BL exceeded that of other female breeds, with exceptions including *Bos primigenius* (Grigson 1978) and domestic cattle (Özkan et al. 2019) (Table 2). Parés Casanova and Jordana i Vidal (2008) recorded a mean TL of 533.0 ±37.1 mm in various domestic cattle and 559.7 ±28.7 mm in the Friesian breed (n = 38 females), whereas Özkan et al. (2019) reported a TL of 592.5 ±15.9 mm in domestic cattle. In contrast, this study found a TL of 519.4 ±21.7 mm in Holstein cows, and a TL of 490.59 ±28.08 mm in Holstein bulls was reported by Çakar et al. (2024). When evaluating data from the literature on female cattle breeds (Bartosiewicz 1980; Hayashi et al. 1981; Nıshıda et al. 1983; Hayashi et al. 1988; Bartosiewicz 2006; Körösi 2013; Cabezas Congo et al. 2019; Gambo et al. 2019; Lomillos and Alonso 2020; Neves et al. 2021), it was noted that TL values tended to be lower than those in Holstein cows (Table 2). Previous studies largely conducted measurements on dry skulls, though some utilized fresh skulls or live animals, with methods involving metric rules, threads, calipers, hauptner measuring canes, non-flexible measuring tapes, or photogrammetric equipment. These variations in cranial measurements across cattle breeds are likely influenced by breed-specific size differences and the methodological variations mentioned above.

Upon reviewing studies within the Bovidae family, it was noted that research specifically addressing NL measurements is relatively scarce (Table 2). In Holstein cows, the mean NL measurement (235.3 ±8.5 mm) was found to be larger than that of the Red Pied-Fleckvieh (Bartosıewıcz 1980) but smaller than the Hungarian Grey (Körösi 2013). Körösi (2013) reported an MFL value of 235.9 mm in Hungarian Grey cattle, while Özkan et al. (2019) recorded 233.5 mm in domestic cattle. The MFL value from this study in Holstein cow (234.4 ±9.2 mm) aligns closely with values from Hungarian Grey cattle and domestic cattle (Grigson 1974, 1978, 1980; Hayashi et al. 1981; Nıshıda et al. 1983; Hayashi et al. 1988; Bartosiewicz 2006; Cabezas Congo et al. 2019; Gambo et al. 2019; Neves et al. 2021; Çakar et al. 2024), indicating similar average measurements across these breeds (Table 2). To fully elucidate the impacts of dimensional differences observed between species on these animals, further examination of genetic, environmental, and production traits is recommended.

Parés Casanova and Jordana i Vidal (2008) reported skull width measurements of 235.3 ±14.2 mm in various domestic cattle and 234.8 ±11.5 mm in the Friesian (black and white) breed. In the present study, the skull width of Holstein cows was measured at 225.4 ±8.5 mm. A review of literature on other cattle breeds (Bartosiewicz 1980; Hayashi et al. 1981; Nıshıda et al. 1983; Hayashi et al. 1988; Bartosiewicz 2006; Körösı 2013; Cabezas Congo et al. 2019; Gambo et al. 2019; Özkan et al. 2019; Lomillos and Alonso 2020) indicates that Holstein cows generally have lower mean skull width values than female European bison (Krasınska et al. 2008), domestic cattle (Özkan et al. 2019), and Jersey (Neves et al. 2021). Furthermore, Parés Casanova and Jordana i Vidal (2008) documented a facial length of 400 ±20.5 mm in the Friesian breed, whereas the Holstein cow in this study exhibited a facial length of 288.4 ±17.4 mm, surpassing most other female cattle breeds reported in the literature (Bartosıewıcz 1980; Nıshıda et al. 1983; Hayashi et al. 1988; Körösi 2013; Cabezas Congo et al. 2019; Gambo et al. 2019; Neves et al. 2021) except for domestic cattle (Parés Casanova and Jordana i Vidal 2008; Özkan et al. 2019) (Table 2). The observed differences in skull width and facial length between Holstein cows and Friesians may stem from genetic and environmental factors contributing to dimensional variations, as well as potential methodological disparities.

In this study, the GHOR value for Holstein cows was determined as 162.7 ±8.2 mm (Table 2). This value was higher than those recorded for other female individuals in the literature, with the exception of *Bos primigenius* (190.7 ±13.3 mm) (Grigson 1978) and *Bos taurus* (170.3 ±7.5 mm) (Özkan et al. 2019) (Table 2). The LHOR values reported by Özkan et al. (2019) were 168.4 ±12.5 mm for water buffalo and 131.3 ±7.3 mm for domestic cattle, while in Holstein cows, the LHOR was 124.3 ±7.6 mm, exceeding

values reported for other female cattle breeds (Grigson 1980; Bartosiewicz 2006; Körösı 2013; Gambo et al. 2019), with the exception of water buffalo and domestic cattle. The GBOC value in Holstein cows closely matched that of domestic cattle (Özkan et al. 2019), and the GMB value was similar to that of the Hungarian grey (Bartosiewicz 2006). Additionally, the GBFM value in Holstein cows had a higher mean compared to buffalo and cattle data in the literature (Brudnickı et al. 2012; Körösi 2013; Gambo et al. 2019; Özkan et al. 2019). The HFM value in Holstein cows (39.3 ±2.1 mm) was comparable to Kuri cattle values (Gambo et al. 2019), aligning closely with literature reports (Körösi 2013; Özkan et al. 2019). For the cranial cavity measurements—LCC1 (140.5 ±6.4 mm), LCC2 (116.8 ±4.3 mm), MWCC (103.3 ±4.4 mm), and MHCC (96.6 ±4.7 mm)—no directly comparable data were available in the literature (Table 2).

In this study, an SL value comparable to index values reported for Holstein cows (43.4 ±1.3), Tibetan Gazelle (Zhu 2012), water buffalo, domestic cattle (Özkan et al. 2019), and Criollo cattle (Cabezas Congo et al. 2019) was obtained. The FAI value in Holstein cows (78.3 ±3.7) was lower than those of camels (Yahaya et al. 2012), gazelles (Zhu 2012), and sheep (Karimi et al. 2011; Ömer and Alpak 2012), but higher than values reported for water buffalo, domestic cattle (Özkan et al. 2019), Holstein bulls, and Simmental bulls (Çakar et al. 2024). Özkan et al. (2019) documented FRI values of 94.9 ±6.3 for water buffalo and 98.3 ±6.8 for domestic cattle, while Çakar et al. (2024) reported values of 96.0 ±5.9 and 96.4 ±6.4 for Holstein and Simmental bulls, respectively. In the current study, the FRI value in Holstein cows was positioned between water buffalo and domestic cattle, measuring 96.2 ±3.4. The BI value in Holstein cows (47.8 ±1.8) closely aligned with the domestic cattle measurement of 47.1 ±1.4 (Özkan et al. 2019). Relative to literature values (Yahaya et al. 2012; Kataba 2014; Choudhary and Singh 2015; Gambo et al. 2019; Özkan et al. 2019; Gündemir et al. 2020; Yılmaz et al. 2020; Çakar et al. 2024), Holstein cows exhibited the lowest FMI value (83.1 ±3.2) among *Artiodactyla* samples. Çakar et al. (2024) recorded LLI1 values of 83.6 ±6.1 and 86.4 ±7.7 in Holstein and Simmental bulls, respectively. This study also found that the LLI1 value in Holstein cows was similar to values for water buffalo and domestic cattle (Özkan et al. 2019). For other indices, including CCI1, CCI2, CCI3, CCI4, LLI2, LLI3, LWI1, and LWI2, no comparable data were found in the literature (Table 3).

Craniological studies spanning about 250 years have highlighted both interspecies similarities and skull variations within the *Bovidae* family, noting age- and gender-related changes. It has been observed that cranial dimensions decrease as cattle breeds transition from wild *Bos primigenius* to domesticated *Bos taurus* (Grigson 1978). Balcarcel et al. (2021) reported a 25.6% reduction in brain size in domestic cattle, as measured by some extracranial dimensions, compared to the aurochs. Craniometric analysis on wild Banteng cattle and five Asian local cattle breeds (Bali, Madura, Aceh, Leyte, Korea) suggested that Bali cattle could be a domesticated form of Banteng due to their close morphological relationship (Hayashi et al. 1988). When the hybrids between European bison and domestic cattle were compared with their parents, an increase in the skull size of the hybrids was observed (Krasinska 1988). In a study examining the changes in European bison skulls over time (from 1950 to the present), a year-related decrease in skull size and an increase in skull height in male individuals were detected in almost all of the skulls examined (Szara et al. 2023). Gender differences in skull measurements in European bison were notable between ages 1 and 3 but stabilized after age 5 (Krasińska et al. 2008). In cattle ontogeny, neurocranium measurements generally decrease, except for neurocranium length, with larger changes observed in the viscerocranium (Bartosiewicz 1980a, 1980b). Hungarian grey cattle exhibit age-related morphological changes in facial and frontal bones (Kőrösi 2013). In Jersey cattle (1–58 months), total head length, cranial and nasal length, and cranial width increased with age, while index values decreased ($p<0.05$) (Neves et al. 2021). In this study, a positive correlation was found solely between age and viscerocranium length (VL) in Holstein cows (p: 0.03, r: 0.582) (Table 4). Morphometric assessments in Lidia cattle (4–6 years) indicated a mesocephalic head in males (50.6) and a dolichocephalic head in females (44.6) (Lomillos and Alonso 2020). Criollo cattle (adults) also displayed a dolichocephalic cranial type (M: 36.5, F: 43.1) (Cabezas Congo et al. 2019). Holstein and Polish Holstein-Friesian breeds have been classified as dolichocephalic and fall under the primigenius cranial type (Gulinski 2021). Similarly, in the current study, female Holstein cows (1.5–8 years) exhibited a dolichocephalic head structure, consistent with female Lidia and Criollo cattle, as indicated by the calculated skull index (43.4) (Table 3). These morphological differences across species are likely due to genetic, environmental, and productivity factors.

CONCLUSIONS

Despite limitations such as the restricted age range and number of female animals from a single species and the absence of morphometric data on the cranial cavity of the species, this study offers the first comprehensive reference data on craniometric features in Holstein cows, which had not been previously documented in the literature. These findings could serve as valuable resources for radiological and clinical studies, forensic science, investigations of sexual dimorphism, and zooarchaeological research.

Multidisciplinary studies are needed to investigate craniometric features across various developmental stages and genders, with findings evaluated from clinical, anatomical, and biological perspectives.

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Ethical approval: This study was carried out at Selçuk University. This research was approved by The Selçuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee (SUVDAMEK, Ref No: 2024/077, Tarih: 05/2024)

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RESEARCH ARTICLE

Commercial Lavender (*Lavandula Angustifolia*) Oil's Effects on Buck Spermatozoa during Cryopreservation

Nail Tekin Önder[1]*, Oğuzhan Şahin[1], Muhammet Can Kılıç[1], Seda Gökdemir[2], Serpil Aygörmez[3], Taygun Gökdemir[1], Mustafa Reha Coşkun[2], Savaş Yıldız[1], Yavuz Öztürkler[1]

[1] *Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Kafkas University, Kars, Türkiye*
[2] *Department of Microbiology, Faculty of Veterinary Medicine, Kafkas University, Kars, Türkiye*
[3] *Department of Biochemistry, Faculty of Veterinary Medicine, Kafkas University, Kars, Türkiye*

ABSTRACT

The objective of this study was to assess the impact of lavender oil preparation on the spermatological data, oxidant-antioxidant parameters, and antimicrobial properties of frozen buck semen due to its antioxidant and antibacterial characteristics. To conduct a comprehensive analysis of these impacts, the study established nine experimental groups. Three control groups designed as; a negative control group, a group treated with 1000 IU/mL penicillin and 1000 µg/mL streptomycin (C1), and a group treated with 500 IU/mL penicillin and 500 µg/mL streptomycin (C2). Additionally, groups were created by adding lavender oil to the base diluent. Commercial lavender oil was diluted at a ratio of 10^{-4}. We then formed four groups, each receiving different amounts of this diluted oil: 10 µL/mL, 15 µL/mL, 20 µL/mL, and 25 µL/mL. Simultaneously, two distinct groups were formed by using dosages of 20 µL/mL and 25 µL/mL by adding to the C2 group. The study revealed that lavender oil had protective effects on motility, plasma membrane integrity, and acrosome integrity. Furthermore, lavender oil's antioxidant properties positively affect frozen-thawed semen, and its antibacterial impact was shown when used in conjunction with the C2 group.

Keywords: Antioxidant, Buck semen, Cryopreservation, *Lavandula angustifolia,* Lavender essential oil

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Ticari Lavanta (*Lavandula Angustifolia*) Yağının Kriyoprezervasyon sırasında Teke Spermatozoası Üzerindeki Etkileri

ÖZ

Bu çalışmada, lavanta yağı preparatının spermatolojik veriler, oksidan-antioksidan parametreler ve antioksidan ve antibakteriyel özellikleri nedeniyle dondurulmuş teke spermasının antimikrobiyal özellikleri üzerindeki etkisinin değerlendirmesi amaçlanmıştır. Bu etkilerin kapsamlı bir analizini yapmak için, çalışmada dokuz deney grubu oluşturulmuştur. Üç kontrol grubu; Negatif kontrol grubu, 1000 IU/mL penisilin ve 1000 ug/ml streptomisin (C1) içeren bir grup ve 500 IU/mL penisilin ve 500 ug/mL streptomisin (C2) içeren bir grup oluşturulmuştur. Ek olarak, baz sulandırıcı üzerine lavanta yağı ilave edilen gruplar oluşturulmuştur. Ticari lavanta yağı 10^{-4} oranında seyreltildi. Daha sonra her biri farklı lavanta yağı dozu içeren dört grup oluşturuldu: 10 uL/mL, 15 uL/ml, 20 uL/ml ve 25 uL/mL. Eşzamanlı olarak, C2 grubuna 20 uL/ml ve 25 uL/ml'lik dozlarda lavanta yağı ilave edilerek iki ayrı grup oluşturuldu. Çalışma, lavanta yağının motilite, plazma membran bütünlüğü ve akrozom bütünlüğü üzerinde koruyucu etkileri olduğunu ortaya koydu. Ayrıca, lavanta yağının antioksidan özellikleri dondurulmuş-çözdürülmüş teke sperması üzerine olumlu etkileri olduğu ve C2 grubu ile birlikte kullanıldığında gösterilmiştir antibakteriyel etkisinin olduğu görülmüştür.

Anahtar Kelimeler: Antioksidan, Kriyoprezervasyon, *Lavandula angustifolia*, Lavanta esansiyel yağı, Teke sperması

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ORCID ID; *NTO: 0000-0001-5141-0008, OŞ: 0000-0003-3123-8540, MCK: 0000-0003-0771-3970, SG: 0000-0002-6040-6559, SA: 0000-0002-5675-5096, TG: 0000-0001-6049-7184, MRC: 0000-0002-1441-3995, SY: 0000-0001-6459-6841; YÖ: 0000-0002-0681-9311*

*Corresponding author e-mail: nailtekinonder@gmail.com

INTRODUCTION

Researchers are interested in plant-derived essential oils from aromatic plants because of their antibacterial and antioxidant capabilities. These oils are being studied to assure food safety and protect cellular functions (Danh et al. 2013; Touazi et al. 2018; Valdivieso-Ugarte et al. 2019; Jadczak et al. 2020; Troisio et al. 2024). Lavender oil has various effects including stress and anxiety reduction, promotion of wound healing, treatment of sleep disorders, and use in aromatherapy massage (Cavanagh and Wilkinson 2005; Fismer and Pilkington 2012; Donelli et al. 2019; Samuelson et al. 2020). Furthermore, lavender oil possesses antibacterial, antifungal, and antioxidant properties (Danh et al. 2013).

There are multiple potential sources of contamination while collecting semen using different procedures. Environmental factors and microorganisms introduced into semen may cause adverse impacts on sperm motility, mitochondrial membrane potential, and viability. The impairment of sperm functions and increased microbial load both have the capacity to result in unfavorable outcomes in the female genital tract and increase the risk of reduced fertility (Anel-Lopez et al. 2021). Hence, antibiotics like penicillin, streptomycin, and gentamicin are employed in semen extenders to eliminate the deleterious bacterial action. Still, the presence of reactive oxygen species during the preservation of semen, whether for a short or long period, has negative effects on spermatological parameters. Therefore, semen extenders are supplemented with antioxidants that reduce the harmful effects of reactive oxygen species on spermatozoa (Madeira et al. 2014; Touazi et al. 2018; Ghanem et al. 2023; Troisio et al. 2024).

The objective of this study was to assess the impact of lavender oil, due to its antimicrobial and antioxidant properties, on the microbial load and sperm data following semen freezing. Additionally, the study aimed to investigate the effectiveness of lower doses of antibiotics in semen extenders when combined with lavender oil.

MATERIALS and METHODS

Three Gurcu bucks, aged between 2 and 5 years, were kept and used at Prof. Dr. Ali Riza Aksoy Education, Research and Application Farm, which is located at the Faculty of Veterinary Medicine, Kafkas University in Kars, Türkiye. The collection of sperm samples was conducted by veterinarians in accordance with applicable regulations and guidelines governing animal husbandry and welfare.

Experimental Design

The chemicals used in the study were acquired from Merck (Merck, Darmstadt, Germany) and Sigma (Sigma, USA), unless otherwise specified. In the study, we used commercial *Lavandula angustifolia* oil preparation (Talya, Antalya, Turkey) from a company holding U.S. Food and Drug Administration, TS EN ISO 22000, TS EN ISO 9001:2015, ISO 22716:2008 GMP certificates (Talya Bitkisel). Lavender oil was first dissolved in DMSO at a ratio of 1:5 (Lavender oil volume: Total volume) (Do et al. 2021) and then diluted with base semen extender to make the total dilution ratio 1:10,000. Lavender oil diluted to 10^{-4} was used to make all dosages in lavender oil groups. Base semen extender consisted of 223.7 mmol/L Tris, 55.5 mmol/L fructose, 66.6 mmol/L citric acid, 100.4 mmol/L Trehalose, 4.03 mmol/L EDTA, 4 g/L, 20% egg yolk (v/v) and 6% glycerol (v/v) in distilled water. The study consisted of a total of nine groups: a negative control group (NC) without antibiotics, a group with antibiotics (C1) containing 1000 IU/mL penicillin G potassium (İbrahim Etem, İstanbul, Turkey) and 1000 μg/mL streptomycin sulfate (Menarini, İstanbul, Turkey) in the base extender, an antibiotic group (C2) containing 500 IU/mL penicillin and 500 μg/mL streptomycin, a group in which 20 μL/mL of diluted lavender oil was added to C2 (C20), a group in which 25 μL/mL of diluted lavender oil was added to C2 (C25), a group containing 10 μL/mL of diluted lavender oil (L10), a group containing 15 μL/mL of diluted lavender oil (L15), a group containing 20 μL/mL of diluted lavender oil (L20), and a group containing 25 of diluted μL/mL lavender oil (L25).

The semen collection technique was performed four times using an electro-ejaculator, at a frequency of every other day during non-breeding season. Following the collection, the ejaculates were transported to a water bath maintained at a temperature of 37°C. The evaluation of rapid wave motion and motility was conducted using a phase-contrast microscope (Nikon Eclipse-E400, Tokyo, Japan) equipped with a heated slide set at a temperature of 37°C. Samples exhibiting motility more than 70% and a sperm concentration exceeding $1.5x10^9$ spermatozoa per milliliter were selected for cryopreservation.

Pooled semen was diluted to a concentration of 25 million sperm per milliliter using the appropriate extender. The groups were thereafter chilled to a temperature of 5°C within a time frame of one hour. After being cooled, the sperm samples were allowed to reach equilibrium for a duration of two hours at a temperature of 5°C. The cryopreservation, thawing, and incubation procedures were conducted following the methods described by Yildiz et al. (2015). Three straws were thawed from each group on each study day, and a total of 12 straws were analyzed for each group.

Semen Analysis

The subjective evaluation of sperm motility was conducted using a 400x phase-contrast microscope with a slide warmed to 37°C. The functional integrity of the plasma membrane was assessed using the

hypoosmotic swelling test (HOST), following the approach published by Alcay et al. (2016).

Flow Cytometric Analysis

The analysis was conducted utilizing the Attune NxT Acoustic Focusing Cytometer, manufactured by Invitrogen in the United States. The fluorescence was quantified using a 480 nm excitation wavelength with a 10 nm excitation bandwidth. The emitted light was filtered using a 530/30 nm filter (BL-1) and a 695/40 nm filter (BL-3). The measurements were recorded using Attune NxT software v2.7 (Thermo Fisher). Following the utilization of forward and side scatter light signals to isolate the cell population, the mean fluorescence intensity of the analyzed sperm cells was quantified. The experiment contained a total of 10,000 sperm cells, with a flow rate of 12.5 µl/minute.

The acrosome integrity was assessed using the fluorescein isothiocyanate–conjugated peanut agglutinin (PNA)/propidium iodide (PI) dual-staining approach. The mitochondrial membrane potential was assessed using Rhodamine 123. Flow cytometric analysis was conducted using the methodology published by Gürler et al. (2016).

Biochemical analysis

Thawed semen samples were subjected to spermatological analyses after centrifugation them at 800 g for 10 minutes, resulting in the separation of the supernatant. The malondialdehyde (MDA) level was assessed following the protocol established by Placer et al. (1966), while the reduced glutathione (GSH) level was evaluated using the method described by Sedlak & Lindsay (1968) by using spectrophotometer (Epoch, Biotek, USA). As an MDA standard, 1, 1, 3, 3-Tetramethoxypropane was used, and the results were reported as nmol/mL protein. During the GSH analysis, the samples underwent precipitation using a 10% solution of trichloracetic acid, followed by centrifugation at a speed of 1000 g for 5 minutes. The reaction mixture consisted of 0.5 mL of semen supernatant, 2 mL of tris hydroxymethyl aminomethane buffer (0.4 M; pH 8.9), and 0.1 mL of l 5,5'-dithio-bis-2-nitrobenzoic acid. The solution was maintained at room temperature for a duration of 5 minutes, and subsequently measured at a wavelength of 412 nm using the spectrophotometer. The GSH values were quantified and reported in units of µmol/mL.

Microbiological analysis

For the microbiological analysis, 250 µL semen straws were delivered to the microbiology laboratory under cold chain at 4 ºC. The liquid was transferred into sterile microtubes and then subjected to dilution for the purpose of culture. To achieve this objective, a 10-fold dilution was prepared and then 100 µl of the diluted sample was plated on Plate Count agar up to a 10^5 dilution. The cultures were incubated at 37°C for 18-24h. All analyses were performed in triplicate. Following the incubation period, the number of colonies was determined and the data was recorded. The study results were reported using the logarithm of colony forming units per milliliter (Log_{10} cfu/mL).

Statistical analysis

The power analysis of the study was conducted using the progressive motility results from the control and 0.5 mg/ml zinc oxide nanoparticles groups in the research by Khalique et al. (2023), with a power of 95% and a significance level of 5%. The G*Power® software (Version 3.1.9.7, developed by Franz Faul, University of Kiel, Germany) was used for power analysis.

The statistical analysis was conducted using IBM SPSS version 28. The Shapiro-Wilk test was employed to evaluate the normality of the data. The data were presented as the mean value plus or minus the standard error. The statistical significance of the differences between subdivided groups was assessed using one-way ANOVA followed by Duncan's post hoc test. The Kruskal-Wallis test was employed to analyze data with a distribution that is not normal. Statistical significance was determined for P values below 0.05.

RESULTS

Table 1 presents the rates of motility, plasma membrane integrity, acrosome integrity, acrosome integrity in live cells, and mitochondrial membrane potential. The results for MDA and GSH are provided in Table 2. The findings of the microbiological analysis are shown in Table 3.

Upon thawing, it was found that the L20 and L25 groups exhibited greater preservation of motility compared to the NC group ($p<0.05$), while the C20 and C25 groups shown higher preservation of motility than the C2 group ($p<0.05$). No statistically significant difference was found in the motility values of the L10 and NC groups ($p>0.05$). Also, no statistically significant difference was observed among the motility of the NC, C1, and C2 groups ($p>0.05$). The C20, C25, L20, and L25 groups had higher plasma membrane integrity values compared to the NC, C1, and C2 groups ($p<0.05$).

Significantly greater acrosome integrity values were seen in all groups that used lavender oil ($p<0.05$). Upon analyzing the acrosomal integrity rates in viable cells, it was shown that the acrosome was maintained intact in all groups that included lavender oil ($p<0.05$). The study did not find any significant disparity in the results of mitochondrial membrane potential between the groups ($p>0.05$).

The MDA analysis revealed that the C20, C25, L15, L20, and L25 groups had lower MDA values compared to the NC, C1, and C2 groups ($p<0.05$). A significant difference was also found in MDA values between the L10 group and the NC groups ($p<0.05$). No significant difference in MDA values was found between lavender oil containing groups ($p>0.05$). Glutathione

peroxidase levels were lower in the NC compared to the L10, L15, L20, and L25 groups and lower in C1 and C2 groups compared to the C20, C25 ($p<0.05$). Analysis of the GSH values from the L10, L20, and L25 groups revealed a significant increase in GSH levels in the L20 and L25 groups ($p<0.05$).

The C1 group exhibited the least microbial development following the culture. The C20 and C25 groups had lower levels of growth compared to the C2 group. The NC, L10, L15, L20, and L25 groups did not significantly differ from each other, and more growth was observed as compared to all antibiotic-containing groups.

Table 1. Effect of lavender oil on sperm parameters

Measurements / Groups	Motility (%)	HOST (%)	A (%)	A-P (%)	M (%)
NC	26.25±1.22^{a}	54.17±1.00^{a}	41.13±1.92^{a}	25.91±1.72^{a}	91.35±1.22
C1	27.92±1.61^{ab}	53.58±1.26^{a}	42.18±2.34^{a}	26.05±1.92^{a}	89.79±1.07
C2	29.58±1.99^{ac}	54.50±1.34^{a}	43.26±1.75^{a}	29.86±1.51^{a}	89.60±1.14
C20	35.83±1.49^{d}	60.25±2.38^{bc}	50.52±1.21^{b}	36.26±1.51^{b}	89.91±1.80
C25	37.92±1.89^{d}	63.00±2.71^{c}	52.36±1.83^{b}	37.88±1.97^{b}	91.37±1.34
L10	29.17±1.21^{ac}	53.17±0.84^{a}	53.24±2.27^{b}	36.05±1.31^{b}	91.60±1.06
L15	30.83±0.89^{bce}	57.50±1.31^{ab}	55.63±3.55^{b}	36.25±1.59^{b}	89.14±2.76
L20	32.73±0.66^{cde}	59.70±0.72^{bc}	54.24±3.24^{b}	39.24±3.28^{b}	90.58±1.43
L25	37.08±0.97^{d}	63.50±1.96^{c}	55.83±3.78^{b}	41.49±3.03^{b}	88.99±2.01

$^{a\text{-}e}$: Values with different superscripts in the same column are significantly different ($P < 0.05$). HOST: Plasma Membrane Functional Integrity, A: Total Acrosome Integrity, A-P: Acrosome integrity with Intact Plasma Membrane, M: Total Mitochondrial Membrane Potential

Table 2. Antioxidant effect of lavender oil

Measurements / Groups	MDA (nmol/mL)	GSH (µmol/mL)
NC	120.20±7.79^{a}	12.63±0.68^{a}
C1	114.62±11.00^{ac}	14.11±0.80^{ab}
C2	115.63±7.22^{ac}	14.05±0.37^{ab}
C20	90.58±5.39^{b}	16.58±0.89^{cd}
C25	90.60±6.06^{b}	16.45±0.70^{cd}
L10	97.74±9.89^{bc}	14.92±0.54^{bc}
L15	93.00±10.02^{b}	15.95±0.49^{bcd}
L20	93.88±4.56^{b}	17.28±0.61^{d}
L25	93.15±6.88^{b}	17.06±0.48^{d}

$^{a\text{-}d}$: Values with different superscripts in the same column for each times are significantly different ($P < 0.05$). MDA: Malondialdehyde, GSH: Reduced glutathione

DISCUSSION

In context of the adverse effects associated with synthetic antioxidants, essential fatty acids possessing antioxidant properties have come forward as a significant alternative (Cornwell et al. 1998; Yang et al. 2010). Lavender oil, an essential oil, has demonstrated significant antioxidant activity in free radical scavenging tests. The lipid peroxidation test using linoleic acid, a significant constituent of lavender oil, demonstrated a 58% reduction in peroxidation (Yang et al. 2010). In order to reduce sperm damage during freeze-thaw processes, various enhancements

are implemented in semen extenders (Üstüner et al. 2022; Önder et al. 2023a; Aktar et al. 2024; Önder et al. 2024). Antioxidants are frequently utilized in extenders for this purpose (Avdatek et al. 2018; Bucak et al. 2019; İnanç et al. 2023; Ustuner et al. 2024). In line with these informations, in our study, including lavender oil into the extender enhanced the motility after freezing. While the composition of lavender oil may vary depending on the extraction method (Danh et al. 2013), our research has shown that the commercial lavender preparation helps maintain motility and plasma membrane integrity in NC, L20 and L25 groups and C2, C20 and C25 groups when evaluated individually. However, although the lower doses of lavender oil (L10 and L15) did not show a positive effect on plasma membrane integrity, the first observed positive effects on motility were detected in the L15 group. Semen may contain a diverse range of microorganisms (Moce et al. 2022; Moreira et al. 2022). The presence of these microorganisms have an adverse effect on sperm quality. However, some antibiotics used for this purpose have an adverse effect on the quality of sperm. (Santos and Silva 2020; Moreira et al. 2022). The study revealed notable disparities in bacterial proliferation among the NC, C1, and C2 groups. However, it observed that microbial propagation did not impact motility and plasma membrane integrity. Moce et al. (2022) noted that the quality of sperm can be affected positively or negatively by different types of bacteria. In our study, it was suggested that this difference, contrary to other studies, could also be due to species differences within the microbiota.

Table 3. Antimicrobial effect of lavender oil

Measurements	Microbiological Results (Log_{10} cfu/mL)
Groups	
NC	4.63±0.06[a]
C1	2.53±0.13[b]
C2	3.83±0.18[c]
C20	3.26±0.14[d]
C25	3.42±0.11[d]
L10	4.63±0.62[a]
L15	4.66±0.04[a]
L20	4.60±0.08[a]
L25	4.50±0.05[a]

[a-d]: Values with different superscripts in the same column for each times are significantly different ($P < 0.05$).

The study found that the addition of lavender oil in adequate quantities to both the group treated with antibiotics and the control group without antibiotics helped maintain the integrity of the acrosome during the semen freezing procedure. Various studies have demonstrated that the use of antioxidants in sperm extenders helps to preserve the integrity of the acrosome (Alcay et al. 2016; Falchi et al. 2020; Toker et al. 2023; Ustuner et al. 2023). On the current basis, the results derived from our investigation exhibit comparable harmony with prior studies on antioxidants. However, despite positive findings compared to the control groups, we found that the mitochondrial membrane potential was similar across all groups. Studies indicate an association with motility and mitochondrial membrane potential (Önder et al. 2023b). According to the results of our study, lavender oil did not have any beneficial effects on mitochondrial membrane potential despite the increased motility. Nevertheless, more thorough research is thought to be needed before a definitive conclusion can be made.

The sperm freezing process leads to the generation of reactive oxygen species, which in turn cause damage to both the structural integrity and functional capabilities of the sperm. The assessment of MDA, resulting from lipid peroxidation, is employed to assess the intensity of oxidative stress (Motlagh et al. 2014). In addition, the assessment of reduced glutathione is employed to evaluate the efficacy of the antioxidant defense system (Kumar et al. 2024). The supposed antioxidant, antibacterial, and other properties of lavender oil are mostly attributed to substances such as linalool, linalyl acetate, 1,8-cineole, cis and trans-ocimene, terpinen-4-ol, and camphor (Kıvrak 2018). In the present study, adding lavender oil to the control groups (NC and C2) resulted in a decrease in MDA levels and an increase in GSH levels. This suggests that lavender oil had a consistent effect on that previous information.

Essential oils have been reported to operate through a multiple mechanism by damaging the cell membrane and suppressing the expression of specific genes (Zych et al., 2024). Several investigations in the literature have assessed the synergistic effects of essential oils, such as

lavender oil, with antimicrobial agents. In these investigations, the interactions between various essential oils and antimicrobial agents are focused on their effects on specific bacterial species (Owen & Laird, 2018). Zych et al. (2020) reported that lavender oil and enrofloxacin had a strong synergistic effect against *Escherichia coli*. Evaluation of the microbiological analyses, in our study, revealed that the inclusion of lavender oil significantly impacted only the groups supplied with low doses of antibiotics. However, when we compared the lavender groups of interest to the negative control group, we found no significant difference. This implies that the dosage used may have been insufficient to detect the antibacterial impact and could have potentially interacted synergistically with the penicillin and streptomycin combination.

CONCLUSION

In conclusion, the study demonstrates that lavender oil is effective in preserving semen through cryopreservation. Additionally, commercially available preparations may serve as a convenient alternative for use.

Author's Contributions: The study was designed, and the manuscript was written by NTO. TG conducted assessments of post-thaw motility and HOST. NTO examined the flow cytometry results. SY and YÖ conducted a semen analysis throughout the process of semen collection. MCK and OS conducted semen collection and cryopreservation and were involved in all semen analysis procedures. SG and MRC performed the microbiological analysis. SA performed MDA and GSH analysis. NTO conducted the statistical analysis.

Ethical Approval: The Scientific Ethical Committee of Kafkas University in Kars, Turkey has granted approval for all matters related to the experimental settings and assessment methodologies (2024-105).

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

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RESEARCH ARTICLE

Pharmacokinetics of Meloxicam Following Repeated Intravenous Administrations in Sheep

Orhan ÇORUM[1*], Duygu DURNA ÇORUM[1], Kamil ÜNEY[2], Ayşe ER[2], Muammer ELMAS[2]

[1]Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Hatay, Türkiye
[2]Selçuk University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Konya, Türkiye

ABSTRACT

The purpose of this study was to investigate the pharmacokinetics of meloxicam following repeated intravenous injection in sheep. Five male merino sheep were used in the study. Meloxicam was administered intravenously to sheep at 0.5 mg/kg once daily for 5 days. Meloxicam analysis from plasma samples was performed by high-pressure liquid chromatography. Non-compartmental analysis was used to establish the pharmacokinetic parameters. Following the first dose of meloxicam, the values for the plasma concentration at the first sampling time ($C_{0.08}$), area under the plasma concentration-time (AUC_{0-24}), elimination half-life ($t_{1/2\lambda z}$), total body clearance (Cl_T), and volume of distribution at steady state (V_{dss}), were 2.53 µg/mL, 12.30 hour*µg/mL, 8.97 hour, 0.03 L/hour/kg, and 0.40 L/kg, respectively. Compared to the first dose, AUC_{0-24} and $C_{0.08}$ increased, Cl_T and V_{dss} decreased after the last dose administration. Following the first and final administration, the $t_{1/2\lambda z}$ values were similar. The accumulation levels of R_1 and R_2 were 2.48 and 2.81, respectively. In conclusion, repeated administration of meloxicam in sheep caused pharmacokinetic changes and accumulation. Therefore, the safety and therapeutic effect of meloxicam after repeated administration in sheep should be established.

Keywords: Intravenous, Meloxicam, Pharmacokinetics, Repeated dose, Sheep

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Koyunlarda Meloksikamın Tekrarlanan İntravenöz Uygulamasını Takiben Farmakokinetiği

ÖZ

Bu çalışmanın amacı, koyunlarda meloksikamın tekrarlanan intravenöz enjeksiyonunu takiben farmakokinetiğini belirlemektir. Araştırma 5 baş Merinos ırkı erkek koyun üzerinde gerçekleştirildi. Meloksikam koyunlara günde bir defa 5 gün boyunca 0.5 mg/kg dozunda intravenöz olarak uygulandı. Plazma örneklerinden meloksikam analizi yüksek basınçlı sıvı kromatografisi ile gerçekleştirildi. Farmakokinetik parametreleri kompartmansız analiz ile belirlendi. Meloksikamın ilk dozundan sonra ilk örnekleme zamanındaki plazma konsantrasyonu ($C_{0.08}$), plazma konsantrasyon-zaman eğrisi altında kalan alan (AUC_{0-24}), eliminasyon yarı ömrü ($t_{1/2\lambda z}$), toplam vücut klirensi (Cl_T) ve kararlı durum dağılım hacmi (V_{dss}) değerleri, sırasıyla 2.53 µg/mL, 12.30 saat*µg/mL, 8.97 saat, 0.03 L/saat/kg ve 0.40 L/kg olarak bulundu. İlk doza göre, son doz uygulamasından sonra AUC_{0-24} ve $C_{0.08}$ artarken, Cl_T ve V_{dss} azaldı. İlk ve son uygulamayı takiben $t_{1/2\lambda z}$ değerleri benzerdi. R_1 ve R_2 birikim seviyeleri sırasıyla 2.48 ve 2.81 idi. Sonuç olarak, koyunlarda meloksikamın tekrarlanan uygulanması farmakokinetik değişikliklere ve birikime neden oldu. Bu nedenle, koyunlarda tekrarlanan uygulamadan sonra meloksikamın güvenliği ve terapötik etkisi belirlenmelidir.

Anahtar kelimeler: Damar içi, Farmakokinetik, Koyun, Meloksikam, Tekrarlanan doz

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ORCID ID; *OÇ: 0000-0003-3168-2510, DDÇ: 0000-0003-1567-991X, KÜ: 0000-0002-8674-4873, AE: 0000-0002-6900-0055, ME: 0000-0001-9059-7151*
*Corresponding author e-mail: orhancorum46@hotmail.com

INTRODUCTION

Sheep are a significant livestock species extensively cultivated for their products, including meat, milk, and wool (Li et al. 2021). In recent years, sheep meat output has risen to 9.78 million tons, constituting 2.93% of total red meat production (Cordeiro et al. 2022). Sheep are susceptible to several disorders characterized by pain and inflammation, including lameness, castration, musculoskeletal pain, foot rot, mastitis, pneumonia, and enteritis, throughout their lives (Corum et al. 2018). Therefore, analgesic drugs are commonly used in sheep.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are extensively utilized in animals due to their anti-inflammatory, analgesic, and antipyretic properties. They show their effects by inhibiting the cyclooxygenase (COX) enzymes responsible for prostaglandin synthesis (Gates et al. 2005). The COX enzyme is present in two forms: COX-1 (constitutive) and COX-2 (inducible). NSAIDs that are more efficient against COX-2 enzymes cause less gastrointestinal adverse effects. Meloxicam is in the oxicam class and is one of the most commonly used NSAIDs in animals (Tekeli et al. 2020). Meloxicam has low gastrointestinal adverse effects since it inhibits the COX-2 enzyme (Woodland et al. 2019). Meloxicam is approved for parenteral use in mammals for musculoskeletal disorders, movement disorders, mastitis, septicemia, enteritis, and acute respiratory tract infections (CVMP 2006). It is also used in cases of pain and inflammation in fish, birds and reptiles (Corum et al. 2022a; Coskun et al. 2023a; Sladky 2003). Although meloxicam is not approved for use in sheep in our country, it is approved in some countries (Sim 2016).

The pharmacokinetics of meloxicam has been established in sheep. This research examined the impact of administration route (Woodland et al. 2019), dosage (Gungor et al. 2024), and age (Coskun et al. 2023b) on the single-dose pharmacokinetics of meloxicam. It is recommended to use a single dose of meloxicam in sheep (Sim 2016). However, it has been stated that it can be used repeatedly depending on the severity of pain and inflammation (Anonymous 2024). Drug repeated administration may change pharmacokinetics, hence affecting therapeutic effects as well as side effects (Corum et al. 2022b). Therefore, it is very important to conduct pharmacokinetic studies after repeated administration. Although the pharmacokinetics of meloxicam following repeated oral administration in sheep have been demonstrated (Depenbrock et al. 2021), no information has been found regarding repeated intravenous administration. Because meloxicam has a long half-life (10-24 hours, Coskun et al. 2023b; Gungor et al. 2024) in sheep, it was hypothesized that repeated intravenous administration might alter its pharmacokinetics. This study aims to determine the pharmacokinetics of meloxicam after repeated (once a day for 5 days) intravenous administration of 0.5 mg/kg to sheep.

MATERIALS and METHODS

Animals

The research was conducted on five male Merino sheep (10-12 months old, 55±5 kg weight). The study comprised sheep that were verified to be healthy by anamnesis and clinical assessment. The sheep were transferred to different pens a week before the research and remained there throughout. Both ear tags and numbered collars were used to number the sheep. The sheep were given hay and water continuously, and commercial feed was also given morning and evening, by their age and weight. The Selçuk University Veterinary Faculty Ethics Committee approved the experimental investigation on sheep.

Experimental Design

For drug administration to sheep, their body weights were weighed. The right and left jugular veins were used for drug administration and blood collection, respectively. Meloxicam (Maxicam x 4, Injection Solution, Sanovel, Türkiye) was administered intravenously to sheep at a dose of 0.5 mg/kg once daily for 5 days. Blood samples were taken at 0, 0.08, 0.17, 0.25, 0.33, 0.42, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 hours on the first (1) and last (5) day. Blood samples were taken at 0.08, 1, 8, and 24 hours on other days. Blood samples were collected using a catheter for the first 12 hours of the first and last days and the venipuncture method for the rest of the time and placed in tubes containing heparin. Plasma samples were obtained by centrifuging blood samples at 4000 g for 10 minutes and thereafter kept at -80 °C until the analysis of meloxicam.

HPLC Analysis

Meloxicam concentrations were analyzed by high-performance liquid chromatography (HPLC)-UV employing a previously established methodology (Tekeli et al. 2020). Following the addition of 0.4 mL of methanol (containing 0.1% formic acid) to 0.2 mL of plasma, vortexing was conducted for 45 seconds, followed by centrifugation for 12 minutes at 12,000 g. 0.2 mL of supernatant was transferred to autosampler vials, and 20 µL was injected onto an Inertsil ODS-3 column maintained at 40 °C. Meloxicam was detected with a UV-VIS detector (SPD-20A) adjusted to 355 nm.

Meloxicam was dissolved in NaOH (0.05 M) to achieve a concentration of 0.5 mg/mL. This stock solution was used to prepare working standards of 0.04–10 µg/mL in water. Calibration standards (0.04–10 µg/mL) were prepared in drug-free sheep plasma using working standards. The calibration curves,

spanning from 0.04 to 10 μg/mL, exhibited linearity with a correlation value of 0.9992. Three concentrations (0.1, 1, and 10 μg/mL) were analyzed 5 times daily for 5 days to determine recovery, intra-day and inter-day precision and accuracy. The limit of detection, limit of quantification, and mean recovery values of meloxicam were 0.02 μg/mL, 0.04 μg/mL, and >93%, respectively. The coefficient of variation and bias values were <6 and ±5.2%, respectively.

Pharmacokinetic Calculation

Plasma concentration time curves were plotted for each sheep. The appropriate pharmacokinetic model was determined via visual examination of individual concentration–time curves and the use of Akaike's Information Criterion. The pharmacokinetic parameters were evaluated by non-compartmental analysis using WinNonlin software. The accumulation ratios (R) of meloxicam in plasma were calculated using the formula previously reported (Colburn 1983; Corum et al. 2019).

Statistical Analysis

The pharmacokinetic data were presented as the geometric mean (minimum-maximum). Levene's test analyzed the homogeneity of variance, whereas the Shapiro-Wilk test tested the normality of data distribution. The paired t-test (SPSS 22.0) was employed to analyze statistical differences between the data from the first and last day. A p-value of less than 0.05 was deemed statistically significant.

RESULTS

The plasma concentrations are presented in Figure 1. The plasma concentrations at the first (0.08 hour) and last (24 hour) sampling times of the first and last days were 2.53 and 5.07 μg/mL, and 0.17 and 0.49 μg/mL, respectively.

The pharmacokinetic parameters are presented in Table 1. Following the first dose of meloxicam, the values for the area under the plasma concentration-time (AUC_{0-24}), elimination half-life ($t_{1/2\lambda z}$), total body clearance (Cl_T), and volume of distribution at steady state (V_{dss}) were 12.30 hour*μg/mL, 8.97 hour, 0.03 L/hour/kg, and 0.40 L/kg, respectively. As compared to the first dose, the last dose resulted in an increase in AUC_{0-24} values and a decrease in Cl_T and V_{dss} values. The $t_{1/2\lambda z}$ was similar in the first and last doses. The drug accumulation level on day 5 following intravenous administration of repeated doses was 2.48 for R_1 and 2.81 for R_2.

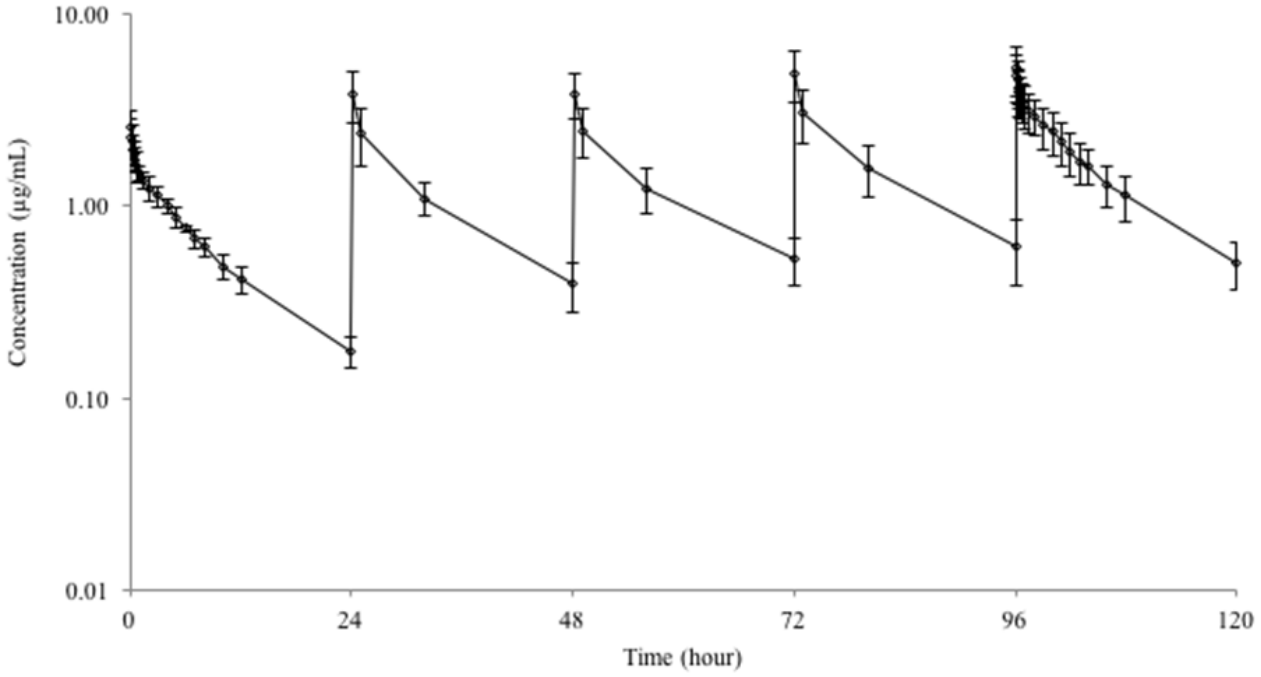

Figure 1: Semi-logarithmic plasma concentration–time curves of meloxicam in plasma after repeated intravenous administrations at the dose of 0.5 mg/kg every 24 hour for 5 days in sheep (mean ± SD, n = 5).

Table 1. Pharmacokinetic parameters for meloxicam in plasma after repeated intravenous administrations at the dose of 0.5 mg/kg every 24 hour for 5 days in sheep (n = 5).

Parameters	First (1) day	Last (5) day
$t_{1/2\lambda z}$ (hour)	8.97 (8.28-10.32)	9.83 (7.91-12.02)
AUC_{0-24} (hour*µg/mL)	12.30 (10.21-13.47)	30.54 (19.53-39.20)*
$AUC_{0-\infty}$ (hour*µg/mL)	14.58 (11.79-16.02)	37.69 (24.00-46.74)*
MRT_{0-24} (hour)	7.28 (6.98-7.72)	7.66 (7.16-8.14)
Cl_T (L/hour/kg)	0.03 (0.03-0.04)	0.01 (0.01-0.02)*
V_{dss} (L/kg)	0.40 (0.37-0.46)	0.18 (0.13-0.27)*
$C_{0.08}$ (µg/mL)	2.53 (2.03-3.35)	5.07 (3.05-6.95)*
R_1	-	2.48 (1.91-2.92)
R_2	-	2.81 (2.40-3.51)

Note: Data were presented as geometric mean (min-max).
*; Value is significantly different from the first day ($p<0.05$).
$t_{1/2\lambda z}$, elimination half-life; AUC, area under the plasma concentration–time curve; MRT, mean residence time; Cl_T, total body clearance; V_{dss}, volume of distribution at steady state; $C_{0.08,}$ plasma concentration at first sampling time, R_1, $AUC_{(0-24)5day}/AUC_{(0-24)1day}$; R_2, $C_{(min)5day}/C_{(min)1day}$. $C_{(min)}$: concentration at 24 hours on the first (1) and last (5) day.

DISCUSSION

The pharmacokinetic changes of meloxicam following repeated oral administration of 1 mg/kg for 10 days were previously demonstrated in sheep (Depenbrock et al. 2021). In this study, the pharmacokinetics of meloxicam after repeated intravenous administration of 0.5 mg/kg to sheep were demonstrated for the first time. Meloxicam dosing on a repeated basis resulted in considerable alterations in pharmacokinetic parameters.

Meloxicam at 0.5 mg/kg dosages was administered intravenously to sheep repeatedly (every 24 hours for 5 days) with no local or systemic adverse pharmacological effects identified. It has been reported that meloxicam is well tolerated in sheep at doses of 0.5–2 mg/kg parenterally or orally (Woodland et al. 2019; Stock et al. 2013). The best route to determine Cl_T and V_d is by intravenous administration, as they have no effect on bioavailability. Therefore, intravenous administration was preferred in this study.

The V_{dss} of meloxicam in sheep after the first and last doses were 0.40 and 0.18 L/kg, respectively. The V_{dss} decreased in the last dose compared to the first dose. Meloxicam is generally low in volume of distribution due to its high (>96%) binding to plasma proteins and its ionization at blood pH (CVMP 2006; Corum et al. 2022a; Gungor et al. 2024). Plasma protein binding is inversely proportional to volume of distribution (Sakai 2009). Since meloxicam is highly bound to plasma proteins, we reasoned that after repeated administration, the binding would reach saturation and the amount of free drug would increase, thus increasing V_{dss}. However, it was unexpected that the V_{dss} decreased. The formula V_d = dose/concentration is used to compute V_d. The last dose concentration of meloxicam is approximately 2 times the first dose concentration. The decrease in V_{dss} after repeated application is probably due to this situation.

The Cl_T of meloxicam in sheep after the first and last doses was 0.03 and 0.01 L/hour/kg, respectively. The Cl_T decreased in the last dose compared to the first dose. Meloxicam undergoes significant metabolism via phase I reactions, predominantly by CYP2C9 enzymes and, to a lesser degree, CYP3A4 enzymes, in animals, with fewer than 10% excreted unaltered in the urine. Meloxicam and its metabolites are excreted in urine and bile (CVMP 2006; Adawaren et al. 2019). The small amount excreted unchanged indicates that metabolic degradation plays an important role in the removal of meloxicam. The decrease in CL of meloxicam with repeated doses may be due to saturation of metabolism. The $t_{1/2\lambda z}$ after the first and last doses was similar. The $t_{1/2\lambda z}$ is a hybrid parameter that is directly proportional to V_d and inversely proportional to Cl_T (Turk et al. 2021). The fact that $t_{1/2\lambda z}$ did not change in this study may be due to the decrease in both V_d and Cl_T values.

Following five days of intravenous meloxicam treatment to sheep at a dosage of 0.5 mg/kg, the accumulation ratios for R_1 and R_2 were 2.48 and 2.81, respectively. The accumulation ratios of medicines are categorized as mild ($1.2 \leq R < 2$), moderate ($2 \leq R < 5$), and high ($R \geq 5$) (Li et al. 2013). The data indicate that intravenous injection of meloxicam every 24 hours for 5 days results in moderate accumulation in the body. However, repeated oral administration to horses and rabbits did not cause accumulation (Toutain et al. 2004; Carpenter et al. 2009). The increase in the accumulation ratio of the drug may also cause toxic effects. Therefore, after repeated administration of meloxicam in sheep, the dosing interval should be extended and adverse effects should be observed.

The therapeutic concentration necessary for meloxicam to provide analgesic and anti-inflammatory actions in sheep remains unidentified. However, effective concentrations for lameness in arthritis in horses and acute paw edema in dogs were reported as 0.13-0.20 µg/mL and 0.21-0.39 µg/mL, respectively (Jeunesse et al. 2011; Toutain and Cester 2004). When these values are considered in sheep, meloxicam maintained an efficacious concentration for 12 hours following the first dose and for 24 hours following the last dose. Nevertheless, it has been reported that NSAIDs accumulate at a higher concentration in the inflammatory site than in plasma due to their strong binding to plasma proteins (Lindemann et al. 2016). Therefore, it is important to evaluate their effectiveness in the inflammatory area, as plasma levels will not reflect their effectiveness.

CONCLUSION

The repeated treatment of meloxicam in sheep reduced excretion and extended retention time. Intravenous treatment of meloxicam to sheep at a dosage of 0.5 mg/kg over 5 days resulted in moderate accumulation. Therefore, the safety and therapeutic effect of meloxicam after repeated administration in sheep should be established.

Conflict of interest: The authors declare that there are no real, potential or perceived conflicts of interest for this manuscript.

Ethical approval: The research was discussed by Selçuk University Experimental Animals Local Ethics Committee on 26.03.2015 and session numbered 2015/03 and ethics committee permission was obtained with decision numbered 2015/30.

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RESEARCH ARTICLE

Evaluation of the Efficacy of a Herbal Formulation Containing Energised Oxygen Derivative and Borage Oil by *In Vitro* and *In Vivo* Studies

Hasan Hüseyin Demirel[1*], Özlem ERDAL ALTINTAŞ[2], Sevim Feyza ERDOĞMUŞ[3]

[1]Department of Laborant and Veterinary Health, Bayat Vocational School, Afyon Kocatepe University, Afyonkarahisar, Türkiye
[2]Department of Nutrition and Dietetics, Faculty of Health Sciences, Afyonkarahisar Health Sciences University, Afyonkarahisar, Türkiye
[3]Department of Basic Pharmaceutical Sciences, Faculty of Pharmacy, Afyonkarahisar Health Sciences University, Afyonkarahisar, Türkiye

ABSTRACT

The use of medicinal and aromatic plants for therapeutic purposes dates back to ancient times. In parallel with technological developments, there has been a notable increase in interest in botanical-based medicinal products. The products developed using advanced formulation techniques are safe, effective and rapid in the treatment of wounds and abrasions. In this study, the cytotoxicity of borage oil was evaluated *in vitro* on human dermal fibroblast cells. The effectiveness of a herbal formulation containing borage oil and energized oxygen molecules on second-degree *in vivo* burn modeling was determined. Acording to cytotoxicity test results showed that Borage oil did not have a toxic effect on human dermal fibroblast cells. It was determined that the herbal formulation was significantly more effective in second-degree burns than the control group. It is thought that the wound healing effect of the herbal formulation occurs thanks to the antimicrobial, antifungal, antioxidant and cell proliferation-increasing effects of Borage oil. The results obtained showed that the herbal formulation has the potential to be used in the treatment of second-degree burns.
Keywords: Energized oxygen molecules, Borage oil, Burn

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Enerjilendirilmiş Oksijen Türevi ve Hodan Yağı İçeren Bitkisel Formülasyonun Etkinliğinin *İn Vitro* ve *İn Vivo* Çalışmalar ile Değerlendirilmesi

ÖZ

Tıbbi ve aromatik bitkiler çok eski çağlardan bu yana tedavi amacıyla kullanılmaktadır. Teknolojik gelişmelere paralel olarak bitkisel içerikli medikal ürünlere olan ilgi giderek artmaktadır. İleri formülasyon teknikleriyle geliştirilen bu ürünler, yara ve yanıkların tedavisinde güvenilir, hızlı ve etkili bir şekilde kullanılabilmektedir. Bu çalışmada, hodan yağının sitotoksisitesi insan dermal fibroblast hücreleri üzerinde *in vitro* olarak değerlendirilmiştir. Hodan yağı ve enerjilendirilmiş oksijen molekülü içeren bitkisel içerikli formülasyonun ikinci derece *in vivo* yanık modellemesi üzerindeki etkinliği belirlenmiştir. Sitotoksisite test sonuçları Hodan yağının insan dermal fibroblast hücreleri üzerinde toksik bir etkisinin bulunmadığını göstermiştir. Bitkisel formülasyonun ikinci derece yanıklarda kontrol grubuna göre anlamlı düzeyde etkili olduğu belirlenmiştir. Bitkisel formülasyonun yara iyileştirici etkinliğinin Hodan yağının antimikrobiyal, antifungal, antioksidan ve hücre proliferasyonunu artırıcı etkisi sayesinde gerçekleştiği düşünülmektedir. Elde edilen sonuçlar; bitkisel formülasyonun ikinci derece yanıkların tadavisinde kullanılabilme potansiyeli olduğunu göstermiştir.
Anahtar kelimeler: Enerjilendirilmiş oksijen molekülleri, Hodan yağı, Yanık

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ORCID ID; *HHD:0000-0002-4795-2266 ÖEA:0000-0003-4680-1738 SFE: 0000-0002-4319-7558*
*Corresponding author e-mail: hdemirel@aku.edu.tr

GİRİŞ

Organizmanın hayati işlevlerini sürdürebilmesi için çeşitli fonksiyonların yerine getirilmesi, deri aracılığıyla sağlanır. Derinin bütünlüğü ve işlevselliği, bir dizi faktör tarafından olumsuz etkilenebilir ve bu durum yara oluşumuna yol açabilir. Yaraların iyileşmesi, derinin işlevinin yeniden kazanılması ve doku bütünlüğünün sağlanması açısından büyük bir öneme sahiptir. Yara iyileşme sürecinde birçok biyokimyasal ve hücresel mekanizma devreye girerken, hücresel yapıların ve doku tabakalarının yeniden oluşumu gerçekleşir. Yanıklar, organizmanın belirli bölgelerinin aşırı ısı, elektrik, kimyasal maddeler ve radyasyon gibi etkenlere maruz kalması sonucunda meydana gelen cilt yaralanmalarıdır (Ordin ve Sütsünbüloğlu 2017). Yanıklar, derinliklerine göre birinci, ikinci, üçüncü ve dördüncü derece olmak üzere dört ayrı kategoriye ayrılır (Markiewicz Gospodarek ve ark. 2022; Noor ve ark. 2022). Birinci derece yanıklar, derinin en üst katmanı olan epidermis tabakasının etkilendiği yaralanmalardır ve bu tür yanıklar genellikle kırmızı, kuru ve ağrılı bir görünüm arz eder. Yaralanmadan sonraki birkaç gün içerisinde ise epitelyumda kabuklaşma gözlemlenebilir. Bu tür yanıklar, birkaç gün içinde iz bırakmadan iyileşebilir (O'Brien ve Billmire 2008). Genellikle sıcak su dökülmesi, alev veya sıcak bir nesneyle temas sonucu oluşan ve derinin hem dermis hem de epidermis tabakasını etkileyen yanıklar ikinci derece yanık olarak sınıflandırılır. Bu tür yanıklar, doku hasarının daha fazla olduğu, ağrılı ve enfeksiyona açık yaralardır (Hussain 2013). Derinin tüm katmanlarının zarar gördüğü yanıklar üçüncü derece yanıklar olup, bu tür yanıkların kendiliğinden iyileşme yeteneği yoktur ve genellikle ağrılıdır. Üçüncü derece yanıklarda cerrahi tedavi uygulanmazsa skar gelişimi, sepsis ve ölüm riski artar. Kas, tendon ve kemiklerin de etkilendiği derin yanıklar ise dördüncü derece yanık olarak adlandırılır ve mutlaka cerrahi müdahale gerektirir (Patel ve ark. 2008; Suha ve Sanam 2022).

Yaraların iyileşme sürecinde uygun çevresel koşulların sağlanması büyük önem taşır. Yara bakımında en sık kullanılan ürünler arasında kremler bulunur. Güçlü antimikrobiyal özelliklere sahip enerjilendirilmiş oksijen molekülleri (EOM), krem formülasyonlarında yer alabilir. Bu moleküller kararsız bileşiklerdir ve belirli bir yarılanma süresi sonunda enerjilerini kaybederek tekrar nötr oksijen moleküllerine dönüşürler. Serbest radikal olmadıkları için, triozon molekülleri olarak bilinen ozondan farklıdırlar. Güneş ışınları veya 254 nm dalga boyundaki ışınlar, oksijen moleküllerini enerjilendirilmiş oksijen türevlerine dönüştürebilir. Oksijen molekülleri, bu enerji ile birbirleri arasında geçici bağlar kurarak enerji depolarlar. Profoks jeneratörü, az miktarda elektrik enerjisi kullanarak havadan elde ettiği oksijenle EOM üretebilir (Tecer ve Gündüz 2021). EOM'ların yağ içinde tutunma oranı yüksek olduğu için çeşitli krem formülasyonlarında baz olarak kullanılabilirler (Okumuş ve ark. 2023).

Tıbbi ve aromatik bitkiler arasında önemli bir grup, yağ içeren bitkilerden oluşur. Özellikle sağlık sektöründe, bu bitkilerden elde edilen sabit yağlara olan ilgi artmıştır. Bu bitkilerden biri olan *Borago officinalis* L. (Hodan), Boraginaceae ailesine aittir. 30-60 cm boyunda, sert tüylü, açık mavi çiçeklere sahip olan bu yıllık otsu bitkinin yaprakları sebze olarak kullanılır (Baytop 1999). Boraginaceae ailesi dünya genelinde 100 cins ve yaklaşık 2000 tür ile temsil edilir ve tropikal, subtropikal ve ılıman bölgelerde yaygın olarak bulunur. Aile üyeleri genellikle yıllık, iki yıllık veya çok yıllık otsu bitkiler olup, nadiren çalı veya ağaç formunda olabilirler. Yaprakları basit, alternan dizilişli ve genellikle sert tüylü, nadiren tüysüzdür (Evans 2002; Tanker ve ark. 2007). Boraginaceae familyasına ait bitkiler genellikle Akdeniz Bölgesi'nde yetişir ve Türkiye florasında bu aileye ait 34 cins ve 315 tür doğal olarak bulunur (Davis 1978, 1988; Özhatay 2011). Bu bitkilerin tohumları, özellikle γ-linolenik asit açısından oldukça zengindir. γ-linolenik asit, linoleik asidin doymamış bir metaboliti olup, başlıca doymamış yağ asitlerinden biridir (Sönmez ve ark., 2018). İçeriğindeki γ-linolenik asit, multiple skleroz, diyabet, kalp hastalıkları, artrit, egzama, immün bozukluklar, kanser ve adet öncesi ağrılar gibi sağlık sorunları üzerinde faydalı etkiler gösteren bir yağ asididir (Gupta ve ark. 2010; Al-Khamees ve ark. 2011). Literatür verilerine göre, Hodan bitkisi, biyolojik aktiviteleri nedeniyle sağlık üzerinde iyileştirici etkiler gösteren bir ajan olarak kullanılmaktadır. Bazı araştırmalar, bu bitkinin solunum, idrar yolu ve cilt hastalıklarının yanı sıra kalp-damar hastalıkları ve iltihaplı durumlarda da destekleyici tedavi olarak kullanılabileceğini ortaya koymuştur (Pieszak ve ark. 2012; Karimi ve ark. 2017). Hodan yağı, hodan çiçeği tohumunun soğuk sıkılması sonucu elde edilen bir yağdır. Protein, mineral ve vitamin bakımından zengin olduğu için sağlık alanında kullanılır. Özellikle cilt sağlığına olan katkılarıyla bilinen hodan yağı, bu konuda oldukça başarılıdır. Yapılan araştırmalar, cildin elastikiyetini artıran kolajen üretimini desteklediğini göstermiştir. Aynı zamanda cilde dolgunluk veren bu yağ, cilt kırışıklıklarını da giderme konusunda oldukça etkilidir (Michalak ve ark. 2023).

Yanıkların tedavisi üzerine çeşitli çalışmalar yapılmasına ve ürünler elde edilmesine rağmen bunların maliyeti yüksek olması, yan etkilere sebebiyet vermesi gibi kullanımlarını kısıtlayan birçok etken vardır. Bu nedenle bu ürünlere alternatif ürünler geliştirilmesi oldukça önemlidir. Bu çalışmada, tıbbi öneme sahip Hodan yağı ve enerjilendirilmiş oksijen molekülleri içeren bitkisel formül ilk kez *in vitro* ve *in vivo* çalışmalar ile değerlendirilmiştir.

MATERYAL ve METOT

EOM içeren Bitkisel İçerikli Formülasyon Hazırlanması

Profoks jeneratörü kullanılarak EOM (Enerjilendirilmiş Oksijen Molekülleri) elde edildi (Tecer ve Gündüz 2021; Okumuş ve ark. 2023). Bu jeneratör, saf oksijeni enerjilendirilmiş oksijen moleküllerine dönüştürme yeteneğine sahiptir. Bu dönüşüm, 254 nm dalga boyunda yoğun UV ışını yayan elektron sintilatörleriyle gerçekleştirilen reaktörlerde sağlanmaktadır. Nano delikler içeren bu sintilatörlerden geçen elektronlar, 254 nm dalga boyunda plazma ışınımı oluşturur. Kapasitör yapısındaki bu reaktörler, belirli bir rezonans frekansında bobinle indüklenir ve LC rezonans devresi sayesinde elektronların nano tüplerden geçişine imkan tanır. Reaktörler, AC 50.000 volt potansiyelinde indüklenerek çalışır. Bu sistemler, tetra oksijen üretmenin yanı sıra, zeytinyağı gibi bitkisel yağlarla da etkileşime girme potansiyeline sahiptir. Bu çalışmada, EOM moleküllerinin üretimi için zeytinyağı kullanıldı. Ayrıca, yara iyileştirme özelliği kazandırmak amacıyla formülasyona Hodan yağı eklendi ve en uygun formülasyonun etkinliği *in vivo* yanık modellemesiyle değerlendirildi (Okumuş ve ark. 2023).

Formül 1: EOM + Hodan yağı (1:1 v/v)
Formül 2: EOM + Hodan yağı (2:1 v/v)
Formül 3: EOM + Hodan yağı (3:1 v/v)
Formül 4: EOM + Hodan yağı (4:1 v/v)
Formül 5: EOM + Hodan yağı (5:1 v/v)

In Vitro Sitotoksisite Testi (MTT)

Hodan yağının HDFa hücre hattı (ATCC, PCS-201012) üzerindeki sitotoksik etkisi, MTT testi ile değerlendirildi. Hücreler, %10 (v/v) oranında fetal buzağı serumu (FBS), %1 (v/v) penisilin-streptomisin ve %1 (v/v) (1 mM) glutamin içeren DMEM (Dulbecco's Modified Eagle Medium) besiyeri kullanılarak, %5 CO_2 ortamda 37 ºC'de inkübe edildi. MTT testi, 96 kuyucuklu mikroplakalar kullanılarak yapıldı. Her kuyucuğa 2×10^4 hücre eklenip 200 µL besiyeri ile 37 ºC'de inkübe edildi. Hücreler %70 veya daha fazla konfluent hale geldikten sonra, 200 µL Hodan yağının iki kat halinde azalan konsantrasyonları eklenerek 24 saat daha 37 ºC'de inkübe edildi. İnkübasyon sonrası her kuyucuğa 20 µL MTT çözeltisi (5 mg/mL, PBS içinde) ilave edilip aynı koşullarda inkübasyona bırakıldı. Ardından MTT çözeltisi uzaklaştırılıp, her kuyucuğa 200 µL DMSO eklenerek 5 dakika inkübasyona devam edildi. Absorbans, ELISA plaka okuyucusunda 570 nm dalga boyunda ölçüldü. Kontrol grubundaki hücrelerin canlılık oranı %100 kabul edilerek, deneysel hücrelerin canlılık oranları aşağıdaki formül ile hesaplandı (Denizot ve Land 1986). EOM için yapılan bir önceki çalışmada, HDFa hücreleri üzerinde sitotoksik etkisi olmadığı belirlenmiştir (Okumuş ve ark. 2023).

% Hücre canlılığı = (örneğin absorbans değeri / kontrolün absorbans değeri)*100

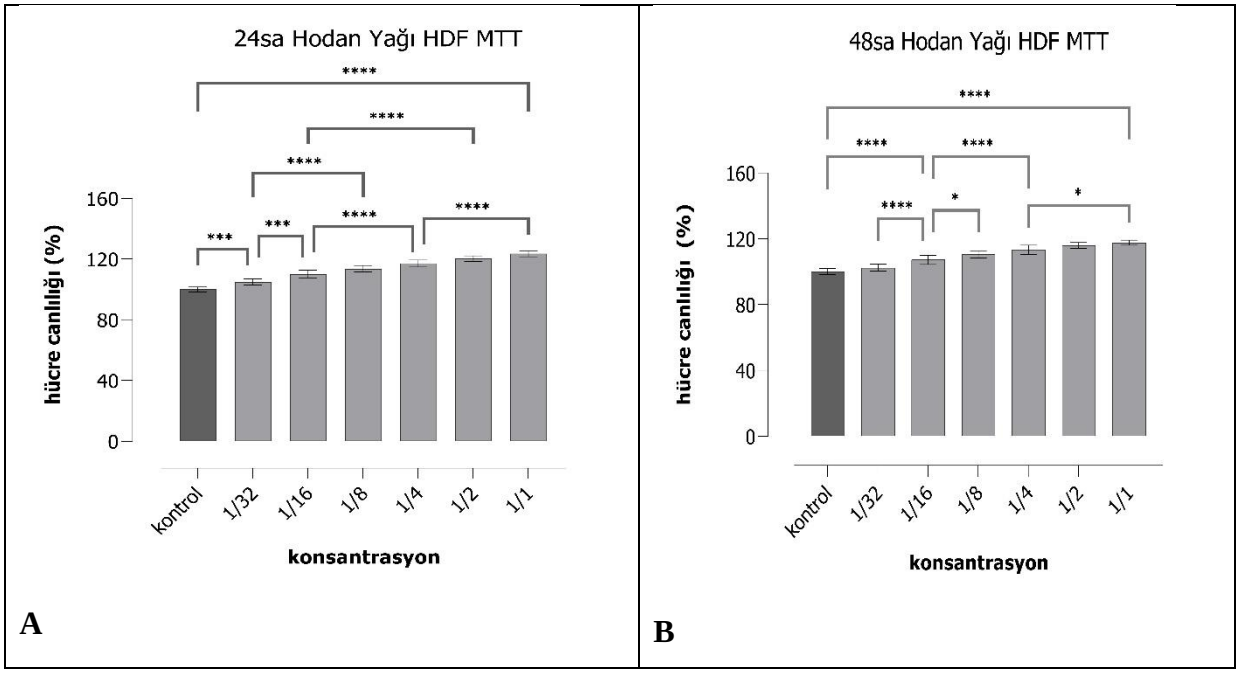
İn Vivo Çalışmalar

Afyon Kocatepe Üniversitesi Deney Hayvanları Ünitesinden temin edilen 18 adet erkek Sprague Dawley cinsi sıçan, 200-300 gram ağırlığında kullanıldı. Hayvanlar, deney sürecine başlamadan önce 7 gün boyunca ortama adapte olmaları için uygun oda sıcaklığında (25°C) ve nem oranı (%50-55) ile barındırıldı. Bu süre zarfında sıçanlara sınırsız miktarda sıçan yemi ve içme suyu sağlandı. Çalışmanın yapılabilmesi için Afyon Kocatepe Üniversitesi Hayvan Deneyleri Yerel Etik Kurulundan (AKUHADYEK) 49533702/261 numaralı onay alındı. İkinci derece yanık modellemesinin değerlendirilmesinde 250-300 g ağırlığında Wistar albino erkek sıçanlar kullanıldı. Deneyde, sıçanlara 12 saat açlık uygulandıktan sonra, 87 mg/kg ketamin ve 13 mg/kg ksilazin ile anestezi sağlandı. Ardından, sıçanların vücut yüzey alanlarının %10'unu geçmeyecek şekilde 1x1 cm boyutunda iki metal plaka 30 saniye boyunca kaynar suda bekletildi ve bu plakalar deneklerin tıraş edilmiş sırtlarına 10 saniye süreyle basılarak ikinci derece yanık oluşturuldu (Okumuş ve ark. 2023). Bu çalışmada, 18 erkek sıçan kullanılarak her biri 6 sıçandan oluşan 3 grup oluşturuldu (Okumuş ve ark. 2023). Negatif kontrol (NK) grubunda hiçbir terapötik ajan kullanılmazken, pozitif kontrol (PK) grubunda yara iyileştirici etkisi bilinen *Centella asiatica* bitkisinin ekstresiyle yapılmış olan Madecassol kremi uygulandı. Üçüncü grupta ise sıçanlara EOM ve Hodan yağı içeren bitkisel formülasyon uygulandı. Yanık oluşumunun ardından, her gruptan 0., 3., 7., 14. ve 21. Günlerde sıçanlar anestezi altına alınarak yanık bölgesinden biyopsi alındı ve örnekler nötral tamponlu formalin ile fikse edildi. Örnekler, ilk olarak akan su altında bir gece bekletildikten sonra, etanol (%50-%100) ve ksilen çözücülerinden geçirildi. Ardından 58 ºC'de erimiş parafinle infiltrasyon yapılarak parafin bloklara gömüldü. Mikrotom (Leica RM2245) kullanılarak parafin bloklardan 5-7 µm kalınlığında kesitler alındı. Ardından örnekler, toplamda 20 dakika süreyle üç kez ksilende bekletildi. Örneklerin hidratasyonu için sırasıyla birinci absolü alkolde 5 dakika, ikinci absolü alkolde, %96'lık alkolde, %80'lik alkolde ve %70'lik alkolde her biri 3 dakika süreyle bekletildikten sonra, distile suda 5 dakika süreyle yıkama işlemi uygulandı. Sonrasında, Harris Hematoksilen ile 10 dakika bekletilerek çekirdek boyaması yapıldı. Boya fazlası, çeşme suyu altında iyice yıkandıktan sonra eozin solüsyonuna alındı ve 5 dakika süreyle boyama işlemi tamamlandı. Boyama sonrası örnekler, sırasıyla %80, %96'lık alkol ve iki kez absolü alkolden geçirilerek, şeffaflaştırma için 5'er dakika süreyle üç farklı ksilende bekletildi ve son olarak entellan ile kapatıldı (Demirel vd, 2023). Boyanmış kesitler, araştırma mikroskobu (Nikon Corporation, Tokyo, Japonya) ile incelenip fotoğraflandı. Çalışma sonuçları, SPSS 22.0 yazılımı (SPSS Software, IBM, ABD) kullanılarak One Way ANOVA testi ile değerlendirildi (Okumuş ve ark. 2023).

BULGULAR

Hodan yağının HDFa hücreleri üzerinde 24, 48 saat boyunca sitotoksik etki göstermediği belirlenmiştir. Hodan yağının konsantrasyon artışına bağlı olarak 24. ve 48. saatlerde % hücre canlılığı değerlerinde artış olduğu tespit edilmiştir. Hodan yağının 1/1 konsantrasyonda kullanıldığında en yüksek hücre canlılığı 24. saatte %123,511±2,00 ve 48. saatte %117,690±1,54 olarak saptanmıştır (Şekil 1). Konsantrasyon artışına bağlı olarak hücre canlılığı değerlerinin de arttığı gözlenmiştir. Graph Pad Prism 9 (GraphPad Software, Inc., USA) programında One Way ANOVA, Tukey's çoklu karşılaştırma testi ile istatistiksel analizleri yapılmıştır ($p \leq 0.05$). EOM ve Hodan yağı formülasyonları krem formu bakımından değerlendirilmiştir. En homojen, sürülebilir formdaki ve nötrol pH'daki formülasyon olan iki numaralı formül *in vivo* çalışmalarda kullanılmıştır (Okumuş ve ark. 2023).

İkinci derece *in vivo* yanık modellemesi üzerinde iki numaralı formülün iyileştirici etkisi test edilmiştir. *İn vivo* deney gruplarının Image J programı kullanılarak ölçülen yanık alanlarının iyileşme yüzdeleri Tablo 1'de belirtilmiştir. Birinci gün alınan biyopsilerde yanık derinliği değerlendirilmiş ve ikinci derece derin yanık oluşturulduğu belirlenmiştir. *İn vivo* deney gruplarının farklı uygulama günlerindeki yanık alanı görüntüleri Şekil 2'de gösterilmiştir. EOM+HY grubu 3., 7., 14. ve 21. günlerde sırasıyla %30,53±3,93, % 57,41±2,27, %80,62±4,83 ve %99,98±0,00 olarak saptanmıştır.



Şekil 1. Hodan yağının HDFa hücre canlılığı üzerindeki etkisi
Figure 1. Effect of borage oil on HDFa cell viability

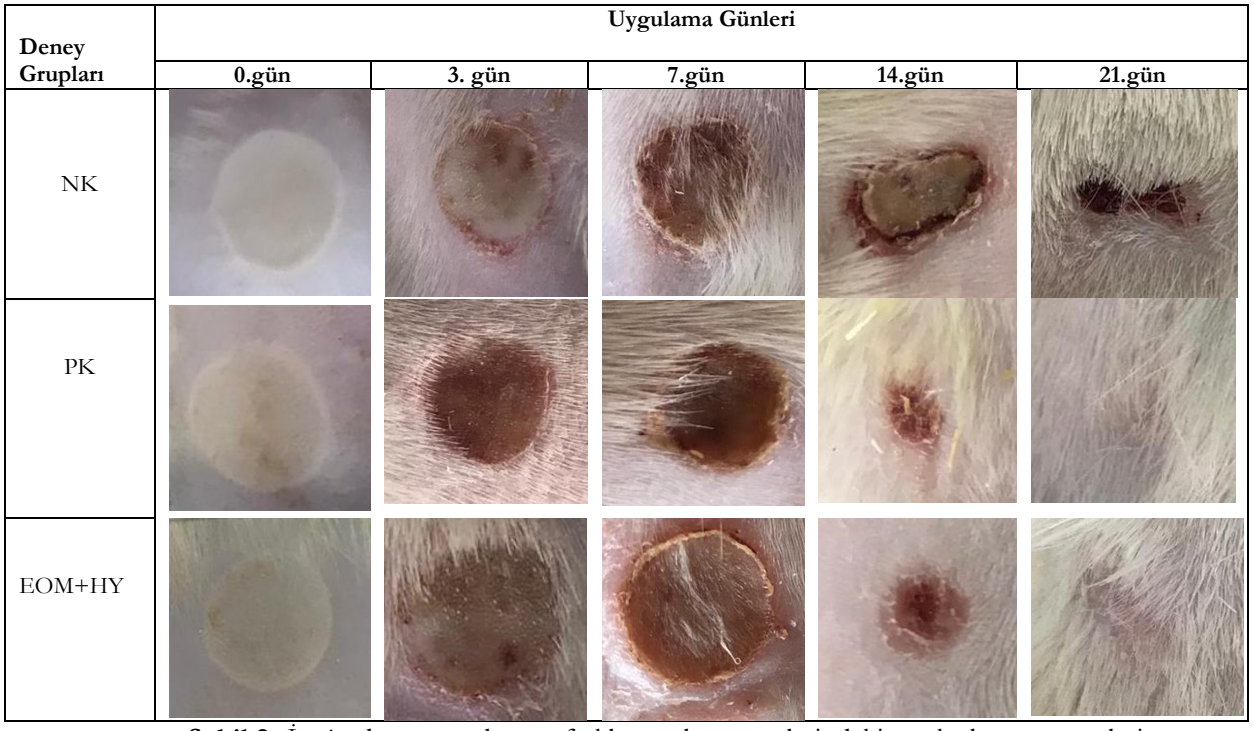
Tablo 1. *İn vivo* deney gruplarının ImageJ programı kullanılarak ölçülen yanık alanlarının iyileşme yüzdeleri
Table 1. Healing percentages of burn areas of *in vivo* experimental groups measured using ImageJ programme

***İn vivo* çalışma grupları (n=10)**	**Uygulama Günleri**			
	3. gün	**7. gün**	**14. gün**	**21. gün**
NK	14,87±2,29^{c}	29,26±2,74^{d}	52,76±2,41^{c}	80,89±3,38^{b}
PK	37,17±5,83^{a}	55,43±5,72^{ab}	90,83±1,42^{a}	99,98±0,00^{a}
EOM+HY	30,53±3,93^{b}	57,41±2,27^{a}	80,62±4,83^{b}	99,98±0,00^{a}
p değeri	0.000	0.000	0.000	0.000

NK: Negatif Kontrol, **PK:** Pozitif Kontrol, **EOM+HY:** Enerjilendirilmiş oksijen molekülleri ve hodan yağı içeren bitkisel formül
a, b:,c,,d: Aynı sütunda farklı harfleri taşıyan yanık iyileşmesi P<0,05 değerleri istatistiksel açıdan önemlidir.
Ortalama ± standard sapma; n=6

Histopatolojik Değişiklikler

Birinci gün alınan biyopsilerde yanık derinliği değerlendirilmiş ve ikinci derece derin yanık oluşturulduğu belirlenmiştir. *İn vivo* deney gruplarının farklı uygulama günlerindeki yanık alanı görüntüleri ve istatistik değerlendirmeleri Şekil 2 ile Tablo 2'de gösterilmiştir. Deney gruplarındaki hayvanlara ait bitkisel formülasyonun reepitelizasyon, neovaskülarizasyon, granulasyon, kollajen, inflamatuar hücre düzeyleri üzerine etkileri histopatolojik olarak ayrıntılı bir şekilde tanımlanmış ve Tablo 2 ile Şekil 3'de gösterilmiştir. Negatif kontrol grubu histopatolojik olarak incelendiğinde 3. günde epidermis ve dermis tabakalrının hemen hemen çoğunluğunun yanıktan etkilendiği ve nekrotik dokuların oluştuğu belirlendi. 7. günde dermiş ve epidermis sınırında kopmaların oluştuğu, inflamatuar hücre oluşumlarının şekillendiği dikkati çekti. 14. günde dermis epidermis sınırında çok sayıda inflamatuar hücre oluşumuyla karakterize granulasyon dokusu oluşumu dikkati çekti. Yer yer kollajen ve fibrositlere de rastlandı. Ancak hakim hücrelerin çoğunluğu inflamatuar nitlikte olduğu belirlendi. Neovaskülarize alanlar da dikkati çekti. 21. gün incelendiğinde ise inflamatuar hücrelerin sayısı 14. güne göre azalırken fibrositlere ve kollajen oluşumlarının şekillendiği dikkati çekti. Ayrıca kısmi bir epitalizasyonun oluştuğu fark edildi. Pozitif kontrol grubu dokuları incelendiğinde 3. günde dermiş ve epidermisin yanıktan etkilendiği kıl folikülü ve yağ ile ter bezlerinde de hasarların oluştuğu tespit edildi. Buna ilaveten nekrotik doku olumu da dikkat çekmekteydi. 7. günde dermiş ve epidermisteki nekrotik dokuda ayrılmalar göze çarparken, fibrotik ve inflamatuar hücrelerin oluşumu da dikkati çekti. 14. günde kabuk yapısının ortadan kalktığı ve fibrotik dokuların çoğalma, inflamatuar hücreleirin ise azalma eğiliminde olduğu belirlendi. Ayrıca en üst katmanda epitelizasyonun da fark edilir derecede oluştuğu saptandı. 21. gün incelendiğinde ise; dermişte birbirine sıkı bir şekilde bağlanmış kollajen lifler dikkati çekti. İnflamatuar hücre sayısının azaldığı görüldü. Epidermis alanında yer yer karatinize epitelin oluştuğu tespit edildi. EOM+HY grubu incelendiğinde ise; 3. günde dermis ve epidermisi içine alan ikinci derece yanık oluşumunun şekillendiği belirlendi. Epidermis ve dermiste nekrotik dokuların oluşumu ile birlikte inflamatuar hücreler ve neovaskülarize alanlar izlendi. 7. günde, nekrotik dokuda azalma ile birlikte fibrotik ve inflamatuar hücrelerin varlığı dikkati çekti.14. günde granulasyon dokusunun arttığı ve yoğun kollajen oluşumu görüldü. 21. günde inflamasyonun azaldığı yoğun bir fibrotik doku tespit edildi. Ayrıca epitelizasyonun çoğunluğunun tamamlanmış olduğu fark edildi. Negatif kontrol, pozitif kontrol ve enerjilendirilmiş oksijen molekülleri ve hodan yağı içeren bitkisel formül grubu dokularında reepitelizasyon, neovaskülarizasyon, granulasyon, kollajen, inflamatuar hücre düzeyleri istatistiksel açıdan incelendiğinde aralarındaki farkın anlamlı olduğu tespit edilmiştir ($p<0.05$).



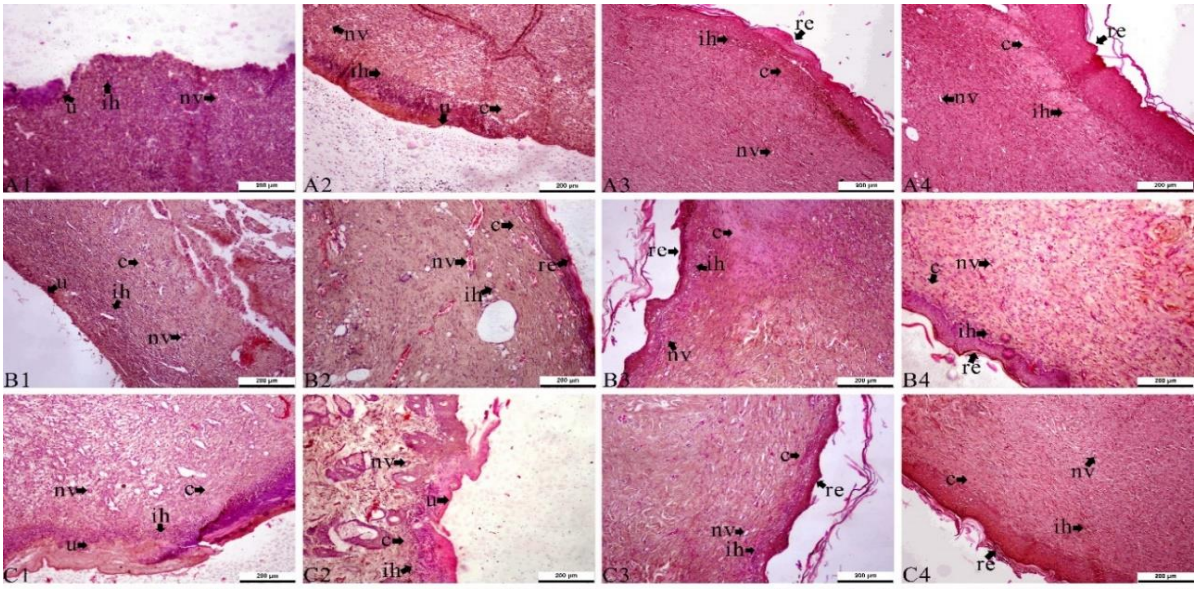
Şekil 2. *İn vivo* deney gruplarının farklı uygulama günlerindeki yanık alanı görüntüleri
Figure 2. Burn area images of *in vivo* experimental groups on different application days
NK: Negatif Kontrol**, PK:** Pozitif Kontrol**, EOM+HY:** Enerjilendirilmiş oksijen molekülleri ve hodan yağı içeren bitkisel formül

Tablo 2. Bitkisel formülasyonun reepitelizasyon, neovaskülarizasyon, granulasyon, kollajen, inflamatuar hücre düzeyleri üzerine etkileri.

Table 2. Effects of herbal formulation on reepithelialisation, neovascularisation, granulation, collagen, inflammatory cell levels.

İn Vivo **Grupları (n=6)**	**Reepitelizasyon**	**Neovaskülerizasyon**	**Granulasyon**	**Kollajen**	**İnflamatuar Hücre**
NK-3. gün	0,16±0,40^{g}	0,50±0,54^{c}	2,16±0,40^{ab}	1,01±0,16^{g}	2,83±0,40^{a}
NK-7. gün	0,33±0,51^{g}	0,83±0,75^{c}	2,01±0,12^{bc}	1,16±0,40^{fg}	2,66±0,51^{ab}
NK-14. gün	1,01±1,16^{defg}	2,01±0,89^{b}	1,50±0,54^{cde}	1,50±0,54^{defg}	2,50±0,54^{abc}
NK-21.gün	0,83±0,75^{defg}	2,33±0,81^{ab}	0,33±0,81^{gh}	1,66±0,81^{cdefg}	2,50±0,54^{abc}
PK-3.gün	0,33±0,51^{g}	1,01±0,63^{c}	2,01±0,63^{bc}	2,16±0,98^{abcde}	2,16±0,75^{abcd}
PK-7.gün	1,51±0,54^{cdef}	2,33±0,81^{ab}	1,01±0,16^{ef}	2,33±1,03^{abcd}	2,33±0,51^{abc}
PK-14.gün	2,66±0,51^{ab}	3,01±0,96^{a}	0,83±0,40^{fg}	2,83±0,40^{a}	2,01±0,16^{bcde}
PK-21.gün	2,83±0,41^{a}	2,83±0,40^{ab}	0,00±0,00^{h}	2,33±0,81^{abcd}	1,33±0,51^{f}
EOM+HY-3.gün	0,66±0,52^{efg}	0,83±0,41^{c}	2,66±0,51^{a}	2,16±0,75^{abcde}	2,50±0,54^{abc}
EOM+HY-7.gün	0,83±0,40^{defg}	2,16±1,32^{ab}	1,33±0,51^{def}	2,50±0,83^{abc}	2,33±0,52^{abc}
EOM+HY-14. gün	2,50±0,83^{abc}	2,66±0,52^{ab}	1,16±0,40^{def}	2,66±0,51^{ab}	1,83±0,40^{cdef}
EOM+HY-21. gün	2,51±0,84^{abc}	2,83±0,41^{ab}	0,00±0,00^{h}	2,50±0,83^{abc}	1,33±0,52^{f}
p değeri	0.000	0.000	0.000	0.000	0.000

NK: Negatif Kontrol, **PK:** Pozitif Kontrol, **EOM+HY:** Enerjilendirilmiş oksijen molekülleri ve hodan yağı içeren bitkisel formül
$^{a, b:,c,d,e,f,g,h:}$ Aynı sütunda farklı harfleri taşıyan yanık iyileşmesi (P<0,05) değerleri istatistiksel açıdan önemlidir.
Ortalama ± standard sapma; n=6



Şekil 3. Erkek ratların deri dokularında enerjilendirilmiş oksijen türevi ve hodan yağı içeren bitkisel formülasyonun etkinliğinin *in vitro* ve *in vivo* çalışmalar ile değerlendirilmesi. Tüm şekiller H&E ile boyanmıştır. Orijinal büyütme oranı olarak 10x ve 200 µm kullanılmıştır. (A) Negatif kontrol grubunu, (B) Pozitif Kontrol (C) Enerjilendirilmiş oksijen molekülleri ve hodan yağı içeren bitkisel formül (1) 3.gün, (2) 7. Gün, (3) 14. gün, (4) 21.gün yanık uygulanan ratları göstermektedir. **re:**reepitelizasyon, **nv:** neovaskülarizasyon, **u:**ulkus, **ih :** inflamatuar hücre, **c:** kollajen birikim alanlarını göstermektedir.

Figure 3. Evaluation of the efficacy of herbal formulation containing energised oxygen derivative and borage oil in skin tissues of male rats by in vitro and in vivo studies. All figures were stained with H&E. 10x and 200 µm were used as original magnification. (A) Negative control group, (B) Positive Control (C) Herbal formulation containing energised oxygen molecules and borage oil (1) 3rd day, (2) 7th day, (3) 14th day, (4) 21st day burned rats**. re:** reepithelialisation, **nv**: neovascularisation, **u:** ulcus**, ih**: inflammatory cell, **c:** collagen deposition areas.

TARTIŞMA

Son yıllarda yara ve yanıkların tedavisinde yan etkilere sebebiyet vermeyen bitkisel içerikli ürünlerin kullanımı daha çok tercih edilmektedir (Hajialyani ve ark. 2018). Tıbbi ve aromatik bitkilerin kullanımıyla ilgili her geçen gün yeni bilgilerin keşfedilmesi, biyoteknolojik gelişmeler ile ileri formülasyon teknolojisilerinin kullanılması ile birlikte bitkisel içerikli medikal preparatlar geliştirilmektedir. Bu ürünler, tıbbi ve aromatik bitkiler sahip oldukları biyoaktif bileşenler sayesinde antioksidan, antimikrobiyal, antiinflamatuar, aneljezik v.b. etkileri ile yara ve yanıkların tedavisinde daha etkili olabilmektedir. Bu çalışma kapsamında, sağlık alanında yaygın olarak kullanılan Hodan yağı ve antimikrobiyal etkinliğe sahip EOM içeren bitkisel içerikli formülasyonun etkinliği değerlendirilmiştir.

Hodan bitkisi, karaciğer hücrelerini etkileyen toksik pirrolizidin alkaloidleri (likopsamin, supinidin, amabilin ve intermedin) içerdiğinden harici kullanım için tavsiye edilir (Pieszak ve ark. 2012). Fitokimyasal analizler hodan otunun karbonhidratlar, yağ asitleri, fitosteroidler, polifenoller (vanilik, p-kumarik, p-hidroksibenzoik, gentisik, kafeik, sinapik, rosmarinik ve klorojenik asitler, kersetin, isorhamnetin ve kaempferol), tanenler, saponinler içerdiğini göstermektedir. Mukoid bileşikleri, organik asitler (askorbik, malik, sitrik, asetik ve laktik asit), tokoferoller, allantoin, mineral tuzları ve vitaminler bakımından zengin olduğunu göstermiştir (Asadi-Samani ve ark. 2014; Abu-Qaoud ve ark. 2018; Kareem ve Hamad 2020). Yapılan çalışmalar Hodan bitkisinin biyoaktif madde içeriği nedeniyle antioksidan, antiinflamatuar, yaşlanma karşıtı, UV koruyucu, yatıştırıcı veya yumuşatıcı etkileriyle topikal cilt ürünlerinde kullanılabilir olduğunu göstermiştir (Asadi-Samani ve ark. 2014; Zemmouri ve ark. 2019). Hodan bitkisinde bulunan önemli bir bileşik grubu, serbest radikalleri temizleme yetenekleriyle bilinen polifenollerdir. Fenolik asitler, flavonoidler ve bunların türevleri dahil olmak üzere fenolik bileşiklerin antioksidan özellikleri, aromatik bir halkaya bağlı hidroksil gruplarının varlığıyla ilişkilidir. Bitki polifenolleri cilt fonksiyonu için önemli maddeler olarak kabul edilir. Polifenoller, kollajen ve elastin liflerinin hidrolizini katalize eden cilt kollajenazı ve elastazda bulunan enzimlerin aktivitesini inhibe eder (Michalak ve ark. 2022). Hodan tohumlarından elde edilen Hodan yağı, çeşitli endüstrilerde kullanılan değerli bir hammaddedir. Soğuk preslenmiş yağ olarak veya besin takviyesi formunda küresel pazarda değerli bir stoktur. Cilt sağlığı ve genel refah için çok önemli olan γ- linolenik asit gibi esansiyel yağ asitleri açısından zengindir. Ayrıca yağda alfa lipoik asit, flavonoidler, selenyum ve çeşitli vitaminler de bulunur (Pieszak ve ark. 2012; Schäfer ve ark. 2022). Hodan tohumu yağında γ- linolenik asit varlığından dolayı potansiyel anti-inflamatuar etkilere sahip olduğu gösterilmiştir (Asadi-Samani ve ark. 2014). Hodan yağı, antiinflamatuar, antimikrobiyal, yatıştırıcı, besleyici ve nemlendirici özellikleri nedeniyle egzama, sedef hastalığı ve sivilce tedavisini desteklemesi nedeniyle kozmetikte geniş uygulama alanı bulmaktadır. Ayrıca atopik dermatit tedavisinde önemli olan uygun hidrolipid bariyerinin korunmasına da katkıda bulunur. Farmasötik kullanımları, kardiyovasküler destekten romatoid artritin tedavisine yardımcı olmaya ve hatta potansiyel olarak kanser riskini azaltmaya kadar uzanır. Hodan yağının zihinsel performansı arttırdığı ve omega-3 yağ asitlerine atfedilen depresyon gibi zihinsel bozuklukların riskini azalttığı bilinmektedir (Pieszak ve ark. 2012; Obiedzińska ve ark. 2012; Kaźmierska ve ark. 2017).

Michalak ve ark. (2023); *Borago officinalis* bitkisinin metanol ve su-metanol özütlerinin fenolik profilleri ve biyolojik aktivitelerini analiz etmişlerdir. Flavonoidler (astragalin, kaempferol 4-glukozit, rutosid ve vitexin) ve fenolik asitler (kafeik, klorojenik, 3,4-dihidroksifenilasetik, ferulik, p-hidroksibenzoik, protokatekuik, rosmarinik ve siringik) olmak üzere on iki bileşik saptanmıştır. İnsan keratinositleri (HaCaT) ve fibroblastlar (BJ) üzerinde yapılan *in vitro* testlerin sonuçları, özütlerinin cilt hücrelerindeki reaktif oksijen türlerinin hücre içi seviyesini azaltabildiğini göstermiştir. Protein denatürasyonunun, lipoksijenaz aktivitesinin ve proteinaz aktivitesinin inhibisyonunu değerlendirmek için yapılan testler, Hodan özütlerinin anti-inflamatuar özelliklere sahip olduğunu göstermiştir. Ayrıca, bitkinin metanol özütü, yaşlanma karşıtı özelliklerin göstergesi olan hem kolajenaz hem de elastaz aktivitesinde güçlü bir inhibisyonu göstermiştir. Sonuçlar, Hodan bitki özlerinin cilt hücresi koruması bağlamında faydalı özelliklere sahip değerli biyoaktif bileşiklerin kaynağı olduğunu göstermiştir.

Başka bir çalışmada Farahpour ve ark. (2012), Hodan özütünün sıçan derisi yara iyileşme modelinde etkileri, histopatolojik çalışmalar ile değerlendirilmiştir. Sıçanlara %1,5 hodan merhemi, öserin-vazelin ve kontrolden oluşan 3 grup oluşturulmuştur. Tüm sıçanlara 21 gün boyunca günlük olarak topikal merhemler uygulanmıştır. Histopatolojik inceleme sonuçları, test gruplarının yara boyutunun kontrol grubuyla karşılaştırıldığında erken dönemde küçüldüğünü göstermiştir. Hodan ile histopatolojik değerlendirmede diğer gruplarla, kontrol ve plaseboyla karşılaştırıldığında anlamlı sonuçlar elde edilmiştir. Mevcut çalışma Hodan bitki özütünün yara iyileşme sürecini destekleyebildiğini göstermiştir.

Bu çalışmalara benzer olarak bizim çalışmamızda Hodan yağı ve EOM içeriğine sahip bitkisel formül ikinci derece yanık modellemesi üzerinde yara iyileştirici etkinliği test edilmiş olup sonuçlar kontrol grubuna elde edilen formülasyonun yara iyileştirici etkisinin olduğunu ve piyasada yara iyileştirici etkinliğiyle bilinen ve pozitif kontrol olarak kullanılan kremin etkinliği ile yakın değerlere sahip olduğunu göstermiştir. *İn vivo* deney gruplarının yanık alanlarının

iyileşme oranları; 7. günün sonunda bitkisel formül uygulanan grupta %57,41±2,27 iyileşme belirlenmişken bu oran pozitif kontrol grubunda %55,43±5,72 ve negatif kontrol grubunda ise %29,26±2,74 olarak belirlenmiştir. 14. günün sonunda bitkisel formül uygulanan grupta %80,62±4,83 iyileşme belirlenmişken bu oran pozitif kontrol grubunda %90,83±1,42 ve negatif kontrol grubunda ise %52,76±2,4 olarak saptanmıştır. 21. günün sonunda ise bitkisel formül uygulanan grupta %99,87±0,00 iyileşme belirlenmişken bu oran pozitif kontrol grubunda %99,98±0,00 ve negatif kontrol grubunda ise %80,89±3,38 olarak saptanmıştır.

Yara iyileşme süreci; inflamasyon, proliferasyon, rejenerasyon ve hücresel yanıtın yer aldığı karmaşık bir süreçtir. Yara iyileşme sürecinde, keratinositler, fibroblastlar, endotelyal hücreler, makrofajlar ve plateletler büyük rol oynamaktadır. Özçelik (2009) tarafından gerçekleştirilen çalışmada, 2. derece yanık yaralarında yara bölgesine enjekte edilen trombositten zengin plazmanın fibroblast aktivasyonunu ve kollajen oluşumunu artırdığı iddia edilmektedir. Bu çalışmada, birinci gruptaki histopatolojik örnekler incelendiğinde, 14. günde fibroblast aktivasyonunun başladığı gözlemlenmiştir. 21. günde ise fibroblast aktivasyonuna bağlı olarak belirgin bir kollajenizasyonun geliştiği ortaya konmuştur. Öte yandan, 14. günde kontrol grubu örnekleri değerlendirildiğinde, fibroblast aktivasyonunun henüz başlamadığı tespit edilmiştir. Yapılan başka bir çalışmada Subrahmanyam (1998), 2. derece yanık yaralarının tedavisinde bal kullanımının etkinliğini ortaya koymaktadır. Araştırmanın 7. gününde epitelizasyonun gerçekleştiği, 21. günde ise tamamlandığı vurgulanmaktadır. Ayrıca, Yüksel (2012) tarafından sıçanlar üzerinde gerçekleştirilen bir diğer çalışmada, %0,9'luk luk sodyum klorür, %1'lik gümüş sülfadiazin ve %10'luk povidon iyot kullanıldığı belirtilmektedir. Bu çalışmanın sonuçlarına göre, 7., 14. ve 21. günlerde yapılan değerlendirmelerde, epitelizasyon ve kollajenizasyon açısından gruplar arasında anlamlı bir farkın olmadığı ifade edilmektedir. Buz (2012) tarafından gerçekleştirilen çalışmada, 2. derece yanık yaralarının (parsiyel kalınlıkta) tedavisi amacıyla taurin, L-karnitin ve glutatyon gibi mezoterapik ajanlar kullanılmıştır. Bu çalışma sonucunda, uygulanan mezoterapik ajanların kontrol grubuna kıyasla epitelizasyon ve kollejenizasyon süreçlerini artırdığı gözlemlenmiştir. Ancak, 22. günde elde edilen örneklerin değerlendirilmesi sonucunda, taurin, L-karnitin ve glutatyonun kollejenizasyon ve epitelizasyon üzerinde yüzeysel bir etki yarattığı ifade edilmiştir. Bizim çalışmamız da yapılan incelemelerde bitkisel formülasyonun reepitelizasyon, neovaskülarizasyon, scar ve ulkus, kollajen, inflamatuar hücre düzeyleri açısından birbirleri ile karşılaştırıldığında anlamlı bir farkın oluştuğu ve literatürdeki çalışmaları destekler nitelikte olduğu belirlenmiştir.

Bu çalışmada 3,7,14 ve 21. günde alınan örneklerden yapılan histopatolojik incelemeler sırasında; EOM+HY uygulandığı olgulardan alınan örneklerde epitelizasyonun kontrol grubuna göre daha önce başladığı 7. günde, devam ettiği ve 21. günde tamamlandığı ortaya konulmuştur. EOM+HY grubu pozitif kontrol grubu ile yakın iyileşme süreci göstermiştir. Ayrıca kontrol grubunda ise, epitelizasyonun yetersiz düzeyde olduğu 21. günde epitelizasyonun henüz tamamlanmadığı saptanmıştır. Bu şekilde EOM+HY uygulanan yanık yaralarında yara iyileşme evrelerinin epitel onarımının negatif kontrole göre daha hızlı bir şekilde oluşturduğu belirlenmiştir.

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SONUÇ

Bitkisel içeriğe sahip medikal ürünler ucuz ve kolay bulunabilirliği, yan etkilerinin olmaması veya çok az olması nedeniyle sentetik ilaçlara alternatif ürünler olarak değerlendirilmektedir. Bu çalışma kapsamında Hodan yağı ve enerjilendirilmiş oksijen molekülleri içeren bitkisel içerikli formülasyonun etkinliği ilk kez ikinci derece *in vivo* yanık modellemesi üzerinde değerlendirilmiştir. Çalışma sonucunda elde edilen veriler; bitkisel formülasyonun ikinci derece yanıklar üzerinde pozitif kontrole yakın derecede etkili olduğunu ve ilaç geliştirme çalışmalarından kullanılabilme potansiyeli bulunduğunu göstermiştir. Bu iyileştirici etkinin bitkisel formülasyon içeriğindeki biyoaktif bileşenlerden kaynaklı antimikrobiyal, antifungal ve antioksidan etkisi sayesinde yara iyileşmesini desteklediği düşünülmektedir. Bu durum, yara/yanık iyileşmesinde kullanılan tıbbi öneme sahip bitkilerin, reepitelizasyon ve kolajenizasyonu hızlandırıcı maddelerle birlikte kullanımının yanık tedavisine önemli bir katkı sunacağını göstermektedir. Bu çalışma kapsamında elde edilen sonuçların klinik araştırmalar ile desteklenerek ürünün ticarileştirilebilmesi ve böylece karşılaşılan klinik vakalarda yanıkların daha kısa sürede iyileşmesine katkı sağlayacığı düşünülmektedir.

Çıkar çatışması: Yazarların bildirecekleri herhangi bir çıkar çatışması yoktur.

Yazarların Katkı Oranları: Yazarlar bu makaleye eşit oranda katkı sağlamışlardır.

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RESEARCH ARTICLE

Microbiological Quality of a Traditional Turkish Food Kokorec in Türkiye

Süleyman ÖNER[1], Murat METLİ[2]*, Fulya TAŞÇI[3]

[1]Hotel, Restaurant and Catering Services, Milas Vocatinal School, Muğla Sıtkı Koçman University, Milas, Muğla, Türkiye

[2]Department of Food Hygiene and Technology, Faculty of Milas Veterinary Medicine, Muğla Sıtkı Koçman University, Milas, Muğla, Türkiye

[3]Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Türkiye

ABSTRACT

Kokorec, widely consumed in many regions of Türkiye, is popular traditional offal food. This study aimed to investigate the general microbiological quality of kokorec. A total number of 100 kokorec samples, 50 raw, and 50 cooked-spiced, were analyzed for the aerobic colony count (AAC), *Enterobacteriaceae*, enterococci, yeast, moulds, enterobacters, coliforms, *E. coli, Bacillus cereus*, Staphylococci and Micrococci. In the raw kokorec samples, it was determined that the mean level of AAC $1.8x10^7$ cfu/g, *Enterobacteriaceae* $7.9x10^4$ cfu/g, coliforms $1.4x10^5$ cfu/g, *E. coli* $4.4x10^4$ cfu/g, enterococci $1.0x10^5$ cfu/g, staphylococci and micrococci $1.5x10^5$ cfu/g, yeast $3.0x10^2$ cfu/g, mould $2.0x10^2$ cfu/g. *E. coli* were found positive in 18 (36%) out of 50 raw kokorec samples. However, *B. cereus* could not be isolated in any of the raw kokorec samples. On the other hand, in cooked-spiced kokorec samples, the average level of AAC was $3.2x10^5$ cfu/g, *Enterobacteriaceae* $7.1x10^4$ cfu/g, coliforms $2.4x10^4$ cfu/g, *E. coli* $1.1x10^3$ cfu/g, enterococci $2{,}4x10^6$ cfu/g, staphylococci and micrococci $1.1x10^3$ cfu/g, *B. cereus* $8.2x10^4$ cfu/g, yeast $5.9x10^2$ cfu/g, mould $2.1x10^1$ cfu/g. In cooked-spiced kokorec samples, *E. coli* was found at a rate of 4% and *B. cereus* was found at a rate of 20%. As a result of this research conducted on kokorec samples offered for consumption, it was determined that the microbiological quality was low because it could be contaminated with undesirable microorganisms at different levels during processing and consumption. Therefore, the consumption of kokorec, which is widely consumed in every region of Türkiye, carries a high risk potential for public health.

Keywords: Kokorec, Microbiological quality, Traditional offal food

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Türkiye'de Geleneksel Türk Yemeği Kokorecin Mikrobiyolojik Kalitesi

ÖZ

Türkiye'nin birçok bölgesinde yaygın olarak tüketilen kokoreç, popüler bir geleneksel sakatat yemeğidir. Bu çalışma kokorecin genel mikrobiyolojik kalitesini araştırmayı amaçlamıştır. 50'si çiğ, 50'si pişmiş-baharatlı olmak üzere toplam 100 kokoreç örneğinde aerobik koloni sayısı, *Enterobacteriaceae*, enterokok, maya, küf, enterobakter, koliform, *E. coli*, *Bacillus cereus*, stafilokok ve mikrokoklar yönünden analiz edildi. Çiğ kokoreç örneklerinde aerobik koloni sayısının ortalama $1{,}8x10^7$ kob/g, *Enterobacteriaceae*'nin $7{,}9x10^4$ kob/g, koliformların $1{,}4x10^5$ kob/g, *E. coli*'nin $4{,}4x10^4$ kob/g, enterokokların $1{,}0x10^5$ kob/g, stafilokoklar ve mikrokokların $1{,}5x10^5$ kob/g, maya sayısının $3{,}0x10^2$ kob/g, küf sayısının $2.0x10^2$ kob/g olduğu belirlendi. 50 çiğ kokoreç örneğinin 18'inde (%36) *E. coli* pozitif tespit edildi. Ancak çiğ kokoreç örneklerinin hiçbirinde *B. cereus* izole edilemedi. Öte yandan pişmiş-baharatlı kokoreç örneklerinde; aerobik koloni sayısının ortalama $3{,}2x10^5$ kob/g, *Enterobacteriaceae*'nin $7{,}1x10^4$ kob/g, koliformların $2{,}4x10^4$ kob/g, *E. coli*'nin $1{,}1x10^3$ kob/g, enterokokların $2{,}4x10^6$ kob/g, stafilokoklar ve mikrokokların $1{,}1x10^3$ kob/g, *B. cereus*'un $8.2x10^4$ kob/g, maya sayısının $5{,}9x10^2$ kob/g, küf sayısının $2{,}1x10^1$ kob/g olduğu tespit edildi. Pişmiş-baharatlı kokoreç örneklerinde *E. coli* %4, *B. cereus* %20 oranında bulunmuştur. Tüketime sunulan kokoreç örnekleri üzerinde yapılan bu araştırma sonucunda, işleme ve tüketim sırasında istenmeyen mikroorganizmalarla farklı düzeylerde bulaşabileceği için mikrobiyolojik kalitesinin düşük olduğu belirlendi. Bu nedenle Türkiye'nin her bölgesinde yaygın olarak tüketilen kokoreç tüketimi, halk sağlığı açısından bir risk potansiyeli taşımaktadır.

Anahtar kelimeler: Kokoreç, Mikrobiyolojik kalite, Geleneksel sakatat yemeği

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ORCID ID; SÖ: 0000-0002-0593-9610, MM: 0000-0001-9247-3347, FT: 0000-0002-4117-7406

*Corresponding author e-mail: muratmetli@gmail.com

INTRODUCTION

Türkiye is a country located in the Anatolian peninsula in southwestern Asia and the Eastern Thrace regions of southeastern Europe. With this location, it serves as a cultural bridge between Asia and Europe. In addition, Türkiye has a wide variety of cultures that show the basic characteristics of Central Asian, Ottoman, Western and Islamic cultures and traditions. This cultural structure has enabled the creation of a rich content of Turkish cuisine, influenced by Turkish, Ottoman, Arabic, Greek and Persian cuisines. Also, Turkish cuisine has been influenced by those above cuisine and other neighbouring cuisines, as well as western European cuisines. Various local meat products such as döner kebab, roasted meat, sausage, pastrami, raw meatballs and kokorec are traditionally produced in Türkiye. (Anonymous 1, 2024; Kılıç, 2009).

In the production of many foods available in Türkiye, old practices and traditional methods have not been abandoned. Kokorec is one of these food items and offal dishes that have been produced and consumed in Türkiye for a long time. Kokorec is a traditional offal nourishment of the Balkans and Anatolia consisting mainly of cattle, lamb or goat intestines, often wrapping seasoned offal. The intestines of lambs of suckling age are used more. The small intestine is cleaned especially thoroughly and is filled with mesenteric fat. Small intestines prepared in this way are usually wrapped on a horizontal skewer and roasted on a charcoal, gas or electric grill. (Figure 1). Then, the chopped insides of the kokorec are mixed with chopped tomatoes and green peppers, and cooked in a large pan by adding hot red pepper and thyme. Using two spatulas, the cook constantly stirs and chops the mixture. When the preparation is finished, the food is kept warm on the grill until someone orders the service. Sometimes it is served in a piece of bread with adding some tomatoes or spices in it. It can also be served in half a loaf of bread or in a sandwich bread, plain or garnish, almost always with thyme and red pepper (Anonymous 2, 2024; Küçükkömürler and Koluman 2021). (Figure 2).

During the preparation of kokorec, intestinal microflora, inadequate cleaning, improper storage conditions, insufficient heat treatment, as well as long waiting times for consumption in the environment, may pose a microbiological risk to public health (Bilgin et al, 2008; Kara et al, 2013). Recently, concerns about food hygiene have increased consumers' awareness about food safety. Consumers' concerns about food safety and high demand for traditional food products are confronting. Although kokorec is popular traditional offal nourishment and widely consumed in all regions of Türkiye, only a few studies have been reported on the microbiological propertis of kokoreç recently. Therefore, the study aimed to determine the microbiological quality of kokoreç sold in different provinces of Türkiye.

Figure 1: The raw kokorec in Turkey

Figure 2: The cooked-spiced kokorec in Turkey

MATERIALS and METHODS

In this study, a total number of 100 kokorec samples, 50 raw and 50 cooked-spiced, were obtained from different provinces of Turkey. Samples were collected in sterile plastic bags and transported to the laboratory in a cold chain. Portions of kokorec (10 g) were transferred to a sterile stomacher bag with 90 mL of maximum recovery diluent (0.85% NaCl+0.1% peptone) (Merck 1.12535) and blended for 2 min in a stomacher (Masticator, IUL Instruments-Spain). Serial decimal dilutions were repeated using the same diluents up to 10^{-8}. Samples of 10^{-1} to 10^{-8} dilutions in 0.1 ml were then spread on the surface of agars.

Coventional microbiological methods and media were used to reveal total aerobic count, *Enterobacteriaceae*, coliforms, *E. coli*, enterococci, yeast, mold, *Micrococcus-Staphylococcus* and *B. cereus*. (Table 1).

The statistical analysis

Minitab-16 was used to calculate the mean, standard deviation, minimum value and maximum value of the data obtained.

RESULTS

In the present study, the presence of microorganisms isolated from raw and cooked-spiced kokorec samples are given in Table 2 and Table 3 respectively. In the raw kokorec samples, it was determined that the mean level of total aerobic count $1.8x10^7$ cfu/g, *Enterobacteriaceae* $7.9x10^4$ cfu/g, coliforms $1.4x10^5$ cfu/g, *E. coli* $4.4x10^4$ cfu/g, enterococci $1.0x10^5$ cfu/g, staphylococci and micrococci $1.5x10^5$ cfu/g, yeast $3.0x10^2$ cfu/g, moulds $2.0x10^2$ cfu/g. *E. coli* were found positive in 18 (36%) out of 50 raw kokoreç samples. Whereas, no *B. cereus* was isolated in all raw kokorec samples.

On the other hand, in cooked-spiced kokorec samples, it was noted that the mean level of total aerobic count $3.2x10^5$ cfu/g, *Enterobacteriaceae* $7.1x10^4$ cfu/g, coliforms $2.4x10^4$ cfu/g, *E. coli* $1.1x10^3$ cfu/g, enterococci $2.4x10^6$ cfu/g, *Micrococcus-Staphylococcus* $1.1x10^3$, *B. cereus* $8.2x10^4$, yeast $5.9x10^2$ cfu/g, moulds $2.1x10^1$ cfu/g. *E. coli* 4% were counted in the cooked-spiced kokorec samples. On the other hand, *B. cereus* was found 20% in the cooked-spiced kokorec samples. The results obtained from the research showed that the hygienic conditions of the the intestine and microbiological quality of the samples were low and never achieved. In this study, Although, *B. cereus* was not isolated in all raw kokorec samples, it was found 20% in the cooked-spiced kokorec samples

Table 1. Groups of Microorganisms and Analysis Methods

Microorganisms	Media	Incubation conditions	Metods
Total Aerobic Count	Plate Count Agar (Merck,1.05463.0500)	30°C 48-72 hour, Aerob	ISO 4833
Enterobacteriaceae	Violet Red Bile Dextrose Agar (Merck, 110275)	37°C 24-48 hour, Anaerob	ISO 7402
Coliform Bacteria	Violet Red Bile (Lactose) Agar (Oxoid, CM 0107)	37°C 24-48 hour, Aerob	ISO 4832
E. coli	Eosine Methylene Blue Agar (Merck, 1.01347.0500)	37°C 24-48 hour, Aerob	ISO 16649-1
Enterococci	Slanetz-Bartley Medium (Oxoid, CM 377)	37°C 18 hour, Aerob	Hartman et al. (1992)
Micrococcus-Staphylococcus	Baird-Parker Agar (Difco, 276840)	37°C 24-48 hour, Aerob	ISO 6888-1
Yeast	Yeast Extract Glucose Chloramphenicol Agar (Merck, 1.1600.0500)	25°C 4-5 days, Aerob	Pichhardt (1993)
Mould	Yeast Extract Glucose Chloramphenicol Agar (Merck, 1.1600.0500)	25°C 4-5 days, Aerob	Pichhardt (1993)
B.cereus	*Bacillus cereus* Selective Agar Base (Oxoid, CM 0617)	30°C 24 hour, Aerob	Lancette and Harmon, 1980

In present study, the mean value of TAMB was detected as $1.8x10^7$ cfu/g in the raw kokorec samples and $3,2x10^5$ in the cooked-spiced kokorec samples. The high count of TAMB found in kokorec samples might be attributed to the number of bacteria in raw kokorec materials, production conditions which were neither modern nor hygienic, unsuitable storage conditions, non-hygienic equipments, and contaminations induced by the environment and personnel. In present study, the mean numbers of yeasts and molds found in raw kokorec samples were $3.0x10^2$ and $2.0x10^2$ cfu/g, respectively.

Table 2. The results of microbiological analysis of raw kokorec samples (n: 50).

Microorganisms	Minimum (cfu/g)	Maximum (cfu/g)	Mean±SD (cfu/g)
Total Aerobic Count	$7.0x10^3$	$3.5x10^8$	$1.8x10^7±2.3x10^6$
Enterobacteriaceae	$<10^1$	$1.0x10^6$	$7.9x10^4±1.7x104$
Coliform Bacteria	$<10^1$	$2.3x10^6$	$1.4x10^5±2.4x10^4$
E. coli	$<10^1$	$3.5x10^6$	$4.4x10^4±5.2x10^4$
Enterococci	$<10^1$	$1.0x10^6$	$1.0x10^5±2.8x10^5$
Micrococcus-Staphylococcus	$<10^1$	$3.2x10^6$	$1.5x10^5±6.1x10^4$
Yeast	$<10^1$	$1.0x10^4$	$3.0x10^2±1.1x10^2$
Mould	$<10^1$	$2.4x10^3$	$2.0x10^2±3.6x10^5$

Table 3. The results of microbiological analysis of cooked-spiced kokorec samples (n: 50).

Microorganisms	Minimum (cfu/g)	Maximum (cfu/g)	Mean±SD (cfu/g)
Total Aerobic Count	$2.5x10^3$	$2.5x10^6$	$3.2x10^5±5.5x10^5$
Enterobacteriaceae	$<10^1$	$1.6x10^6$	$7.1x10^4±3.2x10^5$
Coliform Bacteria	$<10^1$	$6.0x10^5$	$2.4x10^4±1.2x10^5$
E. coli	$<10^1$	$1.0x10^4$	$1.1x10^3±2x10^3$
Enterococci	$<10^1$	$3.0x10^7$	$2.4x10^6±8.2x10^6$
Micrococcus-Staphylococcus	$<10^1$	$1.0x10^4$	$1.1x10^3±2.5x10^3$
B. cereus	$<10^1$	$8.0x10^5$	$8.2x10^4±1.7x10^5$
Yeast	$<10^1$	$7.0x10^3$	$5,9x10^2±1.6x10^3$
Mould	$<10^1$	$2.0x10^2$	$2.1x10^1±4.1x10^1$

On the other way, The mean numbers of yeasts and molds found in cooked-spiced kokorec samples were $5.9x10^2$ and $2.1x10^1$ cfu/g, respectively. According to these results, it is strongly suggested that the need to improve hygienic conditions, and storage conditions in the manufacturing of this product are necessery. It is also recommended that consumers should eat these products well-cooked.

We detected that the mean of *Enterobacteriaceae*, coliform, and *E. coli* counts were found as $7.9x10^4$, $1.4x10^5$, and $4.4x10^4$ cfu/g in raw kokorec samples; and as $7.1x10^4$, $2.4x10^4$ and $1.1x10^3$ in cooked-spiced kokorec samples, respectively. The presence of *E. coli*, which is closely related to fecal contamination and the presence of enteric pathogens, was observed in 18 out of 50 samples (36%) in raw kokoreç samples. However, *E. coli* were counted 4% in the cooked-spiced kokorec samples. The presence of Enterobacteriaceae bacteria, coliforms and *E. coli* in the samples suggests unhygienic practices during the preparation of kokorec. As a result, kokoreç produced at home or commercially may cause food infections and intoxications. Therefore, these results reveal the need to implement regulatory measures such as good manufacturing practices at all stages of the production chain (preparation of raw intestines, cooking, serving) to ensure the microbiological safety of kokoreç sold openly.

Enterococcus sp. bacteria of this genus are thought to be important as indicators of potential pathogenic microorganisms as they cause spoilage in foods. In the present study, the mean enterococci count was $1.0x10^5$ cfu/g in raw kokorec samples and $2.4x10^6$ cfu/g in cooked-spiced kokorec samples.

In this study mean *Staphylococcus-Micrococcus* count was $1.5x10^5$ cfu/g in raw kokorec samples while mean *Staphylococcus-Micrococcus* count was $1.1x10^3$ cfu/g in cooked-spiced kokorec samples. The main reservoir of *Staphylococcus-Micrococcus* is skin, nasal cavity, and throat in human and animal. The presence of *Staphylococcus-Micrococcus* might be resulted from either insufficient heat treated kokorec, or transmitted from human and animal. As a consequence food products may be orginally become contaminated during or after processing.

DISCUSSION

In these various studies conducted in Turkey, many microorganisms, including spore-forming bacteria, have been identified in spices (Tekinşen and Sarıgöl, 1982; Yıldırım et al., 1997; Aksu et al., 1997; Filiz, 2000; Üner et al., 2000; Çoşkun, 2010). In our study, we found that the presence of B. cereus and other microorganisms increased after the addition of spices to kokoreç samples. Spices used as flavor enhancers in meat products can be contaminated with bacteria, mold and yeast. Processing methods, moisture content and grain size affect the microbial load and diversity of spices (Akgül, 1993).

Yentür et al. (1989) stated that total aerobic count, coliform, *Escherichia coli*, *Staphylococcus*, and yeast-mould counts in cooking kokorec samples as 10^4-10^7; $4.0x10^4$; $7.8x10^2$; $1.0x10^3$ and $1.8x10^6$ cfu/g, respectively in Ankara. Temelli et al. (2002) examined the microbiological quality of a total of 30 kokoreç samples, 10 each raw, cooked and cooked-spiced, from different regions of Bursa. TAMB was 10^5-10^7 cfu/g,

10^4-10^5 cfu/g and 10^5-10^6 cfu/g in raw, cooked and spice-added kokoreç, respectively; coliform bacteria counts were 10^4-10^7 cfu/g, <1.0 x 10^1-10^4 cfu/g and 10^4-10^5 cfu/g in raw, cooked and spiced kokorecs, respectively; *E. coli* counts were 10^1-10^6 cfu/g, <1.0x10^1 cfu/g and <1.0x10^1 cfu/g in raw, cooked and post-cooked kokorecs with spices added, respectively; *Enterobacteriaceae* numbers were 10^4-10^6 cfu/g, 10^2-10^4 cfu/g and 10^3-10^5 cfu/g in raw, cooked and post-cooked kokorecs with spices added, respectively; Enterococcus numbers were 10^3-10^5 cfu/g, 10^2-10^4 cfu/g and 10^2-10^4 cfu/g in raw, cooked and spiced kokorecs after cooking, respectively; *Staphylococcu*s and micrococci counts were 10^3-10^6 cfu/g, 10^2-10^4 cfu/g and 10^3-10^5 cfu/g in raw, cooked and post-cooked kokorecs with spices added, respectively; yeast and mold counts were found to be 10^3-10^6 cfu/g, <1.0x10^2-10^4 cfu/g and 10^2-10^4 cfu/g in raw, cooked and post-cooked kokorecs with spices added, respectively.

Hampikyan et al (2008) reported that total aerobic count, coliform, *E. coli*, *S. aureus* counts in 15 kokorec samples as 5.3x10^3- 7.0 x10^5, <1.0x10^1- 2.1x10^4, <1.0x10^1-6.6 x10^2, <1.0x10^2- 4.8x10^3, respectively in Istanbul. (Kara et al. 2013) determined that TAMB, *Enterobacteriaceae*, coliform, *Escherichia coli*, *Enterococcus* spp., *Micrococcus-Staphylococcus*, yeast-mould counts in 50 kokorec samples as 6.29, 4.35, 2.43, 2.10, 4.17, 2.85, 5.89 log kob/g, respectively in Afyon. Kılıç (2016) found that TAMB, total coliform bacteria and yeast-mold numbers in raw kokoreç collected from 10 different restaurants in Isparta were 2.5 x10^7, 1.3x10^5, 1.5x10^5 cfu/g, respectively; TAMP, total coliform bacteria and yeast-mold numbers in cooked kokorec were 5.3x10^3, 1.0x10^1, 1.0x10^1 cfu/g, respectively; TAMP, total coliform bacteria and yeast-mold numbers in spicy-cooked kokorec were found to be 1.1x10^6, 5.7x10^5, 5.5x10^3 cfu/g, respectively.

Bilgin et al. (2016) investigated the microbiological qualities of raw, grilled and tandoor-cooked kokoreç. Accordingly, total aerobic count coliform bacteria and *S. aureus* in raw kokoreç were 3.8x10^7, 2.2x10^4, 3.2x10^3 cfu/g, respectively; total aerobic count, coliform bacteria, *S. aureus* in grilled kokoreç were 1.2x10^3, 5.7x10^1, 1.2x10^2 cfu/g, respectively; In tandoor-cooked kokoreç, total aerobic count, coliform bacteria and *S. aureus* were detected as 2.3x104, 8.6x101, 3.1x102 cfu/g, respectively. Akgöl et al. (2023) examined the microbiological quality of cooked plain and cooked spicy kokoreç samples taken from 3 different restaurants in Elâzığ. They found, on average, TAMB 3.92, 4.03, coliforms 2.04, 2.49, *Staphylococcus-Micrococcus* 1.65, 2.04, yeast-mold 1.16, 2.10 and $\log_{10}$ cfu/g in cooked plain and cooked spicy kokorec, respectively. These different results in the studies may be due to the difference in the microbial load of the spices used, personnel hygiene and storage conditions. Although there are many studies carried out regarding the microbiology of kokorec, in Türkiye, there has been still no national standart established for kokorec in Turkish Food Standarts. Because of the manufacturing technique and the hygienic concerns on raw material, it has been believed that kokorec is not suitable food in terms of safety and consumer health. Therefore, it is recommended to keep kokorec at temperatures between 60°C and 74°C before serving. In addition, time-temperature integrators are increasingly used in the packaging of long-term chilled foods to inform consumers about the cooling conditions to which foods are exposed throughout the distribution chain and to help them make food safety decisions. (Tache and Carpentier, 2014).

CONCLUSION

As a result of this research conducted on kokorec samples, which are very popular among street delicacies in Türkiye, it was determined that they could be contaminated with pathogenic and spoilage microorganisms at different levels during processing and consumption and that their microbiological quality was low. Hence, the consumption of the kokorec carries a high potential risk for the public health. For this reason, compliance with hygienic rules during the preparation and presentation of kokorec for consumption is very important in terms of food safety and public health. In order to produce uninterruptedly safe products "from farm to table", HACCP and GMP rules must be followed in all chains from production to consumption. In addition, traditional kokorec production methods need to be transformed into methods using modern technologies while preserving the familiar taste. At the end, EU prohibeted the consuption of kokoreç prepared unhygiene condition, So, On the way of join to the EU for Türkiye, it is higly important to put some legal standars for kokoreç.

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