Official Publication of
The Afyon Kocatepe University
Faculty of Veterinary Medicine

Kocatepe Veterinary Journal

2019, 12 (2), June

http://www.kvj.aku.edu.tr

ISSN: 1308-1594
e-ISSN: 2147-6853

DergiPark AKADEMİK

Kocatepe Veterinary Journal
2019 June 12 / 2

Official Publication of
The Afyon Kocatepe University

ISSN: 1308-1594 e-ISSN: 2147-6853

Kocatepe Veterinary Journal is International an Peer-Reviewed Journal and published four times a year.

Kocatepe Veterinary Journal;
indexed in TUBİTAK-ULAKBİM TR-Dizin, Turkey Citation Index, CAB Abstract, ResearchBib, SIS (Scientific Indexing Services), CiteFactor, CrossRef, Index Copernicus, Google Scholar, SJIFactor

Addressed:
Kocatepe Veterinary Journal, Afyon Kocatepe University, Faculty of Veterinary Medicine, 03200, Afyonkarahisar, TURKEY.

Tel: +90 272 214 9309 Fax: +90 272 214 9309 E-mail: kvj@aku.edu.tr

www.kvj.aku.edu.tr ***http://dergipark.gov.tr/kvj***

****Only accepts online submission****

RESEARCH ARTICLES

CASE REPORTS

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):110-115

DOI: 10.30607/kvj.493259

RESEARCH ARTICLE

Detection of *Mycoplasma bovis* Infection in Cattle Mammary Tissue by Immunofluorescence and qRT-PCR Methods

Serdar ALTUN[1*], Selçuk ÖZDEMİR[2]

[1]Atatürk University, Faculty of Veterinary Medicine, Department of Pathology, Erzurum, Turkey
[2]Atatürk University, Faculty of Veterinary Medicine, Department of Genetic, Erzurum, Turkey

ABSTRACT

Mastitis in cattle causes important economic losses in all over the world and *Mycoplasma bovis* is one of the important agent among pathogens shown to be responsible for this disease. The aim of this study was to determine the presence and prevalence of *Mycoplasma bovis* among the pathological agents implicated in mastitis by immunofluorescence staining and qRT-PCR methods in cattle mammary tissue. For this aim, 120 mammary samples with or without macroscopic lesions as this agent causes subclinical mastitis, were collected. Mastitis was diagnosed in 78 of 120 cases. Chronic mastitis cases in 56 and, acute mastitis in 22 cases were histopathologically diagnosed. Fluorescent positivity was determined 28/120 according to the results of immunofluorescence staining for all sections. Immunpositive signs of *Mycoplasma bovis* were commonly observed in samples with chronic mastitis. These results were confirmed by qRT-PCR. According to these results the presence of *Mycoplasma bovis* agent that an important threat to herd health is 23,3%.

Keywords: Cattle, Subclinical Mastitis, *Mycoplasma bovis*, qRT-PCR, Immunoflourescence.

***

Sığır Meme Dokularında *Mycoplasma bovis* Enfeksiyonunun İmmunofloresan ve qRT-PCR Yöntemleriyle Araştırılması

ÖZ

Sığırlarda Mastitis tüm dünyada önemli ekonomik kayıplara neden olmaktadır. *Mycoplasma bovis* ise bu hastalıktan sorumlu olarak gösterilen patojenler arasında en önemli bakteriyel etkenlerden biridir. Bu çalışmanın amacı, sığır meme dokusunda oluşan mastitisde rol oynayan patolojik ajanlar arasında Mycoplasma bovis'in varlığı ve prevalansını immunofloresan boyama ve qRT-PCR yöntemleriyle ve saptamaktır. Bu amaçla, etkenin subklinik mastitise yol açtığı düşünülerek, makroskobik olarak lezyonlu ya da lezyonsuz olunmasına bakılmaksızın 120 adet meme örneği toplandı. Toplanan 120 örneğin 56 kronik ve 22 akut olmak üzere toplam 78 örnekte histopatolojik olarak mastitis tanısı kondu. Tüm kesitlere uygulanan immünofloresan boyama sonuçlarına göre floresan pozitifliği 28/120 olarak belirlendi. *Mycoplasma bovis* immünopozitiflikleri daha yoğun olarak kronik mastitisli örneklerde gözlendi. Bu sonuçlar örnklere uygulanan qRT-PCR sonuçları ile uyumludur. Bu sonuçlara göre, Erzurum'da sürü sağlığı için önemli bir tehdit olan *Mycoplasma bovis* etken varlığı %23,3 oranında tespit edilmiştir.

Anahtar Kelimeler: Sığır, Subklinik mastitis, *Mycoplasma bovis*, qRT-PCR, İmmunfloresan.

To cite this article: Altun S. Özdemir S. Detection of Mycoplasma bovis Infection in Cattle Mammary Tissue by Immunofluorescence and qRT-PCR Methods. Kocatepe Vet J. (2019) 12(2):110-115.

Submission: 07.12.2018 **Accepted**: 03.03.2019 **Published Online**: 25.03.2019
ORCID ID; SA: 0000-0002-8735-7031, SÖ: 0000-0001-7539-0523
*Corresponding author e-mail: serdar.altun@atauni.edu.tr

INTRODUCTION

Bovine mycoplasmosis caused by *M. bovis* has an important place in mastitis disease and cause serious economic losses in cattle worldwide. Mastitis caused by *M. bovis* is highly contagious and results in a rapid decline in milk production (Giovannini et al. 2013; Amram et al. 2013). This agent is associated with a variety of disease involving pneumonia, arthritis, keratoconjunctivitis, otitis media (Tamada et al. 2002; Adamu et al. 2013). *M. bovis* is a high pathogen, especially in young calves less than 4 months, but it always threat animals with other virus such as *Bovine Respiratory Syncytial Virus*, *Parainfluenza Virus Type* 3, *Bovine Herpes Virus*, *Infectious Bovine Rhinotracheitis Virus*, *Bovine Viral Diarrhea Virus* and bacterium such as *Mannheimia haemolytica serotype A*, *Pasteurella multocida* and *Histophilus somni* (Aebi et al. 2012; Amram et al. 2013). Besides the severity of disease increases by various factors like stress, environmental conditions, immunodeficiency. Treatment application such as vaccination and using antibiotics was reported to be ineffective. In recent years *M. bovis* is shown as the most important pathogen with increasing prevelance values. *M. bovis* infection in animals results in an annual economic loss of body weight gain was reported to be $ 32 million in the United States. At the same time, $ 100 million loses reported due to mastitis bigger than due to pneumonia or loss of body weight. *M. bovis* which is highly contagious agents can be found in milk, body fluids, reproductive discharges, semen. Chronically infected cows defined as carrier animals play important role in transferring. *M. bovis* passes from cattle to calves with milk by systemic infection without any clinical signs, therefore this agent threat calves health directly (Nicholas et al. 2002 Haapalaa et al. 2018). Because of the nonspecific clinical signs and pathological lesions of *M. bovis* only laboratory diagnosis by culture and serological methods is neccesary for identification. Because of false results due to contamination and long survival time of antigen in blood. In recent years PCR and immunohistochemistry methods have successfully and effectively been employed for *M. bovis* (Radelli et al. 2008; Karahan et al 2010; Wooley et al. 2012). In this study, we aimed to investigate presence and prevalence of *M. bovis* infection in mastitis cases in Erzurum.

MATERIAL and METHOD

Sampling

The material of this study (120 samples) was obtained from slaughterhouses in Erzurum Province in Turkey. Half of each received mammary tissue samples were stored for 1 day to be fixed in a 10% buffered formalin solution for histopathology and immunohistochemistry and half of each sample were stored -20 ^{o}C for qRT-PCR. Routine histopathological process was performed in Shandon Citadel 2000 (USA) tissue system. After the routine histopathology procces, all sample were poured into paraffin for blocking and prepared microtome sections in 5μm by using rotary microtome (Leica RM 2255). Haematoxylin Eosın staining applied to all sections. Slides were examined under the light microscopy (Olympus BX52 with DP72 camera attachment).

Immunofluorescence staining method

After the routine histopathology process, Paraffin sections 4 μm were taken on to lysine-coated slides. These slides put in the oven for deparaffinization in 57° C for 1 hour. For indirect immunofluorescence staining, paraffin sections in 4 μm were placed on lysine-coated slide after primary Anti-rabbit *M. bovis* polyclonal antibody, Pendik Veterinary Control Institute) antibody application which was performed according to the protcol, 1\ 50 diluted seconder immunofluorescence antibody Goat Anti-Rabbit IgG H&L (FITC) (Cat No: ab 6717, 1/100 dilution, Abcam, Cambridge, UK) dropped 12 μl by using micropipette to each slides and waited 45 min. in the darkness. After standing in the dark, slides were washed with distilled water and covered with mounting medium (glycerol, 9 volumes; PBS 1 volume). All slides were examined in florescence microscope (Carl Zeiss axioskop A1 with Calibri 2 led fluorescence attachment).

Total RNA Isolation

Total RNA isolation was realized from the collected mammary tissue samples through the utilization of Trizol (Invitrogen, USA). Total RNA isolation was realized in line with the manufacturers's protocoles. Following the total RNA isolation, the RNA concentration was measured by virtue of NanoDrop (Epoch Microplate Spectrophotometer, USA). RNAs were run in a 1.5% agarose gel in 1XTBE solution for one hour at 80 volts with a view to control total RNA quality and visualized by gel imaging system and their RNA quality was determined.

DNase I treatment and cDNA Synthesis

DNase I (Thermo Scientific, USA) was performed against DNA contamination in isolated RNA samples. Dnaz I treatment was performed in line with the protocol provided in the kit. Subsequently, 1 μg was taken from these RNAs and cDNA was synthesized through utilization of the miScript Reverse Transcription Kit (Qiagen, Germany) in line with the protocol provided. The purity and quantity of the obtained cDNA was measured by virtue of spectrophotometer (Epoch Microplate Spectrophotometer, USA), and the cDNAs were diluted at the same ratios. Subsequently, the cDNA samples were stored at -20 ^{0}C for utilization in Real Time PCR studies.

Real-time PCR

qRT-PCR was performed through utilization of the CFX96 BioRad device in order to detect *M.Bovis*. The *β-actin* gene was employed for internal control. Master mix content created in real time PCR experiments is as follows: Syber Green 2X Rox Dye Master mix (Qiagen Germany), forward and reverse primers designed for genes, cDNAs as template and nuclease-free water. Reaction conditions and primer sequences of the genes (Fan et al. 2018) are shown in Table 1.

Statistical analysis

IBM SPSS 20 program was performed for statistical analysis. The Cp values of each virus were evaluated using a linear mixed model (Thonur et al. 2012).

RESULTS

In this study 120 cow mammary tissue were collected from the slaughterhouse. Mastitis was diagnosed in 78 of 120 (65%) cases. Chronic mastitis cases in 56 and, acut mastitis in 22 cases were histopathologically diagnosed. Fluorescent positivity was determined 28/120 according to the results of immunofluorescence staining for all sections. Positive immun signs of *M. bovis* were observed in 24 samples with chronic mastitis, 4 samples with acute mastitis. qRT-PCR test for *M. bovis* agent extracted from mammary tissue were applied, 28 out of 120 lung tissue samples examined were positive at qRT-PCR test. For positive reactions of immunofluorescence stain frequently cytoplasm of inflammatory cells was first target also epithelium and exudate in the lumen of mammary gland.

Histopathology

Histopathological analysis of 56/120 samples were diagnosed with chronic mastitis and 22/120 cases were diagnosed with acute mastitis. In chronic cases, mononuclear cell infiltration in interstitial tissue(Fig.1 A,B) and interalveolar septum thickness due to increased connective tissue were observed. İn acute cases, presence of exudates containing neutrophil leukocytes in alveoli of mammary gland was observed.

Macrophage infiltration with mononuclear cells was observed. Besides, diffuse and common plasma cell infiltration (Fig.1C) was the most striking finding in *M.bovis* positive tissues.

Immunofluorescence staining results

Positive staining for *M. bovis* antigen was detected in 28 out of 120 by indirect immunofluorescence. *M. bovis* antigen has been localized in the cytoplasm of macrophages around the alveoli and lümen of alveoli containing inflammatory cells in exudate. Positive reactions for immunofluorescence were located in frequently cytoplasm of inflammatory cells and epithelial cells.

qRT-PCR Results

A ten-fold serial dilution of each of the in vitro transcribed RNAs of *M. bovis* was triplicate analysis. M. bovis was detected 20 of 120 samples. *M. bovis* nucleic acid signals were shown in Figs. 2 and 3.

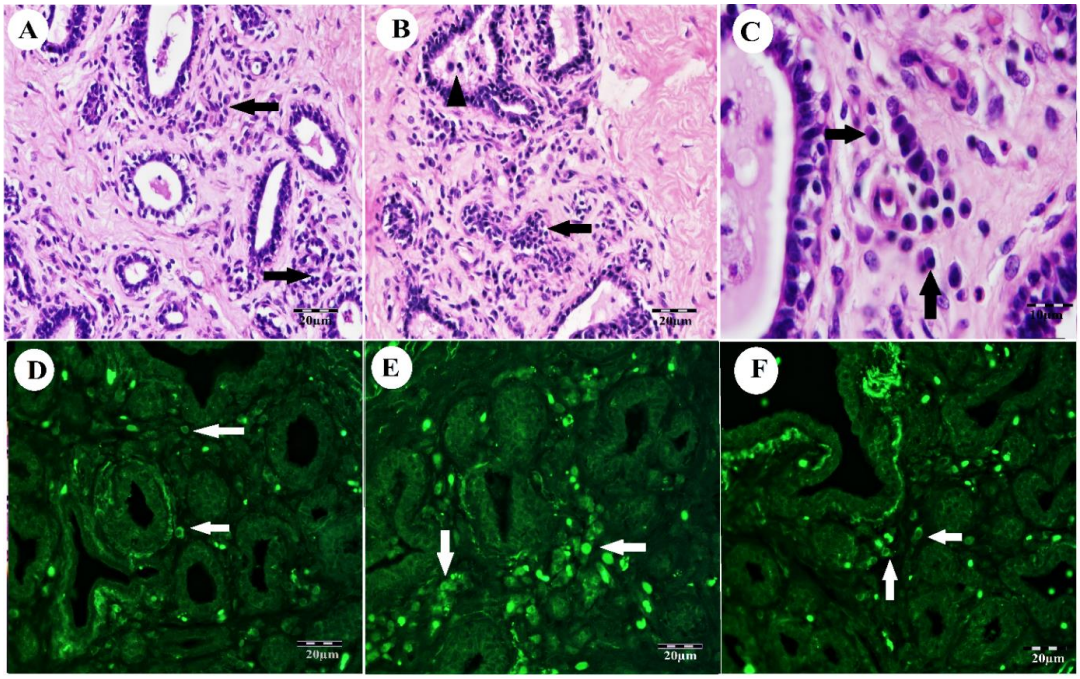


Figure 1. A) Mononuclear (macrophage) cell infiltrations in the interstitial space (arrows). H & E. 20 µm. **B)** Plasma cell infiltrations (arrows), H & E. 20 µm. **C)** Plasma cell accumulation (arrows). Macrophages in gland (arrowhead). H & E. 20 µm. **D)** *M. bovis* positivity in cytoplasm of mononuclear cells (arrows). IF. 20 µm **E)** Positivity (arrows) in mononuclear cells. IF. 20 µm. **F)** Positive reaction for *M.bovis* (arrows) in mononuclear cells. IF. 20 µm

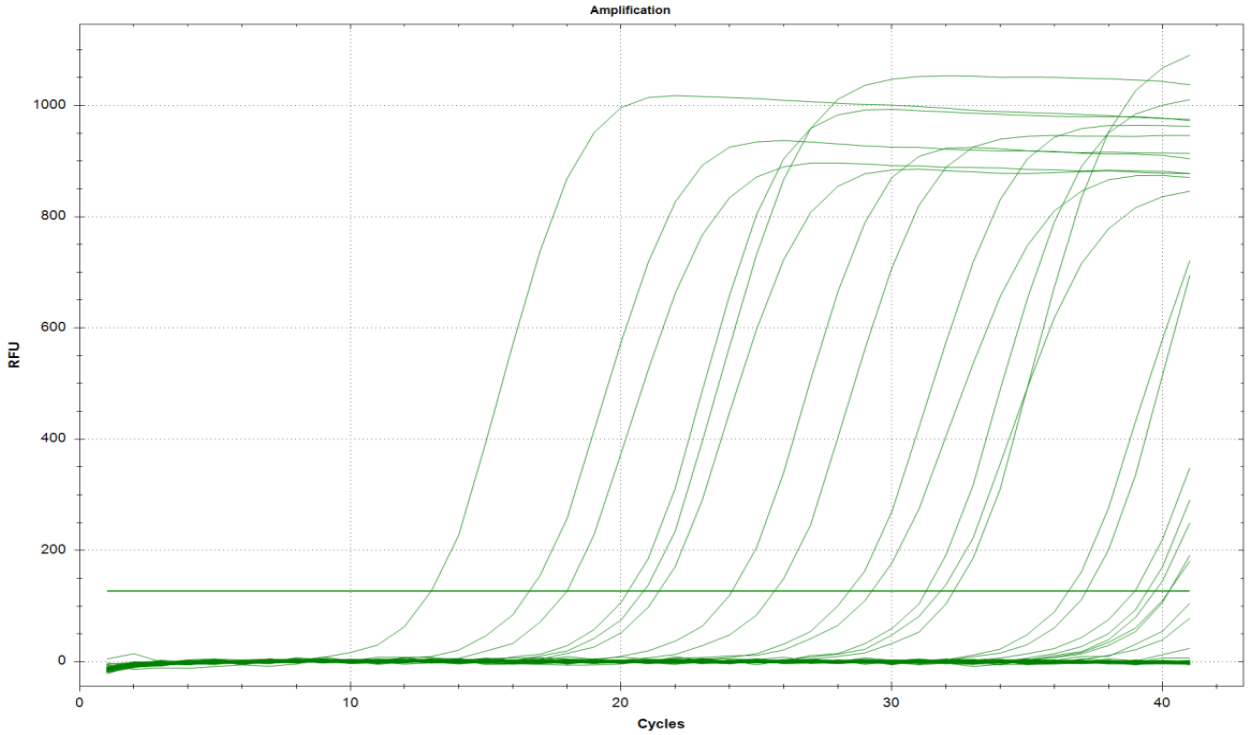


Figure 2. *M. bovis* nucleic acid signals in mammary tissues.

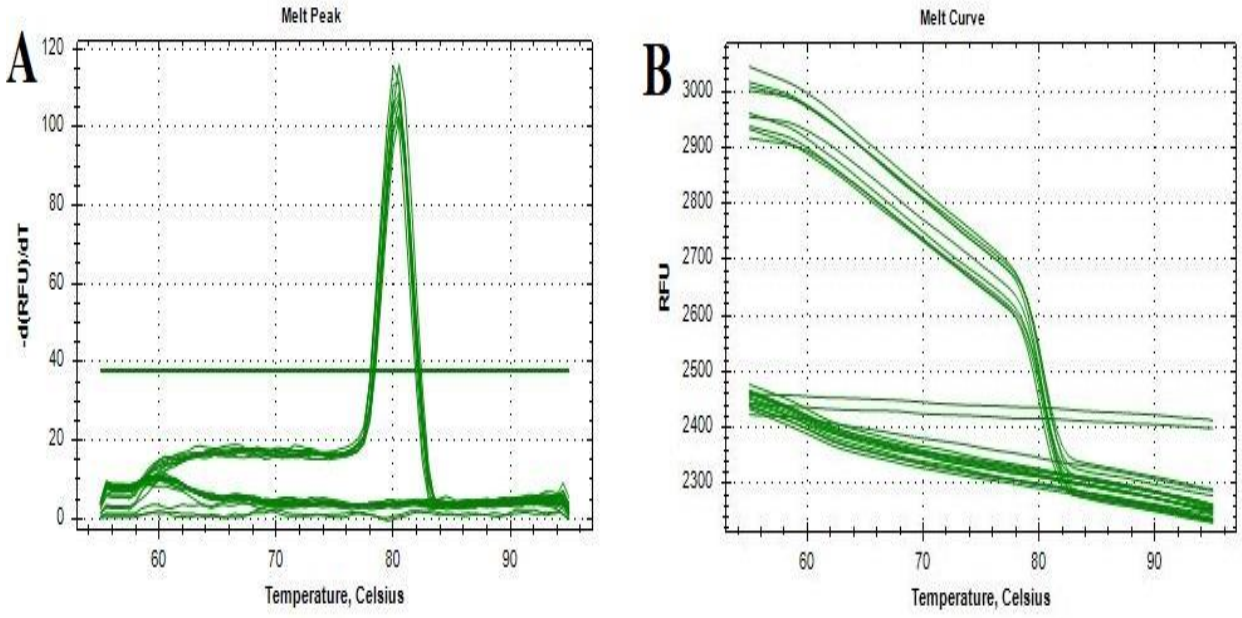


Figure 3. *M. bovis* primer melt paek and melting curve analysis.

Table 1. Primer sequences of *M. bovis*/uvrC

Gen Name	Primer sequences	Annealing	Reference
***M. bovis*/uvrC**	F:CCTGTCGGAGTTGCAATTGTT R:CGGTCAACTTCAACTTGAATTTG	60	Fan et al., 2018

DISCUSSION

Mastitis diseases in cattle is widespread throughout the world, as are diseases which often results in Turkey (Karahan et al. 2010). Mycoplasmal agents are reported to be the most common cause of mastitis with the increasing prevalence values. A lot of studies usually built on serological tests carried out in the presence of *M. bovis* in the world; in France (Grand et al. 2002) 10-20 %, England (Ayling et al. 2005) 22%, United States (Soehnlen et al. 2011) 41.1%. In our country, it is noted that a small number of studies on this subject as reported of mycoplasmal mastitis in cattle, the prevalence of *M. bovis* was reported to be 7.5 % in the Marmara region and (Erdağ et al. 1998). In this study the presence of *M. bovis* factors in cattle with mastitis was found in 23.3 % by immunofluorescence and qRT-PCR.

Although the pathogenesis of the disease is not fully explained, the data reported in different studies are as follows; In a study conducted by investigating *M. bovis* in tissues by immunohistochemical methods. *M. bovis* cause necrosis in the cells by providing oxidative stress and nitrate stress markers via increasing the production of H_2O_2 (Hydrogen peroxide) (Schott et al. 2013). *M. bovis* infection was found to slow cellular metabolism in an experimental study. In addition, decreased neutrophil degranulation and ROS (Reactive Oxygen Species) factors caused by decreasing the growth of leukocytes, the immune system was seen to suppress the immunosuppression and therefore it is revealed that *M. bovis* infections are chronic. In experimental studies, it has been found that *M. bovis* agents have been placed in the cytoplasm of phagocytic cells such as tissue macrophages and can hold onto the surface of macrophages and proliferate here (Kleinschmidt et al. 2013). Obtained findings of this study especially immunofluorescence staining results support these informations.

In recent studies, diagnostic methods such as PCR, culture, ELISA, SDS-PAGE, nucleic acid hybridization were compared and PCR techniques were described as more sensitive and specific method than any other technique. Besides, many researchers have used immunohistochemistry successfully in operation to demonstrate the presence of the agent in lesion. Last research claim that the first step to control and to prevent the spread of mycoplasmal disease is recognizing but mycoplasmal agents are known to be difficult in the early and rapid diagnosis due to difficulty in isolation and identification (Giovannini et al. 2013).
This disease not only threatens the cows with mastitis but also threatens the calves due to their subclinical progression in adults and contaminating milk (Stipkovits et al. 2000; Rosetti et al 2010).

In conclusion our findings demonstrate a high prevalence of *M. bovis* infection in adult cattle in the east of Turkey. Suitable environment conditions for the animals waiting to be able to create an infectious disease in the normal flora and subclinically infected cows of the factors show that the continuity of this threat. There is a critical need for preventative strategies in the farm for this pathogen. According to the results obtained, RT-PCR is an effective method for identification but optimizing processes in the RNA extraction phase for detection *M. bovis* factor by using this method hold an important place for obtaining reliable results.

ACKNOWLEDGEMENT

This study was prepared by using the data obtained from the project supported under TUBITAK 3001 (116O618).

REFERENCES

Giovannini S, Zanoni MG, Salogni C, Cinotti S, Alborali GL. Mycoplasma bovis infection in respiratory disease of dairy calves less than one month age. Resaerch in Veterinary Sciences, 2013; 95: 576-579.

Amram E, Freed M, Khateb N, Mükula I, Blum S, Spergser J, Sharir B, Ozeri R, Harrus S, Lysnyansky I. Multiple locus variable number tandem repeat analysis of Mycoplasma bovis isolated from local and imported cattle, The Veterianary Journal, 2013; 197: 286-290.

Tamada Y, Ohura K, Kato K, Kimura K, Maeda T. Mycoplasma bovis infection in calves with ear drops. Journal of the Hokkaido Veterinary Medical Association. 2002; 46: 308-311.

Adamu JY, Wawegama NK, Browning GF, Markham PF. Membrane proteins of Mycoplasma bovis and their role in pathogenesis. Research in Veterinary Science, 2013; 95: 321-325.

Aebi M, Bodmer M, Frey J, Pilo P. Herd-specific strains of Mycoplasma bovis in outbreaks of mycoplasmal mastitis and pneumonia. Veterinary Microbiology, 2012; 157: 363–368.

Nicholas RAJ, Ayling RD, Stipkovits LP. An experimental vaccine for calf pneumonia caused by Mycoplasma bovis: clinical, cultural, serological and pathological findings, Vaccine, 2002; 20: 3569-3575.

Karahan M, Kalin R, Atil E, Çetinkaya B. Detection of Mycoplasma bovis in cattle with mastitis and respiratory problems in eastern Turkey. Veterinary Record, 2010; 166: 827-829.

Radelli E, Luini M, Loria GR, Nicholas RAJ, Scanziani E. Bacteriological, serological, pathological and immunohistochemical studies of Mycoplasma bovis respiratory infection in veal calves adult cattle at slaughter. Research in Veterinary Sciences, 2008; 85: 282-290.

Wooley L K, Fell S, Gonsalves JR, Walker MJ, Djordjevic SP, Jenkins C, Eamens GJ. Evaluation of clinical, histological and immunological changes and qPCR detection of Mycoplasma hyopneumoniae in tissue during the eraly stages of mycoplasmal pneumonia in pigs after experimental challenge with two field isolates. Veterinary Microbiology, 2012; 161: 186-195.

Grand DL, Calavas D, Brank M, Cıttı C, Rosengarten R, Be´zılle P, Poumarat F. Serological prevalence of Mycoplasma bovis infection in suckling beef cattle in France Veterinary Record, 2002; 150: 268-273.

Ayling R, Nicholas R, Hogg R, Wessels J, Scholes S, Byrne W, Hill M, Moriarty J, Brien T. Mycoplasma bovis isolated from brain tissue of calves, 2005; 156: 391-392.

Soehnlen MK, Kunze ME, Karunathilake E, Henwood BH, Karyawasam S, Wolfgang DR, Jayarao BM. İn vitro antimicrobial inhibition of Mycoplasma bovis isolates submitted to the Pennsylvania Animal Diagnostic Laboratory using flow cytometry and broth microdilution method. Journal of Veterinary Daignostic İnvestigation, 2011, 23(3); 547-551.

Erdağ O, Erdoğan G, Türkaslan J, Gürel A. Buzağı ve dana pneumonilerinde Mycoplasma ve bakteriyel etkenlerin izolasyon, identifikasyon ve antibiyotik duyarlılıkları. Animal Information, 1995; 112: 115-119.

Schott C, Cai H, Parker L, Bateman K G, Caswell L. Hydrogen peroxide produciton and free radical mediated cell stress in Mycoplasma bovis pneumonia. Journal of Compharative Pathology, 2013; 1-11.

Kleinschmidt S, Spergser J, Rosengarten R, Hewicker Trautwein M. Long-term survival of Mycoplasma bovis in necrotic lessions and in phagocyctc cells as demonstrated by transmission and immunogold electron microscopy in lung tissue from experimantally infected calves. Veterinary Microbiology, 2013; 162: 949-953.

Stipkovits L, Ripley P, Varga J, Pálfi V. Clinical study of the disease of calves associated with Mycoplasma bovis infection. Acta Veterinaria Hungarica, 2000; 48(4):387-95.

Rosetti BC, Frey J, Pilo P. Direct detection of Mycoplasma bovis in milk and tissue samples by real-time PCR. Moleculer and Cellular Probes, 2010; 24: 321-323.

Haapalaa V, Pohjanvirtab T, Vähänikkiläb N, Halkilahtic J, Simonend H, Pelkonenb S, Soveria T, Simojokia H, Autiob T. Semen as a source of Mycoplasma bovis mastitis in dairy herds. Veterinary Microbiology, 2018; 216: 60–66.

Fan Q, Xie Z, Xie Z, Xie L, Huang J, Zhang Y, Zeng T, Zhang M, Wan S, Luo S, Liu J, Deng X. Development of duplex fluorescence-based loop-mediated isothermal amplification assay for detection of Mycoplasma bovis and bovine herpes virus 1. J Virol Methods. 2018; 261:132-138.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2): 116-121

DOI: 10.30607/kvj.495736

RESEARCH ARTICLE

Dermatophytes Isolated From Dogs and Cats Suspected Dermatophytoses in Istanbul, Turkey Within A 15-Year-Period: An Updated Report

Belgi DIREN SIĞIRCI*[1], Kemal METİNER[1], Baran ÇELİK[1], Beren BAŞARAN KAHRAMAN[1], Serkan İKİZ[1], A. Funda BAĞCIGIL[1], N. Yakut ÖZGÜR[1], Seyyal AK[1]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Istanbul University Cerrahpasa, TR 34320 Avcılar, Istanbul – TURKEY

ABSTRACT

The present research was aimed to determine the prevalence of dermatophytes isolated from symptomatic dogs and cats, within a 15-year-period, in the city of Istanbul, Turkey. Dermatological specimens were collected from 1504 dogs and 846 cats, which were presented clinical signs of ringworm. Direct microscopy and mycological cultures were performed. The fungal growth rate was detected at 8.2% and 22.8% from dogs and cats, respectively. *Microsporum canis* was the most frequently isolated species followed by *Trichophyton* spp., *M. gypseum*, *T. mentagrophytes*, *M. nanum*, other *Microsporum* spp. moreover *T. tonsurans*. The cats less than two-year age and more than ten-year age showed a statistically significant higher isolation rate of infection ($p < 0.05$). There were no statistically significant differences between the age of the dogs and the dermatophyte isolation rate and between the gender of the dogs and cats and the dermatophyte isolation rate. As a conclusion, the data suggest an updated report on local epidemiology and define potential etiologic agents.

Keywords: Dermatophytoses, Dog, Cat, Mycological Culture, *Microsporum* spp., *Trichophyton* spp.

***

15-Yıllık Periyotta İstanbul Türkiye'de Dermatofitoz Şüpheli Köpek ve Kedilerden İzole Edilen Dermatofitler: Güncellenmiş Rapor

ÖZ

Bu araştırma, İstanbul ilinde 15 yıllık bir süre içinde semptomatik köpek ve kedilerden izole edilen dermatofitlerin yaygınlığını belirlemeyi amaçlamıştır. Dermatolojik örnekler ringworm klinik belirtileri gösteren 1504 köpek ve 846 kediden toplandı. Direkt mikroskopi ve mikolojik kültürler yapıldı. Mantar üreme oranları, köpeklerde % 8.2 kedilerde % 22.8 olarak saptandı. En sık izole edilen tür *Microsporum canis* idi. Bunu *Trichophyton* spp., *M. gypseum*, *T. mentagrophytes*, *M. nanum*, diğer *Microsporum* spp. ve *T. tonsurans* takip etti. İki yaşından küçük ve on yaşından büyük kediler, istatistiksel olarak anlamlı derecede yüksek bir etken izolasyon oranı gösterdi ($p < 0.05$). Köpeklerin yaşı ve dermatofit izolasyon oranları ile kedi ve köpeklerin cinsiyeti ve dermatofit izolasyon oranları arasında istatistiksel olarak anlamlı bir fark bulunmadı. Sonuç olarak, veriler yerel epidemiyoloji üzerine güncel bir rapor sunmakta ve olası etiyolojik ajanları tanımlamaktadır.

Anahtar Kelimeler: Dermatofitler, Köpek, Kedi, Mikolojik Kültür, *Microsporum* spp., *Trichophyton* spp.

To cite this article: Diren Sığırcı B. Metiner K. Çelik B. Başaran Kahraman B. İkiz S. Bağcıgil A.F. Özgür N.Y. Ak S. Dermatophytes Isolated From Dogs and Cats Suspected Dermatophytoses in Istanbul, Turkey Within A 15-Year-Period: An Updated Report. Kocatepe Vet J. (2019) 12(2):116-121.

Submission: 12.12.2018 **Accepted**: 25.03.2019 **Published Online**: 29.03.2019

ORCID ID; BDS: 0000-0001-7153-7428, KM: 0000-0003-4105-5852, BÇ: 0000-0001-9122-0284, BBK: 0000-0002-1736-093X, Sİ: 0000-0001-6502-0780, AFB: 0000-0002-8838-7291, NYÖ: 0000-0001-6052-1324, SA: 0000-0002-6687-8401

*Corresponding author e-mail: belgis@istanbul.edu.tr

INTRODUCTION

Dermatophytoses in companion animals, especially dogs and cats, is a common skin disease caused by keratinophilic dermatophytes. More than 30 species of dermatophytes have been identified; however, *Microsporum canis, Microsporum gypseum* and *Trichophyton mentagrophytes* are the primary etiological agents. Because of the pleomorphic presentation of symptoms, contagious nature, and zoonotic importance, dermatophytoses is recognised as one of the major public health problems worldwide (Moriello et al. 2017, Paterson 2017). It has been emphasised that approximately 20-50 % of human skin infections were caused by zoonotic dermatophytes (Murmu et al. 2015, Weese and Fulford 2010).

Companion animals showed a higher prevalence and considered as the main source of human dermatophyte infections (Khosravi and Mahmoudi 2003, Mancianti et al. 2002, Seker and Dogan 2011). The spread of dermatophytes from animals to humans may usually occur by direct contact or indirectly through infected hair and scales from animals (Khosravi and Mahmoudi 2003). The spreading of dermatophyte infections is crucial to describe the infective routes to determine the possible sources of infection, or to identify the dissemination areas of the pathogens (Kanbe et al. 2003).

Various studies have been documented that the prevalence of dermatophytoses ranges worldwide ranges within 4% to 20% in dogs and more than 20% in cats (Brilhante et al. 2003, Mattei et al. 2014, Moriello et al. 2017, Nichita and Marcu 2010, Paterson 2017). Besides, in Turkey, İlhan et al. (2016), Seker and Dogan (2011) and Tel and Akan (2008) have determined the prevalence of these infections and the ranges were between 8% and 19% in dogs, while 7% and 72% in cats. Most studies have focused that *M. canis* is the ubiquitous dermatophyte isolated from suspected animals. Moreover, *M. canis*, as well as *M. gypseum* and *T. mentagrophytes*, are the fungus responsible for more than 95% of all dermatophytoses cases in companion animals (Mattei et al. 2014). The understanding of ringworm presence is essential for decreasing the transmission of fungal infections to animals and humans. The present study aimed to determine the prevalence of the predominant pathogenic dermatophyte species from symptomatic dogs and cats, within a 15-year-period, to present an updated report on local epidemiology and identify possible pathogens, in the city of Istanbul, Turkey.

MATERIALS and METHODS

Collection of samples

Cases clinically suspected of dermatophytoses and presented at the Department of Internal Medicine were included in the study. At the fifteen-year period, between 2003 and 2017, the samples were obtained from 1504 dogs and 846 cats. Diagnosis of the disease was based on historical data, clinical signs or findings on physical examination. Alopecia and desquamation were reported by veterinary practitioners and consecutively classified as suspected cases of dermatophytoses. Plucked hairs and scraped scales of each animal were collected from the lesions using a sterile lancet by veterinary practitioners and placed in sterile petri dishes. All samples were processed within 2 hours.

Demographic data on patients' sex and age were gathered from each medical record. Three age group were selected for this study; less than two years, 2-10 year, and more than ten years. We did not have age data of 420 dogs and 362 cats, and sex data of 320 dogs and 139 cats did not extract.

Direct microscopic examination

The 'gold standard' diagnostic techniques were applied for identification of dermatophytoses such as direct microscopic examination of clinical specimens (Debnath et al. 2016, Mattei et al. 2014). All samples were examined for fungal elements in 10% potassium hydroxide (KOH) under a light microscope at 40× magnification.

Mycologic culture

The samples were inoculated onto Sabouraud Dextrose Agar (SDA) (HiMedia Laboratories, Mumbai, India, Catalogue No. M063) supplemented with cycloheximide and chloramphenicol, and Dermatophyte Test Medium (DTM) (HiMedia Laboratories, Mumbai, India, Catalogue No. M188). The plates were incubated at 25°C for up to 3 weeks and were observed periodically for the appearance of fungal growth. The identification of the cultures was made according to "dermatophytes identification scheme". The macroscopical examination of cultures was established by the colony morphology, pigmentation and growth rate. The microscopic examination was formed by lactophenol cotton blue staining by their size, shape, presence of septa, the thickness of conidial wall and arrangement of conidial cells around the hyphae (de Hoog et al. 2000, Koneman and Roberts 1985).

Statistical analyses

Chi-square (x^2) test was used to examine the statistical significance of gender and age in the distribution of positive cultures in dogs and cats separately. The cats and dogs, which have age and gender data, were involved in statistical analyses. p value of < 0.05 was considered significant. SPSS 13.0 software was used for statistical analysis. (Özdamar 2003).

RESULTS

Dermatological specimens were collected from 1504 dogs and 846 cats. In dogs, 626 were female while 558 of were male and in cats, 389 were female while 318 of were male. Three hundred twenty-five of the dogs were <2 year, 553 of were between 2 and 10 years while 206 of were >10 years. Two hundred nine of the cats were <2 years, 221of were between 2 and 10 years while 54 of were >10 year.

At the results of the direct microscopic examination of hair samples belonging to 1504 dogs and 846 cats, fungal elements were observed in 56,5 % and 58,2% of clinical specimens, respectively. 60 % of the dog samples and 69,9 % of the cat samples containing fungal elements were also positive for culture.

According to the fungal culture, the colony that were white or yellowish colour; plane, velvety or cottony surface and brown or golden-yellow reverse in SDA were identified as *Microsporum* spp. The appearance of white aerial hyphae and red colour around the colony in DTM demonstrated the presence of *Microsporum* spp. The colony that was powdery to a granular surface; plane, white to cream colour and reverse yellowish brown to reddish-brown in SDA were identified as *Trichophyton* spp. White colonies and a red colour change develop in the medium around the fungal growth in DTM were positive for the presence of *Trichophyton* spp.. Macroscopic appearance of *M.canis* and *T. mentagrophytes* isolates on SDA, and microscopic appearance under a light microscope at a 40× magnification of isolates stained by lactophenol cotton blue are shown in Figure 1.

Overall, dermatophytoses were detected in 317 of 2350 (13.5%) samples. The fungal growth rates were 8.2% and 22.8% from dogs and cats, respectively. *M. canis* was the most frequently isolated species from dogs and cats (64.4%), followed by *Trichophyton* spp., *M. gypseum*, *T. mentagrophytes*, *Microsporum nanum*, other *Microsporum* spp., and *Trichophyton tonsurans*. The distribution of dermatophytes isolated from dog and cat skin scrapings according to the species are shown in Table 1.

Dermatophyte identification was observed mostly in the dogs between 2 and 10 years (n: 53) and in the cats, the maximum identification was detected from the under two years animals (n: 87). Four hundred twenty dogs and 362 cats did not have age data; therefore, these animals were not included in the statistical analysis. The cats less than two-year age and more than ten-year age showed a statistical significance ($p< 0.05$). There were no statistically significant differences between the age of the dogs and the dermatophyte isolation rate. The age and isolation rates of dogs and cats with dermatophytoses are shown in Table 2 and Table 3, respectively.

Dermatophyte identification was observed similarly in male and female dogs and cats. Three hundred twenty dogs and 139 cats did not have sex data; therefore, these animals were not included in the statistical analysis. There were no statistically significant differences between the gender of both dogs and cats, and the dermatophyte isolation rate. The gender and isolation rates of dogs and cats with dermatophytoses are shown in Table 4 and Table 5, respectively.

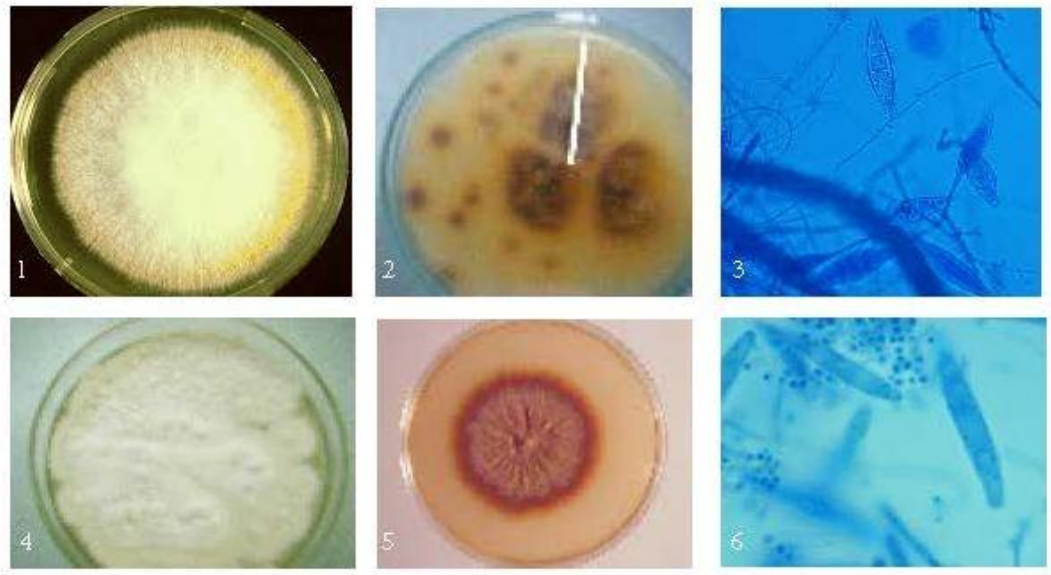


Figure 1. The macroscopic and microscopic appearance of *M.canis* and *T. mentagrophytes* isolates
1.*M.canis* on SDA 2. *M.canis* on SDA, reverse 3. Microscopic appearance of *M.canis* at a 40× 4. *T. mentagrophytes* on SDA 5. *T. mentagrophytes* on SDA, reverse 6. Microscopic appearance of *T. mentagrophytes* at a 40×

Table 1. The distribution of dermatophytes isolated from dog and cat skin scrapings according to the species

Dermatophytes	Dogs	Cats	Total
M. canis	63 (50.8%)	141 (73%)	204 (64.4%)
M. gypseum	9 (7.3%)	28 (14.5%)	37 (11.7%)
M. nanum	7 (5.6%)	3 (1.6%)	10 (3.1%)
Other *Microsporum* spp.	3 (2.4%)	4 (2.1%)	7 (2.2%)
T. mentagrophytes	12(9.7%)	5(2.6%)	17 (5.4%)
T. tonsurans	1 (0.8%)	0 (-)	1 (0.3%)
***Trichophyton* sp.**	29 (23.4%)	12 (6.2%)	41(12.9)
Total	124	193	317

Table 2. The age and isolation rates of dogs with dermatophytoses

Dogs	Dermatophytoses positive	Dermatophytoses negative	Total
<2 year	38 (11.6%)	287 (88.4%)	325 (100 %)
2-10 year	53 (9.5%)	500 (90.5%)	553 (100 %)
>10 year	16 (7.7%)	190 (92.3 %)	206 (100 %)
Total	107	977	1084

Table 3. The age and isolation rates of cats with dermatophytoses

Cats	Dermatophytoses positive	Dermatophytoses negative	Total
<2 year	87 (41.6%)*	122 (58.4%)	209 (100 %)
2-10 year	43 (19.4%)	178 (80.6%)	221 (100 %)
>10 year	29 (53.7%)*	25 (46.3%)	54 (100 %)
Total	159	325	484

* There is a statistical difference ($p < 0.05$) between groups.

Table 4. The gender and isolation rates of dogs with dermatophytoses

Dogs	Dermatophytoses positive	Dermatophytoses negative	Total
Male	50 (8.9%)	508 (91.1 %)	558 (100 %)
Female	55 (8.7%)	571 (91.3 %)	626 (100 %)
Total	105	1079	1184

Table 5. The gender and isolation rates of cats with dermatophytoses

Cats	Dermatophytoses positive	Dermatophytoses negative	Total
Male	66 (20.7%)	252 (79.3 %)	318 (100 %)
Female	93 (23.9%)	296 (76.1 %)	389 (100 %)
Total	159	548	707

DISCUSSION

Dermatophytoses are common worldwide and continue to increase, and thus several reports are available on the prevalence of the infection as varying. Murmu et al. (2016) indicated that the incidence of dermatophytoses in cats was the highest (55.5%) than dogs. Nweze (2011) and Esch and Peterson (2013) who observed a 58-67% occurrence rate in their studies was supported this high prevalence. The prevalence of dermatophytoses in dogs were reported by Brilhante et al. (2003) (14.3%), Nichita and Marcu (2010) (16.8%) and Mancianti et al. (2002) (18.7%). However, Khosravi and Mahmoudi (2003) indicated that 8.2% of samples from dogs were found positive about dermatophytoses. Seker and Dogan (2011) were determined 20.1% as positive for dermatophytes.

In the present study, the dermatophyte isolation rates from dogs and cats were 8.2% and 22.8 %, respectively. Our findings showed roughly similarity with these results. Contrary to this, the studies that had higher results were reported by Faggi et al. (1987), Seker and Dogan (2011) and Moriello et al.

(2017). These differences are not surprising, and it may be originated because of the full range in methodologies. Moreover, the author reported that the prevalence of dermatophytes depends on geographical location, the season of sampling, clinical, and living conditions (Proverbio et al. 2014).

M.canis is a pathogenic fungal species that causes a superficial skin infection called dermatophytoses in domestic carnivores while they can be transmitted to human beings with close contact of the affected animal (Moriello et al. 2017). The cats are reported as the principal reservoir for this pathogen. Nichita and Marcu (2010) observed that the prevalence in cats is usually higher than in dogs. Mancianti et al. (2002), Brilhante et al. (2003) and Cafarchia et al. (2004) reported similar results. According to the results from this study, *M. canis* was the most common causative agent of dermatophyte isolated, and it is in agreement with the reports obtained (Brilhante et al. 2003, Mancianti et al. 2002).

Dermatophytoses studies have been described throughout the world; *M. canis, T mentagrophytes* and *M. gypseum* were jointly responsible for almost all of the infections in dogs and cats. In the present study, the identified dermatophytes were *M. canis* (n=204), *M. gypseum* (n=37), *M. nanum* (n=10), other *Microsporum* sp. (n=7), *T. mentagrophytes* (n=17), *T. tonsurans* (n=1) and other *Trichophyton* sp. (n=41). These data almost correspond to the situation in Turkey where these species are the most common fungus, which has been seen in dogs and cats. Tel and Akan (2008) determined the distribution of isolated strains as 95.9 % *M. canis* and 4.1 % *M. nanum* in cats; 50 % *M. canis,* 18.7 % *T. mentagrophytes*, in dogs in Ankara. Seker and Dogan (2011) indicated that *M. canis* was the most common dermatophyte isolated from dogs (46%) and cats(69.7%), followed by *T. mentagrophytes* (32.4%) in dogs in Ankara and Izmir. Ilhan et al. (2016) showed that the most frequently isolated fungi were *T. terrestre* (4.1%), followed by *M. gypseum* (1.1%), *M. nanum* (1.1%), and *T. mentagrophytes* (0.7%) in cats in Van.

Moriello et al. (2017) identified the predispositions of the development of dermatophytoses in cats and dogs and underlined the being puppies and kittens, lifestyle, free-roaming animals and warm locations for the risk populations. Age was recognised as a predisposing factor by many researchers. Tel and Akan (2008) found the prevalence to be significant ($p \leq 0.01$) in animals that were smaller than one year old. Mattei et al. (2014) determined that the animals younger than one-year-old appear to be susceptible to dermatophytoses. Contrary to these findings, Seker and Doğan (2011) detected no significant difference statistically between the age groups and the prevalence rate. In this study, there was a significant difference in the distribution of positive cultures in cats less than two-year age and more than ten-year age. According to our findings, the higher susceptibility of young and old cats may be related to the immunological condition and deficiency of fungistatic linoleic acid.

Several researchers did not detect any correlation between sex and the presence of infections (Mancianti et al. 2002, Mattei et al. 2014, Seker and Dogan 2011). Therewithal, Pinter et al. (1999) and Cafarchia et al. (2004) have reported that male dogs were most often affected by dermatophyte infections. Also, Iorio et al. (2007) were detected the prevalence rate of dermatophytes in female cats more than male cats and Cafarchia et al. (2004) were reported the prevalence rate of *M. canis* in female cats more than male cats. In the current study, the isolation rate of dermatophytes in female and male animals was not found to be significant.

CONCLUSION

The present study emphasised that fungal infections are ubiquitous in companion animals such as cats and dogs and *M. canis* is usually the first animal-associated fungus causing infections. As a conclusion, the data suggest an updated review of local epidemiology and clarify possible etiologic agents, and this study will provide valuable information on current epidemiological trends for fungal infections in Turkey.

REFERENCES

Brilhante RSN, Cavalcante CSP, Soares-Junior FA, Cordeiro RA, Sidrim JJC, Rocha MFG. High rate of *Microsporum canis* feline and canine dermatophytoses in Northeast Brazil: epidemiological and diagnostic features. Mycopathologia. 2003; 156: 303-308.

Cafarchia C, Romito D, Sasanelli M, Lia R, Capelli G, Otranto D. The epidemiology of canine and feline dermatophytoses in southern Italy. Mycoses. 2004; 47: 508-513.

de Hoog GS, Guarro J, Gené J, Figueras MJ. Atlas of Clinical Fungi, Second Edition, Washington, DC, USA. American Society for Microbiology, 2000.

Debnath C, Mitra T, Kumar A, Samanta I. Detection of dermatophytes in healthy companion dogs and cats in eastern India. Iranian journal of veterinary research. 2016; 17(1): 20.

Esch KJ, Peterson CA. Transmission and epidemiology of zoonotic protozoal diseases of companion animals. Clin. Microbiol. Rev. 2013; 26(1): 58-85.

Faggi E, Saponetto N, Sagone M. Dermatophytes isoles dês carnivores domestiques à Florence (Italie); enquête épidémiologique. Bull Soc Fr Mycol Méd. 1987; 1: 297-302.

Ilhan Z, Karaca M, Ekin IH, Solmaz H, Akkan HA, Tutuncu M. Detection of seasonal asymptomatic dermatophytes in Van cats. Braz J Microbiol. 2016; 47(1): 225-230.

Iorio R, Cafarchia C, Capelli G, Fasciocco D, Otranto D, Giangaspero A. Dermatophytoses in cats and humans in central Italy: epidemiological aspects. Mycoses. 2007; 50: 491-495.

Kanbe T, Suzuki Y, Kamiya A, Mochizuki T, Fujihiro M, Kikuchi A. PCR-based identification of common dermatophyte species using primer sets specific for the DNA topoisomerase II genes. J Dermatol Sci. 2003; 32: 151-161.

Khosravi AR, Mahmoudi M. Dermatophytes isolated from domestic animals in Iran. Mycoses. 2003; 46: 222–225.

Koneman EW, Roberts GD. Practical Laboratory Mycology. 3rd ed. Baltimore: Williams & Wilkins; 1985. Dermatophyte identification schema.

Mancianti F, Nardoni S, Cecchi S, Corazza M, Taccini F. Dermatophytes isolated from symptomatic dogs and cats in Tuscany, Italy during a 15-year-period. Mycopathologia. 2002; 156: 13–18.

Mattei AS, Beber MA, Madrid IM. Dermatophytosis in Small Animals. SOJ Microbiol Infect Dis. 2014; 2(3): 1-6.

Moriello KA, Coyner K, Paterson S, Mignon B. Diagnosis and treatment of dermatophytosis in dogs and cats. Clinical Consensus Guidelines of the World Association for Veterinary Dermatology. Vet Dermatol. 2017; 28(3): 266-268.

Murmu S, Debnath C, Pramanik AK, Mitra T, Jana S, Dey S, Batabyal, K. Detection and characterization of zoonotic dermatophytes from dogs and cats in and around Kolkata. Vet World. 2015; 8(9): 1078.

Nichita I, Marcu A. The Fungal Microbiota Isolated from Cats and Dogs. J Anim Sci Technol. 2010; 43: 411-414.

Nweze EI. Dermatophytoses in domesticated animals. Rev. Inst. Med. Tro. 2011; 53(2): 95-99.

Özdamar K. SPSS ile Biyoistatistik. Kaan kitabevi, Türkiye. 2003.

Paterson S. Dermatophytosis: an update. Companion Anim. 2017; 22(5): 248-253.

Pinter L, Jurak Z, Ukalovic M, Susic V. Epidemiological and clinical features of dermatophytoses in dogs and cats in Croatia between 1990 and 1998. Vet Arhiv. 1999; 69: 261-270.

Proverbio D, Perego R, Spada E, Bagnagatti de Giorgi G, Della Pepa A, Ferro E. Survey of dermatophytes in stray cats with and without skin lesions in Northern Italy. Veterinary medicine international, 2014; 1-4.

Seker E, Dogan N. Isolation of dermatophytes from dogs and cats with suspected dermatophytosis in Western Turkey. Prev Vet Med. 2011; 98: 46–51.

Tel OY, Akan M. Kedi ve köpeklerden dermatofitlerin izolasyonu. Ankara Univ Vet Fak Derg. 2008; 55: 167-171.

Weese JS, Fulford M. editors. Companion Animal Zoonoses. New Jersey: Wiley-Blackwell; 2010. 278–279.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):122-129
DOI: 10.30607/kvj.524391

RESEARCH ARTICLE

Antinociceptive and Anxiolytic-Like Effects of Some Compounds Carrying Benzothiazole Ring

Ümide DEMİR ÖZKAY*, Umut İrfan ÜÇEL, Nazlı TURAN YÜCEL, Özgür Devrim CAN

Anadolu University, Faculty of Pharmacy, Department of Pharmacology, 26470, Eskisehir, Turkey

ABSTRACT

The aim of this study was to examine the antinociceptive and anxiolytic-like effects of some compounds carrying benzothiazole ring. The antinociceptive effects of test compounds, administrated at a dose of 40 mg/kg, were investigated by tail clip, hot plate and acetic acid-induced writhing tests. Hole board and elevated plus maze tests were performed to evaluate the anxiolytic-like effects of the compounds. Rota-rod device was used to assess the motor coordinations of mice. As a result of the experiments, it was determined that compounds 3f, 3g and 3h increased the reaction times of animals in the tail clip and hot plate tests, and compound 3e reduced the writhing number of mice in the acetic acid-induced writhing test. On the other hand, in the hole board test, compounds 3f, 3g and 3h reduced the first head-dipping latencies of mice while increasing the total number of head-dips. In the elevated plus maze test, it has been shown that the same derivatives increase the percentages of open arm entries and time spent in the open arms. In the Rota-rod tests, test compounds did not change the falling time of mice from the rotating mill. All these findings point out that compounds 3f, 3g and 3h exhibit central antinociceptive and anxiolytic-like effects and compound 3e shows a peripheral antinociceptive effect.

Keywords: Benzothiazole ring, antinociceptive, anxiolytic, Rota-rod

***

Benzotiyazol Halkası Taşıyan Bazı Bileşiklerin Antinosiseptif ve Anksiyolitik-Benzeri Etkileri

ÖZ

Bu çalışmanın amacı benzotiyazol halkası taşıyan bazı bileşiklerin antinosiseptif ve anksiyolitik-benzeri etkilerinin incelenmesidir. 40 mg/kg dozda uygulanan test bileşiklerinin antinosiseptif etkileri kuyruk sıkıştırma, sıcak plaka ve asetik asid ile indüklenen kıvranma testleri ile araştırılmıştır. Bileşiklerin anksiyolitik-benzeri etkilerini incelemek için delikli tahta ve yükseltilmiş artı labirent testleri gerçekleştirilmiştir. Farelerin motor koordinasyonlarını değerlendirmek üzere Rota-rod cihazı kullanılmıştır. Deneyler sonucunda 3f, 3g ve 3h kodlu bileşiklerin kuyruk sıkıştırma ve sıcak plaka testlerinde hayvanların reaksiyon sürelerini artırdığı ve 3e kodlu bileşiğin ise asetik asid ile indüklenen kıvranma testinde farelerin kıvranma sayılarını azalttığı belirlenmiştir. Diğer yandan, 3f, 3g ve 3h kodlu bileşikler delikli tahta testinde farelerin ilk baş daldırma sürelerini kısaltırken toplam baş daldırma sayılarını artırmıştır. Yükseltilmiş artı labirent testinde ise aynı türevlerin farelerin açık kola giriş sayılarının ve açık kolda kalma sürelerinin yüzdelerini artırdığı ortaya konulmuştur. Rota-rod testlerinde test bileşikleri farelerin dönen milden düşme sürelerini değiştirmemiştir. Tüm bu bulgular, 3f, 3g ve 3h kodlu bileşiklerin santral antinosiseptif ve anksiyolitik-benzeri etki gösterdiklerine; 3e kodlu bileşiğin ise periferal antinosiseptif etki gösterdiğine işaret etmektedir.

Anahtar Kelimeler: Benzotiyazol halkası, antinosiseptif, anksiyolitik, Rota-rod

To cite this article: Demir Özkay Ü. Üçel U.İ. Turan Yücel N. Can Ö.D. Antinociceptive and Anxiolytic-Like Effects of Some Compounds Carrying Benzothiazole Ring. Kocatepe Vet J. (2019) 12(2):122-129.

Submission: 08.02.2019 **Accepted**: 13.03.2019 **Published Online**: 10.04.2019

ORCID ID; ÜDÖ: 0000-0002-6773-4266, UİÜ: 0000-0002-5470-0139, NTY: 0000-0002-0371-2703, ÖDC: 0000-0002-2260-3174

*Corresponding author e-mail: udemir@anadolu.edu.tr

GİRİŞ

Benzotiyazol, benzen ve tiyazolün kondenzasyonu sonucunda oluşan bisiklik heterosiklik bir halkadır (Hroch ve ark. 2015). Benzotiyazol halka sistemi ilaç geliştirme çalışmalarında kullanılan önemli yapılardan biridir. Yapısında bu halkayı taşıyan çeşitli bileşiklerin antibakteriyel (Singh ve ark. 2013, Thakkar ve ark. 2017, Zha ve ark. 2017), antifungal (Singh ve ark. 2013, Zha ve ark. 2017), antimalaryal (Sarkar ve ark. 2016, Thakkar ve ark. 2017), antiastmatik (Costanzo ve ark. 2003), antikanser (Aouad ve ark. 2018, Osmaniye ve ark. 2018), antiinflamatuar (Muttu ve ark. 2010, Ugwu ve ark. 2018), antidiyabetik (Moreno-Díaz ve ark. 2008, Mariappan ve ark. 2012) ve antioksidan (Karali ve ark. 2010) gibi etkileri bildirilmiştir.
Benzotiyazol türevi çeşitli bileşiklerin santral sinir sistemi (SSS) ile ilişkili farmakolojik aktiviteleri de rapor edilmiştir. Bu halkayı taşıyan bileşiklerin antikonvülzan (Siddiqui ve ark. 2007, Amnerkar ve Bhusari 2010, Hassan ve ark. 2012, Ali ve Siddiqui 2015, Liu ve ark. 2016), antinosiseptif (Gökce ve ark. 2001, Siddiqui ve ark. 2008, Azam ve ark. 2013, Hamdy ve ark. 2013), antidepresan-benzeri (Siddiqui ve ark. 2008, Wang ve ark. 2014), anksiyolitik-benzeri (Sasaki-Hamada ve ark. 2013), nöroprotektif (Carboni ve ark. 2004, Anzini ve ark. 2010, Hassan ve ark. 2012) ve anti-Alzheimer (Keri ve ark. 2013, Demir Özkay ve ark. 2017a) gibi aktiviteleri olduğu bildirilmiştir.

Benzotiyazol halkası taşıyan bileşiklerin SSS üzerine potansiyel farmakolojik etkilerinden hareketle, bu çalışmada Anadolu Üniversitesi Eczacılık Fakültesi Farmasötik Kimya Anabilim Dalı tarafından sentezlenen ve daha önce antidepresan-benzeri etkinlikleri bildirilmiş olan (Demir Özkay ve ark. 2017b) benzotiyazol türevi bazı bileşiklerin olası antinosiseptif ve anksiyolitik-benzeri etkinlikleri araştırılmıştır.

MATERYAL ve METOT

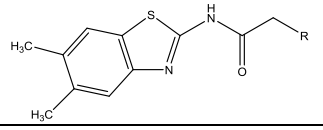
Bileşiklerin Sentezi

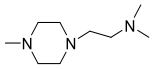
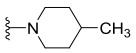
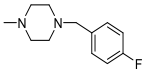
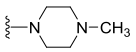
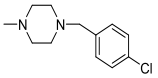
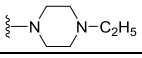
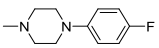
Benzotiyazol türevleri, 2-kloro-N-(5,6-dimetilbenzotiyazol-2-il)asetamid bileşiği ile çeşitli siklik aminlerin potasyum karbonat varlığında aseton içerisinde reaksiyonu sonucunda elde edilmiştir. Bileşiklerin sentez prosedürü ve spektral verileri çalışma grubumuz tarafından rapor edilmiştir (Demir Özkay ve ark. 2017b). Test bileşiklerinin kimyasal yapıları Tablo 1'de sunulmuştur.

Deney Hayvanları

Deneyler BALB/c fareler (30-35 g) ile gerçekleştirilmiştir. Deney hayvanları 24±1°C sıcaklıktaki iyi havalandırılan ve 12 saat aydınlık/12 saat karanlık döngüsüne sahip odalarda barındırılmıştır. Deney hayvanları deneylere başlanmadan en az 48 saat önce laboratuvara getirilmiştir. Deneyler sırasında laboratuvarın sıcaklığı, ses düzeyi ve aydınlatma koşulları sabit tutulmuştur. Bu çalışmanın deneysel protokolü, Anadolu Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu tarafından onaylanmıştır.

Tablo 1. Test bileşiklerinin kimyasal yapıları
Table 1. Chemical structures of test compounds



Bileşik	R	Bileşik	R
3a		3e	
3b		3f	
3c		3g	
3d		3h	

Test Maddelerinin Uygulanması

Deneyler için test bileşikleri 40 mg/kg dozda oral yolla uygulanmıştır (Wang ve ark. 2014). Test bileşikleri çiçek yağı içerisinde çözüldüğü için kontrol grubunda yer alan farelere aynı hacimde çiçek yağı uygulanmıştır. Referans ilaç olarak morfin sülfat (10 mg/kg, i.p) (Kaplancikli ve ark. 2009) ve diazepam (1 mg/kg, i.p) (Can ve ark. 2016) kullanılmıştır.

Analjezi Testleri

Kuyruk Sıkıştırma Testi

Kuyruk sıkıştırma testi, deney hayvanlarının mekanik ağrılı uyarana verdikleri yanıtı değerlendirmek için uygulanan bir testtir. Bu test, kuyruklarına metal bir klamp takılan deney hayvanlarının dönüp klampi ısırma sürelerinin ölçülmesi esasına dayanmaktadır. Deneylerden önce farelere duyarlılık testi uygulanmıştır. Bu testlerde klampe 10 saniyeden kısa sürede yanıt veren fareler deneylere alınmıştır. Farelerin kuyruklarının hasar görmemesi için uyarı 10 saniyeden fazla uygulanmamıştır (D'Amour ve Smith 1941, Demir Özkay ve Can 2013).

Sıcak Plaka Testi

Sıcak plaka testi, deney hayvanlarının termal ağrılı uyarana verdikleri yanıtı değerlendirmek için uygulanan bir testtir. Bu test, 55±1.0 °C'ye ayarlanmış Hot-plate cihazına (Ugo Basile, No. 7280, İtalya) konulan farelerin ayaklarını yalamaya başlama ve/veya zıplama sürelerinin kaydedilmesi esasına dayanmaktadır. Deneylerden önce farelere duyarlılık testi uygulanmış ve 15 saniyeden kısa sürede yanıt veren fareler deneylere alınmıştır. Uyarı 30 saniyeden

fazla uygulanmamıştır (Woolfe ve MacDonald 1944, Demir Özkay ve Can 2013).

Kuyruk sıkıştırma ve sıcak plaka testlerinde ölçülen yanıt sürelerinden maksimum olası etkinin yüzdesini (%MPE) hesaplamak için aşağıdaki formül kullanılmıştır:

$$\%MPE = \frac{(\text{uygulama sonrası süre} - \text{uygulama öncesi süre})}{(\text{cut} - \text{off süresi} - \text{uygulama öncesi süre})} \times 100$$

Asetik asid ile İndüklenen Kıvranma Testi

Asetik asid ile indüklenen kıvranma testi, deney hayvanlarının kimyasal ağrılı uyarana verdikleri yanıtı değerlendirmek için uygulanan bir testtir. Bu testte intraperitonal yolla uygulanan %0.6'lık asetik asid solüsyonu (10 mL/kg) farelerde abdominal ağrı ve kıvranma yanıtı oluşturmaktadır. Test, asetik asid enjeksiyonundan 5 dakika sonra deney hayvanlarının kıvranma davranışlarının 10 dakika süre ile sayılması esasına dayanmaktadır (Koster ve ark. 1959, Demir Özkay ve Can 2013).

Davranış Deneyleri

Delikli Tahta Testi

Farelerin keşif davranışları delikli tahta testi ile değerlendirilmiştir. Testin yapıldığı delikli tahta cihazı (Ugo Basile, No. 6650, Varese, İtalya) 3 cm çapında 16 adet deliğin bulunduğu gri pleksiglas bir panelden (40×40 cm) oluşmaktadır. Cihaz yerden 15 cm yüksekliktedir. Delikli tahta testinde, her bir fare sırtı gözlemciye dönük biçimde cihazın ortasına konulmuş ve cihazı serbestçe keşfetmesine izin verilmiştir. Farelerin ilk baş daldırma süreleri ve toplam baş daldırma sayıları 5 dakikalık süre boyunca kaydedilmiştir (Takeda ve ark. 1998, Can ve ark. 2012a).

Yükseltilmiş Artı Şekilli Labirent Testi

Farelerin anksiyete şiddetleri yükseltilmiş artı şekilli labirent testleri ile değerlendirilmiştir. İki açık kol (35x5 cm) ve iki kapalı koldan (35x5x15 cm) oluşan yükseltilmiş artı şekilli labirent düzeneği (Ugo Basile, No. 40143, Varese, İtalya) yerden 60 cm yüksekliktedir. Bu testte, her bir fare yüzü açık kola dönük şekilde merkez platforma yerleştirilmiş ve 5 dakikalık süre boyunca farelerin açık ve kapalı kollara giriş sayıları ve bu kollarda geçirdikleri süreler kaydedilmiştir (Can ve ark. 2012b, Can ve ark. 2013).

Deney hayvanlarının açık kola giriş sayılarının yüzdesini (%AKGS) ve açık kolda kaldıkları sürelerin yüzdesini (%AKKS) hesaplamak için aşağıdaki formüller kullanılmıştır:

$$\%\ AKGS = \frac{\text{Açık kola giriş sayısı}}{\text{Açık ve kapalı kollara giriş sayısı}}\ x\ 100$$

$$\%\ AKKS = \frac{\text{Açık kolda geçirilen süre}}{\text{Açık ve kapalı kollara geçirilen süre}}\ x\ 100$$

Rota-rod Testi

Farelerin motor koordinasyonları Rota-rod testleri ile değerlendirilmiştir. Deneyler başlamadan önce farelere 16 rpm sabit hızda dönen Rota-rod cihazı (Ugo Basile, no.47600, Varese, İtalya) ile üç ardışık gün boyunca alıştırma (training) yapılmıştır. Dönen milin üzerinde 180 saniyeden daha fazla kalabilen fareler deneylere alınmıştır. Farelerin dönen milin üzerinden düşme süreleri cihaz tarafından kaydedilmiştir (Kaplancikli ve ark. 2009, Demir Özkay ve Can 2013).

İstatistiksel Analiz

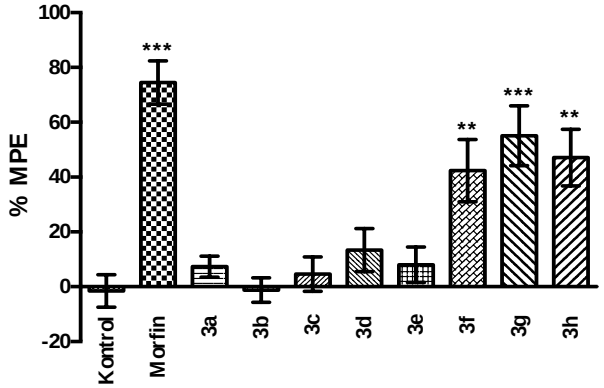
İstatistiksel analiz için Graphpad Prism ver. 6.01 paket programı kullanılmıştır. Deneylerden elde edilen veriler tek yönlü varyans analizi (ANOVA) ve ardından Tukey HSD çoklu karşılaştırma testleri uygulanarak değerlendirilmiştir. Sonuçlar, ortalama ± ortalamanın standart hatası olarak verilmiştir. $p<0.05$ değeri anlamlı kabul edilmiştir.

BULGULAR

Analjezi Deneylerine İlişkin Bulgular

Kuyruk sıkıştırma testine ilişkin bulgular

Test bileşikleri ve morfin uygulamalarının kuyruk sıkıştırma testinde farelerin reaksiyon süreleri üzerine etkileri Şekil 1'de verilmiştir [$F(9,60)=11.77$, $p<0.001$]. Tek yönlü ANOVA testini takiben uygulanan çoklu karşılaştırma testleri, 3f, 3g ve 3h kodlu türevlerin hayvanların %MPE değerlerini kontrol grubunun %MPE değerlerine göre istatistiksel olarak anlamlı biçimde artırdığını, 3a-3e kodlu türevlerin ise etkisiz olduğunu ortaya koymuştur. Referans ilaç morfin uygulamaları da farelerin %MPE değerlerini anlamlı biçimde artırmıştır (Şekil 1).

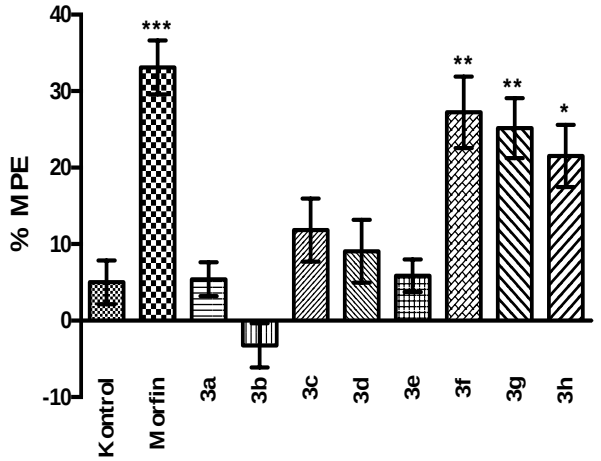


Şekil 1. Test bileşiklerinin (40 mg/kg) ve morfin'in (10 mg/kg) kuyruk sıkıştırma testinde farelerin %MPE değerleri üzerine etkileri. Kontrol grubuna göre anlamlı farklılık $^{**}p<0.01$, $^{***}p<0.001$. Tek yönlü varyans analizi, takiben Tukey HSD çoklu karşılaştırma testi, n=7.

Figure 1. Effects of test compounds (40 mg/kg) and morphine (10 mg/kg) on MPE % values of mice in the tail clip test. Significance against control group $^{**}p<0.01$, $^{***}p<0.001$. One-way ANOVA, post hoc Tukey's test, n=7.

Sıcak plaka testine ilişkin bulgular

Şekil 2'de test bileşikleri ve morfin uygulamalarının sıcak plaka testinde farelerin reaksiyon süreleri üzerine etkileri gösterilmiştir [$F(9,60)=11.20$, $p<0.001$]. Tek yönlü ANOVA testini takiben uygulanan çoklu karşılaştırma testleri, 3f, 3g ve 3h kodlu türevlerin ve referans ilaç morfin'in hayvanların %MPE değerlerini kontrol grubunun %MPE değerlerine göre istatistiksel olarak anlamlı biçimde artırdığını ortaya koymuştur. 3a-3e kodlu türevlerin farelerin %MPE değerlerinde anlamlı bir değişikliğe neden olmadığı belirlenmiştir (Şekil 2).



Şekil 2. Test bileşiklerinin (40 mg/kg) ve morfin'in (10 mg/kg) sıcak plaka testinde farelerin %MPE değerleri üzerine etkileri. Kontrol grubuna göre anlamlı farklılık $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Tek yönlü varyans analizi, takiben Tukey HSD çoklu karşılaştırma testi, n=7.

Figure 2. Effects of test compounds (40 mg/kg) and morphine (10 mg/kg) on MPE % values of mice in the hot plate test. Significance against control group $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. One-way ANOVA, post hoc Tukey's test, n=7.

Asetik asid ile indüklenen kıvranma testine ilişkin bulgular

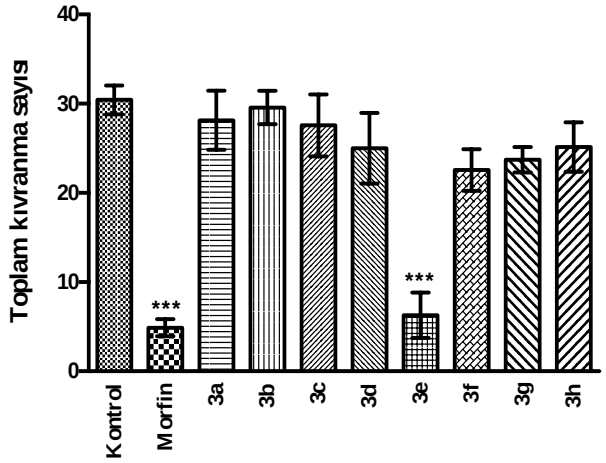
Test bileşikleri ve morfin uygulamalarının asetik asid ile indüklenen kıvranma testinde farelerin kıvranma sayıları üzerine etkileri Şekil 3'de verilmiştir [$F(9,60)=12.51$, $p<0.001$].

Tek yönlü ANOVA testini takiben uygulanan çoklu karşılaştırma testleri, 3e kodlu türevin hayvanların kıvranma sayılarını kontrol grubunun kıvranma sayılarına göre anlamlı biçimde azalttığını, 3a-3d ve 3f-3h kodlu türevlerin ise etkisiz olduğunu ortaya koymuştur. Referans ilaç morfin uygulamaları da farelerin kıvranma sayılarını anlamlı biçimde azaltmıştır (Şekil 3).

Davranış Deneylerine İlişkin Bulgular

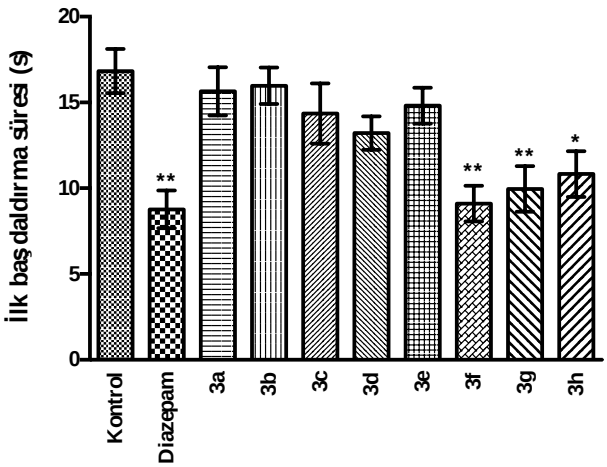
Delikli tahta testine ilişkin bulgular

Şekil 4'de test bileşikleri ve diazepam uygulamalarının delikli tahta testinde farelerin ilk baş daldırma süreleri üzerine etkileri gösterilmiştir [$F(9,60)=5.84$, $p<0.001$]. Tek yönlü ANOVA testini takiben uygulanan çoklu karşılaştırma testleri, 3f, 3g ve 3h kodlu türevlerin ve referans ilaç diazepam'ın hayvanların ilk baş daldırma sürelerini kontrol grubunun ilk baş daldırma sürelerine göre istatistiksel olarak anlamlı biçimde kısalttığını ortaya koymuştur. 3a-3e kodlu türevlerin farelerin ilk baş daldırma sürelerinde anlamlı bir değişikliğe neden olmadığı belirlenmiştir (Şekil 4).



Şekil 3. Test bileşiklerinin (40 mg/kg) ve morfin'in (10 mg/kg) asetik asid ile indüklenen kıvranma testinde farelerin kıvranma sayıları üzerine etkileri. Kontrol grubuna göre anlamlı farklılık $^{***}p<0.001$. Tek yönlü varyans analizi, takiben Tukey HSD çoklu karşılaştırma testi, n=7.

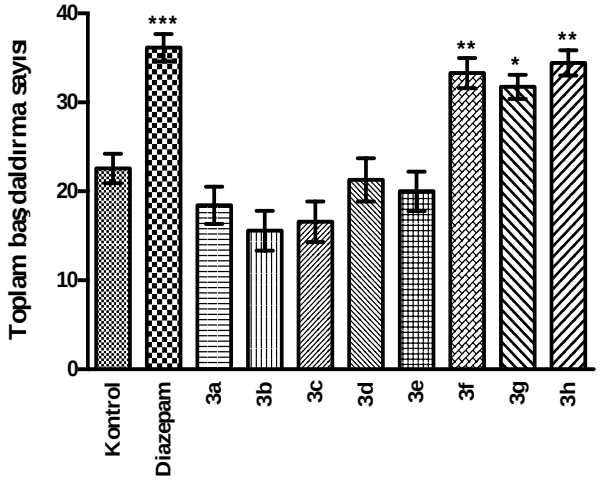
Figure 3. Effects of test compounds (40 mg/kg) and morphine (10 mg/kg) on writhing number of mice in the acetic acid-induced writhing test. Significance against control group $^{***}p<0.001$. One-way ANOVA, post hoc Tukey's test, n=7.



Şekil 4. Test bileşiklerinin (40 mg/kg) ve diazepam'ın (1 mg/kg) delikli tahta testinde farelerin ilk baş daldırma süreleri üzerine etkileri. Kontrol grubuna göre anlamlı farklılık $^{*}p<0.05$, $^{**}p<0.01$. Tek yönlü varyans analizi, takiben Tukey HSD çoklu karşılaştırma testi, n=7.

Figure 4. Effects of test compounds (40 mg/kg) and diazepam (1 mg/kg) on first head-dipping latencies of mice in the hole board test. Significance against control group $^{*}p<0.05$, $^{**}p<0.01$. One-way ANOVA, post hoc Tukey's test, n=7.

Test bileşikleri ve diazepam uygulamalarının delikli tahta testinde farelerin toplam baş daldırma sayıları üzerine etkileri Şekil 5'de verilmiştir [$F(9,60)=17.19$, $p<0.001$]. Tek yönlü ANOVA testini takiben uygulanan çoklu karşılaştırma testleri, 3f, 3g ve 3h kodlu türevlerin hayvanların toplam baş daldırma sayılarını kontrol grubunun toplam baş daldırma sayılarına göre anlamlı biçimde artırdığını, 3a-3e kodlu türevlerin ise etkisiz olduğunu ortaya koymuştur. Referans ilaç diazepam uygulamaları da farelerin toplam baş daldırma sayılarını anlamlı biçimde artırmıştır (Şekil 5).



Şekil 5. Test bileşiklerinin (40 mg/kg) ve diazepam'ın (1 mg/kg) delikli tahta testinde farelerin toplam baş daldırma sayıları üzerine etkileri. Kontrol grubuna göre anlamlı farklılık $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Tek yönlü varyans analizi, takiben Tukey HSD çoklu karşılaştırma testi, n=7.

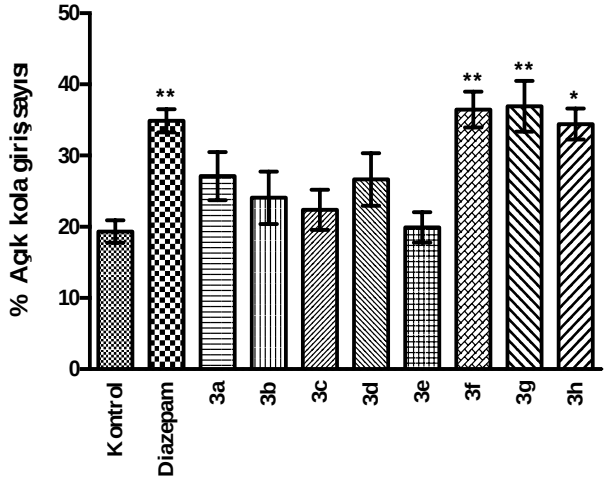
Figure 5. Effects of test compounds (40 mg/kg) and diazepam (1 mg/kg) on total number of head-dips of mice in the hole board test. Significance against control group $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. One-way ANOVA, post hoc Tukey's test, n=7.

Yükseltilmiş artı şekilli labirent testine ilişkin bulgular

Şekil 6'da test bileşikleri ve diazepam uygulamalarının yükseltilmiş artı şekilli labirent testinde farelerin %AKGS değerleri üzerine etkileri gösterilmiştir [$F(9,60)=5.96$, $p<0.001$].

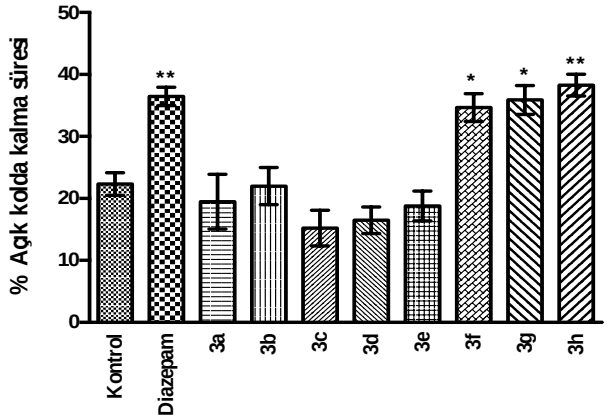
Tek yönlü ANOVA testini takiben uygulanan çoklu karşılaştırma testleri, 3f, 3g ve 3h kodlu türevlerin ve referans ilaç diazepam'ın hayvanların %AKGS değerlerini kontrol grubunun %AKGS değerlerine göre istatistiksel olarak anlamlı biçimde artırdığını ortaya koymuştur. 3a-3e kodlu türevlerin farelerin %AKGS değerlerinde anlamlı bir değişikliğe neden olmadığı belirlenmiştir (Şekil 6).

Test bileşikleri ve diazepam uygulamalarının yükseltilmiş artı şekilli labirent testinde farelerin %AKKS değerleri üzerine etkileri Şekil 7'de verilmiştir [$F(9,60)=12.81$, $p<0.001$]. Tek yönlü ANOVA testini takiben uygulanan çoklu karşılaştırma testleri, 3f, 3g ve 3h kodlu türevlerin hayvanların %AKKS değerlerini kontrol grubunun %AKKS değerlerine göre anlamlı biçimde artırdığını, 3a-3e kodlu türevlerin ise etkisiz olduğunu ortaya koymuştur. Referans ilaç diazepam uygulamaları da farelerin %AKKS değerlerini anlamlı biçimde artırmıştır (Şekil 7).



Şekil 6. Test bileşiklerinin (40 mg/kg) ve diazepam'ın (1 mg/kg) yükseltilmiş artı şekilli labirent testinde farelerin açık kola giriş sayılarının yüzdeleri üzerine etkileri. Kontrol grubuna göre anlamlı farklılık $^{*}p<0.05$, $^{**}p<0.01$. Tek yönlü varyans analizi, takiben Tukey HSD çoklu karşılaştırma testi, n=7.

Figure 6. Effects of test compounds (40 mg/kg) and diazepam (1 mg/kg) on percentages of open arm entries of mice in the elevated plus maze test. Significance against control group $^{*}p<0.05$, $^{**}p<0.01$. One-way ANOVA, post hoc Tukey's test, n=7.



Şekil 7. Test bileşiklerinin (40 mg/kg) ve diazepam'ın (1 mg/kg) yükseltilmiş artı şekilli labirent testinde farelerin açık kolda geçirdikleri sürelerin yüzdeleri üzerine etkileri. Kontrol grubuna göre anlamlı farklılık $^{*}p<0.05$, $^{**}p<0.01$. Tek yönlü varyans analizi, takiben Tukey HSD çoklu karşılaştırma testi, n=7.

Figure 7. Effects of test compounds (40 mg/kg) and diazepam (1 mg/kg) on percentages of time spent by mice in open arms in the elevated plus maze test. Significance against control group $^{*}p<0.05$, $^{**}p<0.01$. One-way ANOVA, post hoc Tukey's test, n=7.

Rota-rod Testine İlişkin Bulgular
Test bileşiklerinin farelerin dönen milden düşme sürelerinde istatistiksel olarak anlamlı bir değişikliğe neden olmadığı görülmüştür (veri gösterilmedi).

TARTIŞMA

Bu çalışmada, benzotiyazol türevi bazı bileşiklerin olası antinosiseptif ve anksiyolitik-benzeri etkileri araştırılmıştır. Bileşiklerin antinosiseptif etkilerini değerlendirmek için kuyruk sıkıştırma, sıcak plaka ve asetik asid ile indüklenen kıvranma testleri yapılmıştır. Anksiyolitik-benzeri etkileri delikli tahta ve yükseltilmiş artı şekilli labirent testleri ile araştırılmıştır. Test bileşiklerinin farelerin motor koordinasyonları üzerine olası etkileri Rota-rod testi ile değerlendirilmiştir.

Kuyruk sıkıştırma ve sıcak plaka testleri santral antinosiseptif etkinliğin araştırılmasında sıklıkla kullanılan testlerdir (Can ve ark. 2012a, Demir Özkay ve Can 2013). Bu çalışmada, kuyruk sıkıştırma ve sıcak plaka testlerinde 3f, 3g ve 3h kodlu test bileşiklerinin uygulanması ile farelerin yanıt sürelerinin uzadığı belirlenmiştir. Bu bulgular, söz konusu türevlerin santral antinosiseptif etkinlik gösterdiklerini ortaya koymuştur. Kuyruk sıkıştırma testinin ağırlıklı olarak spinal düzeydeki nosiseptif iletim ile, sıcak plaka testinin ise daha ziyade supraspinal düzeydeki nosiseptif iletim ile ilişkili olduğu bildirilmiştir (Wong ve ark. 1994, Gabra ve Sirois 2003). Bu nedenle, 3f, 3g ve 3h kodlu test bileşiklerinin antinosiseptif etkilerinin hem spinal hem de supraspinal mekanizmalar ile ilişkili olduğu ileri sürülebilir. Diğer yandan 3f, 3g ve 3h kodlu test bileşiklerinin sırasıyla mekanik ve termal ağrılı uyaran uygulanan kuyruk sıkıştırma ve sıcak plaka testlerinde antinosiseptif etki göstermeleri, söz konusu türevlerin hem mekanik hem de termal ağrılı uyaranın taşındığı nöronal yolakları etkilediğini işaret etmektedir.

Asetik asid ile indüklenen kıvranma testi periferal antinosiseptif etki tarama çalışmalarında sıklıkla kullanılan bir testtir (Kaplancikli ve ark. 2009, Demir Özkay ve Can 2013). Bu testte intraperitonal asetik asid uygulaması, deney hayvanlarında abdominal kasların kasılması, arka bacakların ekstansiyonu ve bedenin uzaması ile karakterize bir reaksiyona neden olmaktadır (Park ve ark. 2012). İntraperitonal irritasyonun asetilkolin, P maddesi, histamin, kininler ve prostaglandinler gibi çeşitli endojen maddelerin salınımını tetiklediği bildirilmiştir. Bu endojen maddelerin vasküler permeabiliteyi artırdıkları, nosisepsiyon eşiğini düşürdükleri ve nonsteroidal antiinflamatuar ilaçlara ve/veya opioid ilaçlara duyarlı nosiseptif nöronları stimüle ettikleri rapor edilmiştir (Coelho ve ark. 2005, Pinheiro ve ark. 2012). Bu çalışmada, 3e kodlu bileşiğin farelerin asetik asid ile indüklenen kıvranma sayılarını azalttığı belirlenmiştir. Söz konusu periferal antinosiseptif etkinin, test maddesinin periferik dokulardaki inflamatuar mediyatörlerin salıverilmesini azaltması ya da reseptörlerini doğrudan bloke edilmesi ile ilişkili olabileceği ileri sürülebilir. Ayrıca bu etkinin nosiseptif eşiğin artması ya da ağrı uyaranının sinir lifine iletiminin engellenmesi ile ilişkili olması da mümkündür (Kasap ve Can 2016).

Çalışma kapsamında benzotiyazol türevlerinin antinosiseptif etkinliğinin yanı sıra anksiyolitik-benzeri etkileri de araştırılmıştır. Anksiyolitik-benzeri etki taramalarında delikli tahta ve yükseltilmiş artı şekilli labirent testleri sıklıkla kullanılmaktadır (Can ve ark. 2012a, Can ve ark. 2012b, Can ve ark. 2013, Can ve ark. 2016). Delikli tahta testinde deney hayvanlarının yeni bir ortamdaki keşif davranışları değerlendirilmektedir (File ve Pellow 1985). Yükseltilmiş artı şekilli labirent testinde ise, deney hayvanları yerden yüksekte olan artı şekilli labirente konulmaktadırlar. Bu düzenekte deney hayvanları açık, dar ve yüksek olan kollardan kaçınmakta ve doğal eğilimleri gereği kapalı ve karanlık kolları tercih etmektedirler (Kumar ve ark. 2007, Can ve ark. 2016). Anksiyolitik ajanların %AKGS ve %AKKS değerlerinde artışa neden olurken, anksiyojeniklerin bu değerleri azalttığı bildirilmiştir (Sampath ve ark. 2011). Bu çalışmada, 3f, 3g ve 3h kodlu test bileşikleri farelerin ilk baş daldırma sürelerini kısaltmış, toplam baş daldırma sayılarını ise artırmıştır. Ayrıca söz konusu bileşiklerin %AKGS ve %AKKS değerlerini artırdığı belirlenmiştir. Bu bulgular, 3f, 3g ve 3h kodlu bileşiklerin anksiyolitik-benzeri etki gösterdiklerine işaret etmektedir.

Deney hayvanlarının motor koordinasyonlarının değerlendirildiği Rota-rod testlerinde test bileşikleri farelerin dönen milden düşme sürelerinde istatistiksel olarak anlamlı bir değişikliğe neden olmamıştır. Bu bulgu, bu çalışmada ortaya konulan farmakolojik etkilerin spesifik olduğunu göstermektedir.

SONUÇ

Deneyler sonucunda, 3f, 3g ve 3h kodlu bileşiklerin santral antinosiseptif ve anksiyolitik-benzeri etki gösterdikleri; 3e kodlu bileşiğin ise periferal antinosiseptif etki gösterdiği saptanmıştır. Bileşiklerin yapıları incelendiğinde, santral etkinlik gösteren 3f, 3g ve 3h kodlu bileşiklerin piperazin halkasının 4. konumunda fenil ya da benzil sübstitüenti taşıdıkları, periferal etkinlik gösteren 3e kodlu bileşiğin ise piperazin halkasının 4. konumunda dimetilaminoetil sübstitüenti taşıdığı görülmektedir. Piperazin halkasının 4. konumunda aromatik yapılar içeren 3f, 3g ve 3h kodlu bileşiklerin yüksek logP değerlerine sahip oldukları çalışma grubumuz tarafından daha önce bildirilmiştir (Demir Özkay ve ark. 2017b). Bu

nedenle, söz konusu bileşiklerin santral etkilerinin lipofilik özellikleri ile ilişkili olduğu ileri sürülebilir.

Bu çalışmanın bulguları benzotiyazol halkası taşıyan bileşiklerin antinosiseptif ve anksiyolitik-benzeri etkilerini bildiren önceki çalışmaları destekler niteliktedir. Diğer yandan, söz konusu farmakolojik etkilere aracılık eden mekanizmaların aydınlatılması için daha detaylı çalışmalara gereksinim duyulmaktadır.

TEŞEKKÜR

Bu çalışmanın yazarları, test maddelerinin teminindeki desteğinden dolayı Prof. Dr. Yusuf ÖZKAY'a ve yapmış olduğu finansal destekten dolayı Anadolu Üniversitesi Bilimsel Araştırma Projeleri Komisyonu'na (Proje No: 1805S200) teşekkürlerini sunar.

KAYNAKLAR

Ali R, Siddiqui N. New benzo[d]thiazol-2-yl-aminoacetamides as potential anticonvulsants: synthesis, activity and prediction of molecular properties. Arch Pharm (Weinheim). 2015; 348(4):254-265.

Amnerkar ND, Bhusari KP. Synthesis, anticonvulsant activity and 3D-QSAR study of some prop-2-eneamido and 1-acetyl-pyrazolin derivatives of aminobenzothiazole. Eur J Med Chem. 2010; 45(1):149-159.

Anzini M, Chelini A, Mancini A, Cappelli A, Frosini M, Ricci L, Valoti M, Magistretti J, Castelli L, Giordani A, Makovec F, Vomero S. Synthesis and biological evaluation of amidine, guanidine, and thiourea derivatives of 2-amino(6-trifluoromethoxy)benzothiazole as neuroprotective agents potentially useful in brain diseases. J Med Chem. 2010; 53(2):734-744.

Aouad MR, Soliman MA, Alharbi MO, Bardaweel SK, Sahu PK, Ali AA, Messali M, Rezki N, Al-Soud YA. Design, Synthesis and anticancer screening of novel benzothiazole-piperazine-1,2,3-triazole hybrids. Molecules. 2018; 23(11): 2788.

Azam MA, Dharanya L, Mehta CC, Sachdeva S. Synthesis and biological evaluation of some novel pyrazolopyrimidines incorporating a benzothiazole ring system. Acta Pharm. 2013; 63(1):19-30.

Can OD, Altintop MD, Ozkay UD, Uçel UI, Doğruer B, Kaplancikli ZA. Synthesis of thiadiazole derivatives bearing hydrazone moieties and evaluation of their pharmacological effects on anxiety, depression, and nociception parameters in mice. Arch Pharm Res. 2012a; 35(4):659-669.

Can OD, Demir Özkay U, Kıyan HT, Demirci B. Psychopharmacological profile of Chamomile (Matricaria recutita L.) essential oil in mice. Phytomedicine. 2012b; 19(3-4):306-310.

Can ÖD, Demir Özkay Ü, Üçel Uİ. Anti-depressant-like effect of vitexin in BALB/c mice and evidence for the involvement of monoaminergic mechanisms. Eur J Pharmacol. 2013; 699(1-3):250-257.

Can ÖD, Turan N, Alyu F. 1,3,5-triaril-4,5-dihidro-1h-pirazol türevi bazı bileşiklerin benzodiazepin reseptörleri aracılıklı anksiyolitik-benzeri etkileri. Cukurova Med J. 2016; 41(2):304-315.

Carboni S, Hiver A, Szyndralewiez C, Gaillard P, Gotteland JP, Vitte PA. AS601245 (1,3-benzothiazol-2-yl (2-[[2-(3-pyridinyl) ethyl] amino]-4 pyrimidinyl) acetonitrile): a c-Jun NH2-terminal protein kinase inhibitor with neuroprotective properties. J Pharmacol Exp Ther. 2004; 310(1):25-32.

Coelho LP, Reis PA, de Castro FL, Gayer CR, da Silva Lopes C, da Costa e Silva MC, de Carvalho Sabino KC, Todeschini AR, Coelho MG. Antinociceptive properties of ethanolic extract and fractions of Pterodon pubescens Benth. seeds. J Ethnopharmacol. 2005; 98(1-2):109-116.

Costanzo MJ, Yabut SC, Almond HR Jr, Andrade-Gordon P, Corcoran TW, De Garavilla L, Kauffman JA, Abraham WM, Recacha R, Chattopadhyay D. Potent, small-molecule inhibitors of human mast cell tryptase. Antiasthmatic action of a dipeptide-based transition-state analogue containing a benzothiazole ketone. J Med Chem. 2003; 46(18):3865-3876.

D'Amour FE, Smith DL. A method for determining loss of pain sensation. J Pharmacol Exp Ther. 1941; 72(1):74-79.

Demir Özkay U, Can OD. Anti-nociceptive effect of vitexin mediated by the opioid system in mice. Pharmacol Biochem Behav. 2013; 109:23-30.

Demir Özkay Ü, Can ÖD, Sağlık BN, Turan N. A benzothiazole/piperazine derivative with acetylcholinesterase inhibitory activity: Improvement in streptozotocin-induced cognitive deficits in rats. Pharmacol Rep. 2017a; 69(6):1349-1356.

Demir Özkay Ü, Kaya C, Acar Çevik U, Can ÖD. Synthesis and antidepressant activity profile of some novel benzothiazole derivatives. Molecules. 2017b; 22(9): 1490.

File SE, Pellow S. The effects of triazolobenzodiazepines in two animal tests of anxiety and in the holeboard. Br J Pharmacol. 1985; 86(3):729-735.

Gabra BH, Sirois P. Beneficial effect of chronic treatment with the selective bradykinin B1 receptor antagonists, R-715 and R-954, in attenuating streptozotocin-diabetic thermal hyperalgesia in mice. Peptides. 2003; 24(8):1131-1139.

Gökce M, Cakir B, Erol K, Sahin MF. Synthesis and antinociceptive activity of [(2-oxobenzothiazolin-3-yl)methyl]-4-alkyl/aryl-1,2,4-triazoline-5-thiones. Arch Pharm (Weinheim). 2001; 334(8-9):279-283.

Hamdy NA, Abdel-Aziz HA, Kamel GM, Fakhr IM. Convenient synthesis, anti-inflammatory, analgesic and ulcerogenic activites of some new bis-hydrazones and pyrazole derivatives. Acta Pol Pharm. 2013; 70(3):469-480.

Hassan MZ, Khan SA, Amir M. Design, synthesis and evaluation of N-(substituted benzothiazol-2-yl)amides as anticonvulsant and neuroprotective. Eur J Med Chem. 2012; 58:206-213.

Hroch L, Aitken L, Benek O, Dolezal M, Kuca K, Gunn-Moore F, Musilek K. Benzothiazoles - scaffold of interest for CNS targeted drugs. Curr Med Chem. 2015; 22(6):730-747.

Kaplancikli ZA, Turan-Zitouni G, Ozdemir A, Can Ov, Chevallet P. Synthesis and antinociceptive activities of some pyrazoline derivatives. Eur J Med Chem. 2009; 44(6):2606-2610.

Karali N, Güzel O, Ozsoy N, Ozbey S, Salman A. Synthesis

of new spiroindolinones incorporating a benzothiazole moiety as antioxidant agents. Eur J Med Chem. 2010; 45(3):1068-1077.

Kasap M, Can, ÖD. Opioid system mediated anti-nociceptive effect of agomelatine in mice. Life Sci. 2016; 163:55-63.

Keri RS, Quintanova C, Marques SM, Esteves AR, Cardoso SM, Santos MA. Design, synthesis and neuroprotective evaluation of novel tacrine-benzothiazole hybrids as multi-targeted compounds against Alzheimer's disease. Bioorg Med Chem. 2013; 21(15):4559-4569.

Koster R, Anderson M, De Beer EJ. Acetic acid for analgesic screening. Fed Proc. 1959; 18:412-415.

Kumar KV, Krishna DR, Palit G. Histaminergic H1 receptors mediate L-histidine-induced anxiety in elevated plus-maze test in mice. Behav Pharmacol. 2007; 18(3):213-217.

Liu DC, Zhang HJ, Jin CM, Quan ZS. Synthesis and biological evaluation of novel benzothiazole derivatives as potential anticonvulsant agents. Molecules. 2016; 21(3):164.

Mariappan G, Prabhat P, Sutharson L, Banerjee J, Patangia U, Nath S. Synthesis and antidiabetic evaluation of benzothiazole derivatives. J Korean Chem Soc. 2012; 56(2):251-256.

Moreno-Díaz H, Villalobos-Molina R, Ortiz-Andrade R, Díaz-Coutiño D, Medina-Franco JL, Webster SP, Binnie M, Estrada-Soto S, Ibarra-Barajas M, León-Rivera I, Navarrete-Vázquez G. Antidiabetic activity of N-(6-substituted-1,3-benzothiazol-2-yl)benzenesulfonamides. Bioorg Med Chem Lett. 2008; 18(9):2871-2877.

Muttu CT, Bhanushali MD, Hipparagi SM, Tikare VP, Karigar A. Microwave assisted synthesis and evaluation of some fluoro chloro 2-N-(substituted Schiff's bases)aminobenzothiazoles derivatives for their antiinflamatory activity. Int J Res Ayurveda Pharm. 2010; 1(2):522-528.

Osmaniye D, Levent S, Karaduman AB, Ilgın S, Özkay Y, Kaplancıklı ZA. Synthesis of new benzothiazole acylhydrazones as anticancer agents. Molecules. 2018; 23(5): 1054.

Park SH, Sim YB, Kang YJ, Kim SS, Kim CH, Kim SJ, Seo JY, Lim SM, Suh HW. Hop extract produces antinociception by acting on opioid system in mice. Korean J Physiol Pharmacol. 2012; 16(3):187-192.

Pinheiro MM, Boylan F, Fernandes PD. Antinociceptive effect of the Orbignya speciosa Mart. (Babassu) leaves: evidence for the involvement of apigenin. Life Sci. 2012; 91(9-10):293-300.

Sampath C, Holbik M, Krenn L, Butterweck V. Anxiolytic effects of fractions obtained from Passiflora incarnata L. in the elevated plus maze in mice. Phytother Res. 2011; 25(6):789-795.

Sarkar S, Siddiqui AA, Saha SJ, De R, Mazumder S, Banerjee C, Iqbal MS, Nag S, Adhikari S, Bandyopadhyay U. Antimalarial activity of small-molecule benzothiazole hydrazones. Antimicrob Agents Chemother. 2016; 60(7):4217-4228.

Sasaki-Hamada S, Sacai H, Sugiyama A, Iijima T, Saitoh A, Inagaki M, Yamada M, Oka J. Riluzole does not affect hippocampal synaptic plasticity and spatial memory, which are impaired by diazepam in rats. J Pharmacol Sci. 2013; 122(3):232-236.

Siddiqui N, Rana A, Khan SA, Ahsan W, Alam MS, Ahmed S. Analgesic and antidepressant activities of benzothiazole-benzamides. Biomed Pharmacol J. 2008; 1(2):297-300.

Siddiqui N, Rana A, Khan SA, Bhat MA, Haque SE. Synthesis of benzothiazole semicarbazones as novel anticonvulsants–the role of hydrophobic domain. Bioorg Med Chem Lett. 2007; 17(15):4178-4182.

Singh MK, Tilak R, Nath G, Awasthi SK, Agarwal A. Design, synthesis and antimicrobial activity of novel benzothiazole analogs. Eur J Med Chem. 2013; 63:635-644.

Takeda H, Tsuji M, Matsumiya T. Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and/or anxiolytic state in mice. Eur J Pharmacol. 1998; 350(1):21-29.

Thakkar SS, Thakor P, Ray A, Doshi H, Thakkar VR. Benzothiazole analogues: Synthesis, characterization, MO calculations with PM6 and DFT, in silico studies and in vitro antimalarial as DHFR inhibitors and antimicrobial activities. Bioorg Med Chem. 2017; 25(20):5396-5406.

Ugwu DI, Okoro UC, Ukoha PO, Gupta A, Okafor SN. Novel anti-inflammatory and analgesic agents: synthesis, molecular docking and in vivo studies. J Enzyme Inhib Med Chem. 2018; 33(1):405-415.

Wang S, Chen Y, Zhao S, Xu X, Liu X, Liu BF, Zhang G. Synthesis and biological evaluation of a series of benzoxazole/benzothiazole-containing 2,3-dihydrobenzo[b][1,4]dioxine derivatives as potential antidepressants. Bioorg Med Chem Lett. 2014; 24(7):1766-1770.

Wong CH, Dey P, Yarmush J, Wu WH, Zbuzek VK. Nifedipine-induced analgesia after epidural injection in rats. Anesth Analg. 1994; 79(2):303-306.

Woolfe G, MacDonald AD. The evaluation of the analgesic action of pethidine hydrochloride (Demerol). J Pharmacol Exp Ther. 1944; 80(3):300-307.

Zha GF, Leng J, Darshini N, Shubhavathi T, Vivek HK, Asiri AM, Marwani HM, Rakesh KP, Mallesha N, Qin HL. Synthesis, SAR and molecular docking studies of benzo[d]thiazole-hydrazones as potential antibacterial and antifungal agents. Bioorg Med Chem Lett. 2017; 27(14):3148-3155.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):130-134

DOI: 10.30607/kvj.511340

RESEARCH ARTICLE

The Effect of Melatonin on Some Coagulation Parameters in Streptozotocin-induced Diabetic Rats

Ercan KESKIN[1], Deniz ULUIŞIK[1*]

[1]Department of Physiology, Faculty of Veterinary Medicine, University of Selcuk, Konya, Turkey

ABSTRACT

The aim of this study is to evaluate the possible protective effects of melatonin on hemostatic parameters in diabetic rats. For this purpose, 32 adult, male, healthy Wistar Abino rats were seperated into four groups. Control group didn't exposure any trial. Melatonin group was treated with 50 mg/kg melatonin by intraperitoneally during 8 weeks. In diabetes group, diabetes was induced by subcutaneous injections of streptozotocin at dose of 40 mg/kg for two days as a single daily dose. Diabetes+Melatonin group was consist of the animals that treated with 50 mg/kg melatonin by intraperitoneally to streptozotocin induced diabetic during 8 weeks. In diabetic rats, the platelet count and fibrinogen level significantly increased compared to control group ($p<0.05$), whereas melatonin application to the diabetic rats caused to decrease in fibrinogen level when compared to diabetic rats ($p<0.05$). Activated Partial Tromboplastin Time (APTT), Prothrombin Time (PT) and International Normalized Ratio (INR) levels significantly shortened in the experimentally diabetes group compared to the control group ($p<0.05$). PT and INR significantly prolonged in diabetic rats with the melatonin treatment compared to diabetic rats ($p<0.05$). In conclusion, the obtained data indicated that administration of melatonin partly ameliorated procoagulant state caused by diabetes in rats.

Keywords: APTT, fibrinogen, diabetes, melatonin, rat

***

Streptozotosin ile Diyabet Oluşturulan Ratlarda Melatonin Uygulamasının Bazı Koagulasyon Parametreleri Üzerindeki Etkisi

ÖZ

Bu çalışmanın amacı streptozotosin ile diyabet oluşturulan ratlarda hemostatik parametreler üzerine melatoninin muhtemel koruyucu etkilerinin belirlenmesidir. Bu amaçla 32 yetişkin erkek sağlıklı Wistar Abino rat dört gruba ayrıldı. Kontrol grubuna herhangi bir uygulama yapılmadı. Melatonin grubuna 8 hafta boyunca intraperitoneal olarak 50 mg/kg melatonin uygulandı. Diyabet grubunda 40 mg/kg streptozotosinin günlük tek doz olmak üzere iki gün subkutan enjeksiyonuyla diyabet oluşturuldu. Diyabet+Melatonin grubuna streptozotosin uygulanarak diyabet oluşturulduktan sonra 8 hafta boyunca intraperitoneal olarak 50 mg/kg melatonin enjekte edildi. Diyabetik ratlarda platelet sayısı ve fibrinojen seviyesi kontrol grubuna göre önemli oranda artarken ($p<0.05$), diyabetik ratlara melatonin uygulaması diyabet grubuna göre fibrinojen düzeyinde azalmaya neden oldu ($p<0.05$). Deneysel diyabet grubundaki APTT, PT ve INR düzeyleri kontrol grubuna göre önemli bir şekilde azaldı ($p<0.05$). Diyabetik ratlara intraperitoneal olarak melatonin uygulaması ile PT ve INR düzeyleri diyabetik ratlara göre önemli bir şekilde uzadı ($p<0.05$). Sonuç olarak, bu çalışmadan elde edilen veriler melatonin uygulamasının ratlarda diyabetten kaynaklanan prokoagulan durumu kısmen düzelttiğini göstermektedir.

Anahtar Kelimeler: APTT, fibrinojen, diyabet, melatonin, rat

To cite this article: Uluışık D. Keskin E. The Effect of Melatonin on Some Coagulation Parameters in Streptozotocin-induced Diabetic Rats. Kocatepe Vet J. (2019) 12(2):130-134.

Submission: 10.01.2019 **Accepted**: 26.04.2019 **Published Online**: 30.04.2019

ORCID ID; DU: 0000-0003-1462-0836, EK: 0000-0003-3839-0414

*Corresponding author e-mail: denizfedai@selcuk.edu.tr

INTRODUCTION

In the worldwide, there is an increase in death due to atherothrombotic disorders result from metabolic disorders such as diabetes mellitus (DM), hypertension and obesity (Sanz and Fuster 2011, Korish et al. 2015). Diabetes mellitus caused by either low insulin level or high insulin resistance is characterized with high hyperglycemia and the other metabolic disorders (Yeom et al. 2016). Millions of people are affected by cardiovascular diseases related to diabetes mellitus (Kakouros et al. 2011). In later stages, diabetes mellitus causes abnormal endothelial function, increase of arterial stiffness, platelet hyperreactivity and hemorheological changes (Wilkinson et al. 2000, Schäfer et al. 2007, Cho et al. 2008, Yeom et al. 2016). Another complication of diabetes is hemostatic disorders. Some studies claim that diabetes increases the tendency to coagulation (Takada et al. 1993, Osende et al. 2001, Creager et al. 2003, Ferreiro et al. 2010, Ferreiro and Angiolillo 2011). These serious disturbances play a crucial role in the etiology of diabetes-related vascular complications including arteriosclerosis and myocardial infarction (Beckman et al. 2002, Loomans et al. 2004, Yeom et al. 2016). Thrombotic disorders and vascular lesions such as microvasculopathy, retinopathy and macroangiopathy, especially in the coronary and cerebral vessels can be life threatening. Rupture of an atherosclerotic plaque promotes platelet activation and locally triggering of the coagulation process that can cause thrombus formation at the region of endothelial damage (Kakouros et al. 2011). It has been stated that cardiovascular and cerebrovascular disorders frequently occurred in diabetes due to micro- and macrovascular complications (American Diabetic Association 2011, Eriksson et al. 2012, Korish et al. 2015).

Many therapeutic approaches have been still studied to alleviate the complications of diabetes. Recently, studies have been focused on melatonin as a therapeutic agent due to its several physiological activities (Carrillo-Vico et al. 2005, Claustrat et al. 2005). In 1958, Lerner's group first isolated melatonin (N-acetyl-5-methoxy-tryptamine) from the bovine pineal gland (Minneman and Wurtman 1976). Melatonin production is regulated by the suprachiasmatic nucleus (SCN) as known the central circadian pacemaker (Carrillo-Vico et al. 2005). It is synthesized in several organs, including the pineal gland, Harder's glands, gastroenteric mucous membrane, retina, platelets and megakaryocytes (Reiter et al. 1988). Melatonin has high lipid and water solubility features, thus it passes easily across cell membranes (Claustrat et al. 2005). Pineal secretion reaches maximum plasma levels around 03:00–04:00 a.m. During the day, its level is low or even undetectable (Follenius et al. 1995, Rodella et al. 2013).

Although, there is a limited data about the effects of melatonin on coagulation, there are various findings about the effect of melatonin on cardiovascular incidents, hemorrhage, activities of some coagulation proteins and fibrinolytic systems regarding in subjected to circadian variations (Pinotti et al. 2005, Montagnana et al. 2009). Some studies reported that melatonin may used for hemostasis bu using in a different route, doses and period to animals and human (Tunali et al. 2005, Tai et al. 2010, Kostovski et al. 2011). It was also suggested that there is a dose-dependent relationship between plasma melatonin level and coagulation activity (Wirtz et al. 2008, Pashalieva et al. 2014).

Based upon these acknowledgements we aimed to determine the effects of melatonin on some coagulation parameters in streptozotocin-induced diabetic rats.

MATERIALS and METHODS

In the study, 32 male, 6 weeks of age, healthy Wistar Abino rats were used. The animals were divided into four groups. All animals were fed with standart rat diet as *ad libitum* during 8 weeks. The Ethical Committee of Selcuk University Experimental Medicine Research and Application Center approved the study protocol (Report no. 2017-15).

- The animals in control group (n=6) didn't exposure any treatment.
- Melatonin group (n=6) was injected with 50 mg/kg melatonin (Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally during 8 weeks.
- Diabetes group (n=10) was induced by subcutaneously injected with streptozotocin (Sigma-Aldrich, St. Louis, MO, USA) at dose of 40 mg/kg in 0.1 M citrate buffer (pH 4.5) for two days as a single daily dose.
- Diabetes+Melatonin group (n=10) was injected with 50 mg/kg melatonin intraperitoneally to the diabetic rats during 8 weeks.

In the cases of streptozotocin-induced hypoglycemia, rats were given 5% dextrose solution as a precaution after 6 h of streptozotocin administration during next 3 days. Diabetes was verified by measuring blood glucose level strips using glucometer (PlusMED Accuro, Taiwan) via the tail vein after one week from streptozotocin injections. Animals, which have a blood glucose level higher than 250 mg/dl, were accepted as diabetic and were included in the experiment. During the experiment, one animal from diabetes group were died due to streptozotocin-induced hypoglycemia.

At the end of the 8 weeks, blood samples were taken from all animals. In these blood samples, platelet, fibrinogen, activated partial thromboplastin time (APTT), prothrombin time (PT), international normalized ratio (INR) levels were determined. Platelet, fibrinogen, APTT, PT, INR levels were determined by using Abbott kits in Abbott analyzer (Abbott Architect i2000).

The data obtained from the study were analyzed by one-way ANOVA (SPSS 19). Differences among the groups were determined by Duncan's multiple range test. Differences were considered significant at $p<0.05$.

RESULTS

The effect of melatonin on coagulation parameters in experimentally induced diabetes in rats were summarized Table 1. In diabetic rats, the platelet count and fibrinogen level significantly increased compared to control group ($p<0.05$, Table 1), whereas intraperitoneally melatonin application to the diabetic rats caused to decrease in both parameters when compared to diabetic rats. The decrease in fibrinogen level was significant ($p<0.05$, Table 1). In this study, APTT, PT and INR levels in the experimentally diabetes group significantly shortened compared to control group ($p<0.05$, Table 1). With the intraperitoneally melatonin application to the diabetic rats, PT and INR significantly prolonged compared to diabetic rats ($p<0.05$, Table 1), while the changes in APTT was not important statistically.

DISCUSSION

In diabetes mellitus, cardiovascular complications and the other metabolic disorders generally coexist and lead to high morbidity and mortality in worldwide. It is frequently seen that prothrombotic conditions such as platelet hyperreactivity, impaired fibrinolysis, endothelial dysfunction and increased coagulation cause these cardiovascular complications in diabetes. (Takada et al. 1993, Osende et al. 2001, Creager et al. 2003). Some mechanisms caused by metabolic and cellular abnormalities have been accepted as a reason of coagulation tendency in diabetes mellitus. These mechanisms and their ethiologies were been categorized as hyperglycemia, insulin deficiency and resistance associated with metabolic conditions and other cellular abnormalities. (Ferreiro et al. 2010, Ferreiro and Angiolillo 2011). In parallel with the knowledge that mentioned above, coagulation parameters were significantly impaired in streptozosin induced diabetic rats in this study. So that, thrombocyte count and fibrinogen level significantly increased in diabetic group compared to control group ($p<0.05$, Table 1), whereas APTT, PT and INR shortened compared to control group ($p<0.05$, Table 1). Ohaeri and Adoga (2006) reported that the increases in platelet count and coagulation factors amount (factor V, VII, VIII, IX and X) were determined in experimentally diabetic rats compared to control rats. In addition, plasma fibrinogen levels were found to be higher as parallel to hyperglycemic degree in many studies conducted with diabetes (Ceriello et al. 1994, Schalkwijk et al. 1999, Dunn and Ariëns 2004). In another study, it has been stated that PT, APTT and coagulation time in diabetic rats were found to be lower than control group, while platelet count and plasma fibrinogen levels in diabetic rats were obtained as higher than control group's level (ElGendy and Abbas 2014). It was suggested that hyperglycemia exposure may induce procoagulant state in diabetes due to hemorheological changes of thrombocytes, platelet aggregation, endothelial dysfunction and increasing levels of tissue factors (Yeom et al. 2016). Another pathway of activation of procoagulant state is that hyperglycemia leads to increasing levels of tissue factor, prothrombin fragments, decreasing factor VII/VIIa and increasing factor VIII (Vaidyula et al. 2006b). In addition, it was reported that hyperglycemia upregulate platelet expression of CD40L and increase monocyte-platelet aggregates as indicative of platelet activation (Vaidyula et al. 2006a, Kakouros et al. 2011).

Melatonin, a signaling molecule for circadian rhythms, plays an important role in many biological processes (Dahm et al. 2006, Kostovski et al. 2011). It was suggested that endogenous melatonin may also produce antithrombotic, antioxidant and anti-inflammatory activity (Carrillo-Vico et al. 2005, Claustrat et al. 2005, Dahm et al. 2006, Ashy and Shroff 2016). Depend on its wide effects, melatonin could be expected to regulate hemostatic events and the effects of melatonin on hemostasis are relatively poorly studied (Kostovski et al. 2011). For this reason, we determined some hemostatic parameters in diabetic rats treated with melatonin. Melatonin treatment significantly decreased plasma fibrinogen levels compared to diabetic group, while PT and INR levels significantly increased when compared diabetic group ($p<0.05$, Table 1). The changes in platelet count and APTT with the melatonin treatment to diabetic rats were not important statistically. In the light of our results, it might be said that melatonin has favorable effects on hemostatic parameters in streptozotocin-induced diabetic rats. These effects of melatonin on hemostasis such as inhibiting procoagulant state were attributed to some mechanisms. Kostovski et al. (2011) reported that melatonin increased tissue factor pathway inhibitor (TFPI) in endothelial cells *in vitro*. In contrary, it was suggested that plasma TFPI level is low when plasma melatonin levels peak in darkness, vice versa (Dahm et al. 2006). Protein C anticoagulant pathway is one of important mechanisms of the anticoagulant system. This pathway includes membrane proteins and circulating proteins (such as protein C, thrombomodulin, endothelial protein C receptor

(EPCR) and protein S) (Castellino and Ploplis 2009, Stancheva et al. 2015). Castellino and Ploplis (2009) stated that melatonin significantly reduced activity of protein C anticoagulant pathway in rats. The beneficial effects of melatonin related to procoagulant state might based on endothelium protective effects (Rodella et al. 2013). In diabetes, increased in plasma glucose levels contribute endothelial dysfunction characterized by proliferating barrier function and adhesion of circulating cells (Ho et al. 2000, Ido et al. 2002, Favaro et al. 2008). Therefore, melatonin with its antioxidant and antiinflamatory properties is being expected to act protective on endothelial dysfunction arise from diabetes. Thus, melatonin activates antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase (Rezzani et al. 2006, Bharti and Srivastava 2009), protects cells from oxidative damage and apoptosis (Jou et al. 2007, Rodella et al. 2013).

Table 1. Effect of melatonin on coagulation parameters in diabetes (Mean±SE)

	Platelet (K/μl)	Fibrinogen (mg/dl)	APTT (sec)	PT (sec)	INR
Control	690.50±27.65^{b}	227.83±22.39^{b}	35.62±3.30^{a}	11.60±0.21^{ab}	1.05±0.02^{a}
Melatonin	711.83±37.62^{b}	193.67±18.79^{b}	36.78±3.18^{a}	11.80±0.10^{a}	1.06±0.01^{a}
Diabetes	827.56±20.71^{a}	313.56±24.37^{a}	24.21±2.80^{b}	9.79±0.29^{c}	0.90±0.02^{b}
Diabetes+Melatonin	747.20±25.44^{ab}	248.30±18.71^{b}	31.59±2.14^{ab}	10.92±0.24^{b}	1.00±0.02^{a}

$^{a-c}$ The difference between mean values with different superscripts in the same column is significant at the $p<0.05$ level.

CONCLUSIONS

As a result, the obtained data from this study indicated that administration of melatonin partly improved procoagulant state caused by diabetes in rats. Nevertheless, the further studies are needed established with different melatonin doses related to hemostatic state in diabetes in the future.

ACKNOWLEDGEMENT

The Ethical Committee of Selcuk University Experimental Medicine Research and Application Center approved the study protocol (Report no. 2017-15).

REFERENCES

American Diabetic Association. National Diabetes Fact Sheet 2011 (released Jan. 26, 2011). http://www.diabetes.org/diabetes-basics/diabetes-statistics; Last accessed date: 10.09. 2012.

Ashy NI, Shroff KV. Evaluation of the potential drug interaction of melatonin and warfarin: a case series. Life Sci J. 2016; 13(6):46-51.

Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. JAMA. 2002; 287(19):2570-2581.

Bharti VK, Srivastava RS. Pineal proteins upregulate specific antioxidant defense systems in the brain. Oxid Med Cell Longev. 2009; 2(2):88-92.

Carrillo-Vico A, Guerrero JM, Lardone PJ, Reiter RJ. A review of the multiple actions of melatonin on the immune system. Endocrine. 2005; 27(2):189-200.

Castellino FJ, Ploplis VA. The protein C pathway and pathologic processes. J Thromb Haemost. 2009; 7(1):140-145.

Ceriello A, Taboga C, Giacomello R, Falleti E, De Stasio G, Motz E, Lizzio S, Gonano F, Bartoli E. Fibrinogen plasma levels as a marker of thrombin activation in diabetes. Diabetes. 1994; 43(3):430-432.

Cho YI, Mooney MP, Cho DJ. Hemorheological disorders in diabetes mellitus. J Diabetes Sci Technol. 2008; 2(6):1130-1138.

Claustrat B, Brun J, Chazot G. The basic physiology and pathophysiology of melatonin. Sleep Med Rev. 2005; 9(1):11-24.

Creager MA, Lüscher TF, Cosentino F, Beckman JA. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: Part I. Circulation. 2003; 108(12):1527-1532.

Dahm A, Osterud B, Hjeltnes N, Sandset PM, Iversen PO. Opposite circadian rhythms in melatonin and tissue factor pathway inhibitor type 1: does daylight affect coagulation?. J Thromb Haemost. 2006; 4(8):1840-1842.

Dunn EJ, Ariëns RA. Fibrinogen and fibrin clot structure in diabetes. Herz. 2004; 29(5):470-479.

ElGendy AA, Abbas AM. Effects of warfarin and L-carnitine on hemostatic function and oxidative stress in streptozotocin-induced diabetic rats. J Physiol Biochem. 2014; 70(2):535-546.

Eriksson M, Carlberg B, Eliasson M. The disparity in long-term survival after a first stroke in patients with and without diabetes persists: the Northern Sweden MONICA study. Cerebrovasc Dis. 2012; 34(2):153-160.

Favaro E, Miceli I, Bussolati B, Schmitt-Ney M, Cavallo Perin P, Camussi G, Zanone MM. Hyperglycemia induces apoptosis of human pancreatic islet endothelial cells: effects of pravastatin on the Akt survival pathway. Am J Pathol. 2008; 173(2):442-450.

Ferreiro JL, Angiolillo DJ. Diabetes and antiplatelet therapy in acute coronary syndrome. Circulation. 2011; 123(7):798-813.

Ferreiro JL, Gómez-Hospital JA, Angiolillo DJ. Platelet abnormalities in diabetes mellitus. Diab Vasc Dis Res. 2010; 7(4):251-259.

Follenius M, Weibel L, Brandenberger G. Distinct modes of melatonin secretion in normal men. J Pineal Res. 1995; 18(3):135-140.

Ho FM, Liu SH, Liau CS, Huang PJ, Lin-Shiau SY. High glucose-induced apoptosis in human endothelial cells is

mediated by sequential activations of c-Jun NH(2)-terminal kinase and caspase-3. Circulation. 2000; 101(22):2618-2624.

Ido Y, Carling D, Ruderman N. Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation. Diabetes. 2002; 51(1):159-167.

Jou MJ, Peng TI, Yu PZ, Jou SB, Reiter RJ, Chen JY, Wu HY, Chen CC, Hsu LF. Melatonin protects against common deletion of mitochondrial DNA-augmented mitochondrial oxidative stress and apoptosis. J Pineal Res. 2007; 43(4):389-403.

Kakouros N, Rade JJ, Kourliouros A, Resar JR. Platelet function in patients with diabetes mellitus: from a theoretical to a practical perspective. Int J Endocrinol. 2011; Article ID 742719:1-14. doi:10.1155/2011/742719.

Korish AA, Abdel Gader AGM, Alhaider AA. Camel milk ameliorates the coagulopathy in streptozotocin diabetic rat model. Int J Dairy Technol. 2015; 68(1):79-87.

Kostovski E, Dahm AE, Iversen N, Hjeltnes N, Østerud B, Sandset PM, Iversen PO. Melatonin stimulates release of tissue factor pathway inhibitor from the vascular endothelium. Blood Coagul Fibrinolysis. 2011; 22(4):254-259.

Loomans CJ, de Koning EJ, Staal FJ, Rookmaaker MB, Verseyden C, de Boer HC, Verhaar MC, Braam B, Rabelink TJ, van Zonneveld AJ. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. Diabetes. 2004; 53(1):195-199.

Minneman KP, Wurtman RJ. The pharmacology of the pineal gland. Annu Rev Pharmacol Toxicol. 1976; 16:33-51.

Montagnana M, Salvagno GL, Lippi G. Circadian variation within hemostasis: an under recognized link between biology and disease?. Semin Thromb Hemost. 2009; 35(1):23-33.

Ohaeri OC, Adoga GI. Anticoagulant modulation of blood cells and platelet reactivity by garlic oil in experimental diabetes mellitus. Biosci Rep. 2006; 26(1):1-6.

Osende JI, Badimon JJ, Fuster V, Herson P, Rabito P, Vidhun R, Zaman A, Rodriguez OJ, Lev EI, Rauch U, Heflt G, Fallon JT, Crandall JP. Blood thrombogenicity in type 2 diabetes mellitus patients is associated with glycemic control. J Am Coll Cardiol. 2001; 38(5):1307-1312.

Pashalieva I, Decheva L, Stancheva E, Nyagolov Y, Negrev N. Melatonin and luzindole-induced effects on integral blood coagulation parameters in rats. Compt Rend Acad Bulg Sci. 2014; 67(9):1269-1274.

Pinotti M, Bertolucci C, Portaluppi F, Colognesi I, Frigato E, Foà A, Bernardi F. Daily and circadian rhythms of tissue factor pathway inhibitor and factor VII activity. Arterioscler Thromb Vasc Biol. 2005; 25(3):646-649.

Reiter RJ, Li K, Gonzalez-Brito A, Tannenbaum MG, Vaughan MK, Vaughan GM, Villanua MA. Elevated environmental temperature alters the responses of the reproductive and thyroid axes of female Syrian hamsters to afternoon melatonin injections. J Pineal Res. 1988; 5(3):301-315.

Rezzani R, Rodella LF, Bonomini F, Tengattini S, Bianchi R, Reiter RJ. Beneficial effects of melatonin in protecting against cyclosporine A-induced cardiotoxicity are receptor mediated. J Pineal Res. 2006; 41(3):288-295.

Rodella LF, Favero G, Foglio E, Rossini C, Castrezzati S, Lonati C, Rezzani R. Vascular endothelial cells and dysfunctions: role of melatonin. Front Biosci (Elite Ed). 2013; 5:119-129.

Sanz G, Fuster V. Polypill and global cardiovascular health strategies. Semin Thorac Cardiovasc Surg. 2011; 23(1):24-29.

Schäfer A, Fraccarollo D, Vogt C, Flierl U, Hemberger M, Tas P, Ertl G, Bauersachs J. Improved endothelial function and reduced platelet activation by chronic HMG-CoA-reductase inhibition with rosuvastatin in rats with streptozotocin-induced diabetes mellitus. Biochem Pharmacol. 2007; 73(9):1367-1375.

Schalkwijk CG, Poland DC, van Dijk W, Kok A, Emeis JJ, Dräger AM, Doni A, van Hinsbergh VW, Stehouwer CD. Plasma concentration of C-reactive protein is increased in type 1 diabetic patients without clinical macroangiopathy and correlates with markers of endothelial dysfunction: evidence for chronic inflammation. Diabetologia. 1999; 42(3):351-357.

Stancheva E, Zarkova A, Pashalieva I, Nyagolov Y, Negrev N. Melatonin inhibits the protein C anticoagulant pathway in rats. Scr Sci Med. 2015; 47(1):57-63.

Tai SH, Chen HY, Lee EJ, Chen TY, Lin HW, Hung YC, Huang SY, Chen YH, Lee WT, Wu TS. Melatonin inhibits postischemic matrix metalloproteinase-9 (MMP-9) activation via dual modulation of plasminogen/plasmin system and endogenous MMP inhibitor in mice subjected to transient focal cerebral ischemia. J Pineal Res. 2010; 49(4):332-341.

Takada Y, Urano T, Watanabe I, Taminato A, Yoshimi T, Takada A. Changes in fibrinolytic parameters in male patients with type 2 (non-insulin-dependent) diabetes mellitus. Thromb Res. 1993; 71(5):405-415.

Tunali T, Sener G, Yarat A, Emekli N. Melatonin reduces oxidative damage to skin and normalizes blood coagulation in a rat model of thermal injury. Life Sci. 2005; 76(11):1259-1265.

Vaidyula VR, Boden G, Rao AK. Platelet and monocyte activation by hyperglycemia and hyperinsulinemia in healthy subjects. Platelets. 2006a; 17(8):577-585.

Vaidyula VR, Rao AK, Mozzoli M, Homko C, Cheung P, Boden G. Effects of hyperglycemia and hyperinsulinemia on circulating tissue factor procoagulant activity and platelet CD40 ligand. Diabetes. 2006b; 55(1):202-208.

Wilkinson IB, MacCallum H, Rooijmans DF, Murray GD, Cockcroft JR, McKnight JA, Webb DJ. Increased augmentation index and systolic stress in type 1 diabetes mellitus. QJM. 2000; 93(7):441-448.

Wirtz PH, Spillmann M, Bärtschi C, Ehlert U, von Känel R. Oral melatonin reduces blood coagulation activity: a placebo-controlled study in healthy young men. J Pineal Res. 2008; 44(2):127-133.

Yeom E, Byeon H, Lee SJ. Effect of diabetic duration on hemorheological properties and platelet aggregation in streptozotocin-induced diabetic rats. Sci Rep. 2016; 6:21913. doi: 10.1038/srep21913.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):135-143
DOI: 10.30607/kvj.478100

RESEARCH ARTICLE

The Presence and Prevalence of Bovine Parainfluenza 3 (BPIV-3), Bovine Papillomaviruses (BPV), Bovine Herpesvirus 1 (BHV-1) in Subclinical Mastitis in Cattle

Serdar ALTUN*[1], Selçuk ÖZDEMİR[2], Yavuz Selim SAĞLAM[1]

[1]Atatürk University, Faculty of Veterinary Medicine, Department of Pathology. Erzurum-Turkey
[2]Atatürk University, Faculty of Veterinary Medicine, Department of Genetics. Erzurum-Turkey

ABSTRACT

In general, bacterial pathogens are the focus of mastitis studies. Symptoms of mastitis may be absent in cases of viral infection, because other clinical symptoms are more dominant. Subclinical mastitis cases cannot be generally diagnosed and as a consequence, they are not investigated very well. This may lead to the inability of viral agents to be predicted in bovine subclinical cases. There are many viral agents associated with bovine mastitis and which may cause subclinical mastitis in dairy cattle. However, since viral infections have not been much investigated in mastitis studies, strategies developed against subclinical mastitis may be inadequate. Bovine Parainfluenza 3 (BPIV-3), Bovine Papillomaviruses (BPV), Bovine Herpesvirus 1 (BHV-1) could cause clinical and subclinical mastitis. The aim of the present study was to investigate the presence and prevalence of BPIV-3, BPV, and BHV-1 viral agents in subclinical mastitis case in Erzurum province. For this purpose, 120 bovine mammary tissues with no macroscopic lesions were collected and these tissues were examined with histopathology and immunofluorescence methods. Thereafter, all viruses were identified with qRT-PCR in mammary tissues. The end of histopathologic process 78 of 120 cases (56 chronic subclinic mastitis, 22 acut mastitis) was found severity of inflammatory changes. According to the results of immunofluorescence staining for all sections. The immun positive signs for BPIV-3 BPV, and BHV-1 was determined in 50 out of 120 cases. qRT-PCR results which compatible with immunofluorescence results showed that BPIV-3 agent was detected by the qRT-PCR in 26/120 samples, BPV-2 agent in 8/120 samples, BHV-1 agent in 16/120 samples, and BPV-1 agent in 7/120 samples (only by qRT-PCR). Coinfection with BPIV-3 and BHV-1 was detected in 5/120 samples, BPIV-3, and BPV-1 in 3/120 samples, BHV-1, and BPV-2 in 2/120 samples. According to our results, although the role of viral agents in mastitis disease has not been clearly elucidated, we have found that viral agents are common in mammary tissues with subclinical mastitis.

Keywords: Subclinical mastit, Bovine Parainfluenza 3, Bovine Papillomaviruses, Bovine Herpesvirus 1, qRT-PCR, Immunofluorescence.

***

Sığırlarda Subklinik Mastitisde Sığır Parainfluenza 3 (BPIV-3), Sığır Papillomavirüsleri (BPV), Sığır Herpesvirüs 1 (BHV-1) Varlığı ve Prevalansı

ÖZ

Genellikle etiyolojik mastitis çalışmaları bakteriyel patojenler üzerine odaklanmıştır. Viral enfeksiyon vakalarında mastit belirtileri, diğer klinik semptomlar daha baskın olduğu için gözden kaçabilmektedir. Subklinik mastitis olguları genellikle teşhis edilemediği için çok iyi araştırılmamıştır. Bu durum sığır subklinik mastitis vakalarında viral ajanların önlenememesine yol açabilmektedir. Sığırlarda mastitis ile ilişkili ve süt sığırlarında subklinik mastite neden olabilecek birçok viral ajan tanımlanmıştır. Bununla birlikte, mastitis çalışmalarında viral enfeksiyonlar çok fazla araştırılmamış olduğundan, subklinik mastitislere karşı geliştirilen stratejiler yetersiz olabilir. Sığır Parainfluenza 3 (BPIV-3), Sığır papillomavirüsleri (BPV), sığır herpes virüsü 1 (BHV-1) klinik ve subklinik mastitlerde rol aldığı düşünülen viral ajanlardır. Bu çalışmada, Erzurum ilindeki subklinik mastitis olgularındaki BPIV-3, BPV ve BHV-1 viral ajanlarının varlığının ve prevalansının araştırılması amaçlanmıştır. Bu amaçla 120 büyükbaş hayvanın makroskobik olarak lezyon izlenmyen meme doku örnekleri toplandı ve bu dokular histopatoloji ve immünofloresan yöntemlerle incelendi. Ayrıca, meme dokularında belirlenen viral etkenlerin varlığı qRT-PCR ile araştırıldı. Toplanan 120 örneğe yapılan histopatolojik değerlendirme sonucunda (56 kronik mastit, 22 akut mastit) 78 örnekte yangısal değişikliklere rastlandı. Tüm dokulara uygulanan immünofloresan boyama sonuçlarına göre 120 olguda kronik mastitis tanısı konan 50 örnekte pozitif reaksiyonlara rastlandı. qRT-PCR sonuçlarına göre immunfloresan boyama sonuçlarıyla uyumlu olarak toplam 50 (%41.6) örnekte etkenlerin DNA varlıkları tespit edildi. BPIV-3 etkenine 26/120, BPV-2 etkenine 8/120, BHV-1 etkenine 16/120 oranında olduğu tespit edildi. Sadece qRT-PCR yöntemi kullanılarak 7/120 oranında BPV-1 etkenine rastlandı. Ayrıca, 5/120 oranında BPIV3 ve BHV-1, 3/120 oranında BPIV-3 ve BPV-1, 2/120 oranında ise BHV-1 ve BPV-2 etkenlerine birlikte rastlandı. Yapılan çalışmadan elde edilen sonuçlara göre, mastitis hastalığında viral ajanların rolü açık bir şekilde aydınlatılmamış olmakla birlikte, viral ajanların subklinik mastitli meme dokularında yaygın olduğunu tespit edilmiştir.

Anahtar Kelimeler: Subklinik mastitis, Sığır Parainfluenza 3, Sığır Papillomavirüsleri, Sığır Herpes virüsü 1, qRT-PCR, İmmünofloresan.

To cite this article: Altun S. Özdemir S. Sağlam Y.S. The Presence and Prevalence of Bovine Parainfluenza 3 (BPIV-3), Bovine Papillomaviruses (BPV), Bovine Herpesvirus 1 (BHV-1) in Subclinical Mastitis in Cattle. Kocatepe Vet J. (2019) 12(2):135-143

Submission: 02.11.2018 **Accepted**: 25.03.2019 **Published Online**: 15.05.2019
ORCID ID; SA: 0000-0002-8735-7031, SÖ: 0000-0001-7539-0523, YSS: 0000-0002-7861-9642
*Corresponding author e-mail: serdar.altun@atauni.edu.tr

INTRODUCTION

Mastitis is defined as an inflammatory response resulting in the infection of mammary tissue. Mastitis has been reported to occur in many mammals, especially in dairy cattle (Gomes and Henriques 2016). Mastitis in domestic dairy cattle causes economic losses. The economic problems caused by mastitis is related to direct and indirect losses. Direct losses are due to treatment costs, unaccountable milk, staff costs, deaths, and recurrence of mastitis. Indirect losses are caused by lower milk yield, lower milk quality, increased separation, decreased animal welfare and other health problems (Petrovski et al. 2006). The great majority of mastitis cases are bacterial and non-bacterial infections such as mycoplasms, fungi, yeasts, virus, and chlamydia (Wellenberg et al. 2002). Mastitis is divided into clinical and subclinical form. In clinical mastitis, the risk of contamination can be stopped to separate the animal from the herd, but the subclinical mastitis continues to flock because the animal does not show clinical symptoms, and mastitis causes the other animals to catch up. As a result, the economic losses is getting bigger (Bogni et al. 2011).

Although the bacterial pathogens are the focus of mastitis studies. There are many viral agents associated with bovine mastitis and which may cause or play important role in subclinical mastitis in dairy cattle (Wellenberg et al. 2002, Kalman et al. 2004). However, since viral infections have not been much investigated in mastitis studies, strategies developed against subclinical mastitis may be inadequate.

Bovine Herpesvirus (BHV-1) (Hage JJ et al. 1998), Bovine Parainfluenza 3 (BPIV-3) virus (Kawakami et al. 1966a,b), Bovine Papillomaviruses (Wellenberg et al. 2002) could cause or play a role in clinical and subclinical mastitis. BHV-1, a member of the *Herpesviridae* family, can lead to serious diseases such as infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious pustular balanoposthitis. Diseases caused by the virus include conjunctivitis, rhinotracheitis, abortion, encephalomyelitis, and mastitis (Underwood et al. 2015). Roberts et al. (1974) isolated BHV-1 from a cattle with mastitis in the USA. In addition, BHV-1 was identified in bovine milk with mastitis in combination with Mycoplasma species (Espinasse et al. 1974, Gourlay et al. 1974). Bilge (1998) reported that BHV-1 was found in one of the milk samples obtained from 96 cows with mastitis. BHV-1 leads to high milk cell counts even if there is no other pathogens that cause the mastitis (Siegler et al., 1984). In addition, it may cause the mastitis when the udder was infected with BHV1, and was isolated from mammary tissues with mastitis in cattle (Greig and Bannister 1965, Gourlay et al. 1974, Roberts et al. 1974).

BPIV-3 virus, a member of the *Paramyxoviridae* family, was isolated from Japanese cattle with nasal secretions and milk (Kawakami et al. 1966a). Furthermore, this virus was identified in milk of 14 of 58 cattle. Although the cattle which BPIV-3 virus was isolated from its milk did not observe any signs of clinical mastitis, somatic cell counts (SCCs) were increased in milk samples. Another study revealed that BPIV-3 virus was found in milk of cattle with typical aseptic mastitis (Kawakami et al. 1966b).

The Bovine Papilloma viruses (BPV) belong to the *Papillomaviridae* family (Olson 1990). BPV-1, BPV-2, and BPV-13 can infect and cause the development of tumors in cattle (Bocaneti et al. 2016). Different types of BPV have been identified into four genera, including Xipapillomavirus, Deltapapillomavirus, Epsilonpapillomavirus, and Dyoxipapillomavirus (Hamad et al. 2016, Lunardi et al. 2013). Since BPV can cause the fibropapillomas in the ductus papillaris, this virus could show a predisposition in cattle with mastitis (Francis 1984).

When literature review is conducted, it is revealed that there is not enough study which is performed on viral agents and mastitis/subclinical mastitis, and there is not enough information about the effect of viral agents on mastitis. From here, the aim of this study is to determine the presence and distribution of viral agents BPIV-3, BPV-1, BPV-2, and BHV-1, by using Immunofluorescence assay and qRT-PCR methods in the mammary tissue of cattle.

MATERIAL and METHOD

Material

The material of this study consisted of 120 mammary tissue samples (average 15 samples collected per week) obtained from slaughterhouses without any selection before slaughtering of animals in Erzurum Province in Turkey. Mammary tissue samples were collected randomly by using lancet without distinction or irrespective of whether it is inflammated or not to determine the presence of agents in cases of subclinical mastitis. Collected samples were brought to the laboratory for routine histopathology, Immunofluorescence assay, and total RNA isolation procedures.

Routine Histopathology

Each received mammary samples were stored for 1 day to be fixed in a 10% buffered formalin solution for histopathology and immunohistochemistry and half of each sample were stored -20 ° C for PCR. The routine histopathological process was performed in Shandon Citadel 2000 (USA) tissue system. After the routine histopathology process, all samples were poured into paraffin for blocking and prepared microtome sections in 5µm by using rotary microtome (Leica RM 2255). All microtome sections

were stained with hematoxylin-eosin (Presnell and Schreibman 1997).

Immunofluorescence Staining Method

After the routine histopathology process, 4 μm of paraffin sections were taken on to lysine-coated slides. These slides put in the oven for deparaffinization in 57° C for 1 hour. For indirect immunofluorescence staining, paraffin sections in 4 μm were placed on lysine-coated slides after primary antibody (Bovine Parainfluenza Virus Type 3 antibody, aPI116J10, BioX, 1/250 dilution; Anti-Bovine Papilloma Virus E2 antibody, ab77174, abcam, 1/250 dilution; BoHV-1 antibody, aIBR16L12, BioX, 1/250 dilution) application which was performed according the same test protocol of immunohistochemistry, 1\ 50 diluted seconder immunofluorescence antibody (Cat No: Goat Anti-Rabbit IgG H&L (FITC) dropped 12 μl by using micropipette to each slide and waited 45 minutes in the darkness. After standing in the dark, slides were washed with distilled water and covered with mounting medium (glycerol, 9 volumes; PBS 1 volume). All slides were examined in florescence microscope (Carl Zeiss Axio Skop A1 with Calibri 2 led fluorescence attachment).

Total RNA Isolation

Total RNA isolation was realized from the collected mammary tissue samples through the utilization of Trizol (Invitrogen, USA). Total RNA isolation was realized in line with the manufacturer's protocol. Following the total RNA isolation, the RNA concentration was measured by virtue of NanoDrop (Epoch Microplate Spectrophotometer, USA). RNAs were run in a 1.5% agarose gel in 1XTBE solution for one hour at 80 volts with a view to control total RNA quality and visualized by gel the imaging system and their RNA quality was determined.

DNase I treatment and cDNA Synthesis

DNase I (Thermo Scientific, USA) was performed against DNA contamination in isolated RNA samples. DNase I treatment was performed in line with the protocol provided in the kit. Subsequently, 1 μg was taken from these RNAs and cDNA was synthesized through utilization of the miScript Reverse Transcription Kit (Qiagen, Germany) in line with the protocol provided. The purity and quantity of the obtained cDNA was measured by virtue of spectrophotometer (Epoch Microplate Spectrophotometer, USA), and the cDNAs were diluted at the same ratios. Subsequently, the cDNA samples were stored at -20 ^{0}C for utilization in Real Time PCR studies.

Real time PCR

qRT-PCR was performed through utilization of the CFX96 BioRad device in order to detect BPIV-3, BHV-1, BPV-1, and BPV-2. The β-actin gene was employed for internal control. Master mix content created in real time PCR experiments is as follows: Syber Green 2X Rox Dye Master mix (Qiagen Germany), forward and reverse primers designed for genes, cDNAs as template and nuclease-free water. The samples were analyzed in Real-Time device following the preparation of master mixes. Reaction conditions and primer sequences of the genes are shown in Table 1. The primer sequences were received from previously conducted studies (Thonur et al. 2012, Pangty et al. 2010, Kubis et al. 2013).

Statistical analysis

IBM SPSS 20 program was performed for statistical analysis. The Cp values of each virus were evaluated using a linear mixed model (Thonur et al. 2012).

RESULTS

Histopathological evaluation

Histopathologic evaluation showed different character and severity of inflammatory changes in 78 of 120 cases. 56/120 samples were diagnosed with chronic subclinic mastitis and 22/120 cases were diagnosed with acute mastitis. In chronic subclinical cases, mononuclear cell infiltration in interstitial tissue and interalveolar septum thickness due to increased connective tissue were observed (Fig.1A, 1B). In acute cases, the presence of exudates containing neutrophil leukocytes in alveoli of mammary gland was observed. Degeneration and necrosis of alveolar epithelial layer, desquamation of gland epithelium were observed as common findings. Nonspecific chronic inflammatory cells such as lymphocyte and macrophage infiltrations (Fig. 1A, 1B, 1C) were detected in histopathological examination of BPIV-3 and BPV positive samples. Mononuclear cells accumulation, inclusion bodies in the cytoplasm of epithelial cells (Fig. 1C) were observed in BHV-1 positive tissues.

Immunofluorescence staining results

In the immunofluorescence staining, positive reactions for viral agents were observed in mammary gland epithelium, especially in mononuclear cells in the region (Figs. 1D, 1E, 1F). According to the results, the fluorescent positive reactions were detected in totally 50 cases with chronic (subclincal) mastitis (26/120 for BPIV-3, 8/120 for BPV-2 and 16/120 for BHV-1).

Integrity of material

A β-actin signal was observed in all mammary samples tested in the qRT-qPCR indicating no evidence of extraction failure or PCR inhibition. (Fig.2)

Real-Time PCR Results

A ten-fold serial dilution of each of the in vitro transcribed RNAs of BPIV-3, BHV-1, BPV-1, and

BPV-2 was triplicate analysis. The standard curves identified with qRT-PCR are shown in Figs. 3A, 3B, and 3C. All four targets were analyzed simultaneously and no evidence of cross reactivity between primers was detected. BPIV-3 agent was detected by the qRT-PCR in 26/120 samples, BHV-1 agent in 16/120 samples, BPV-1 agent in 7/120 samples, and BPV-2 agent in 8/120 samples. Coinfection with BPIV-3 and BHV-1 was detected in 5/120 samples, BPIV-3, and BPV-1 in 3/120 samples, BHV-1 and BPV-1 in 2/120 samples (Table 2). BPIV-3, BHV-1, BPV-1, and BPV-2 nucleic acid signals were shown in Figs. 4A, 4B, 4C, and 4D.

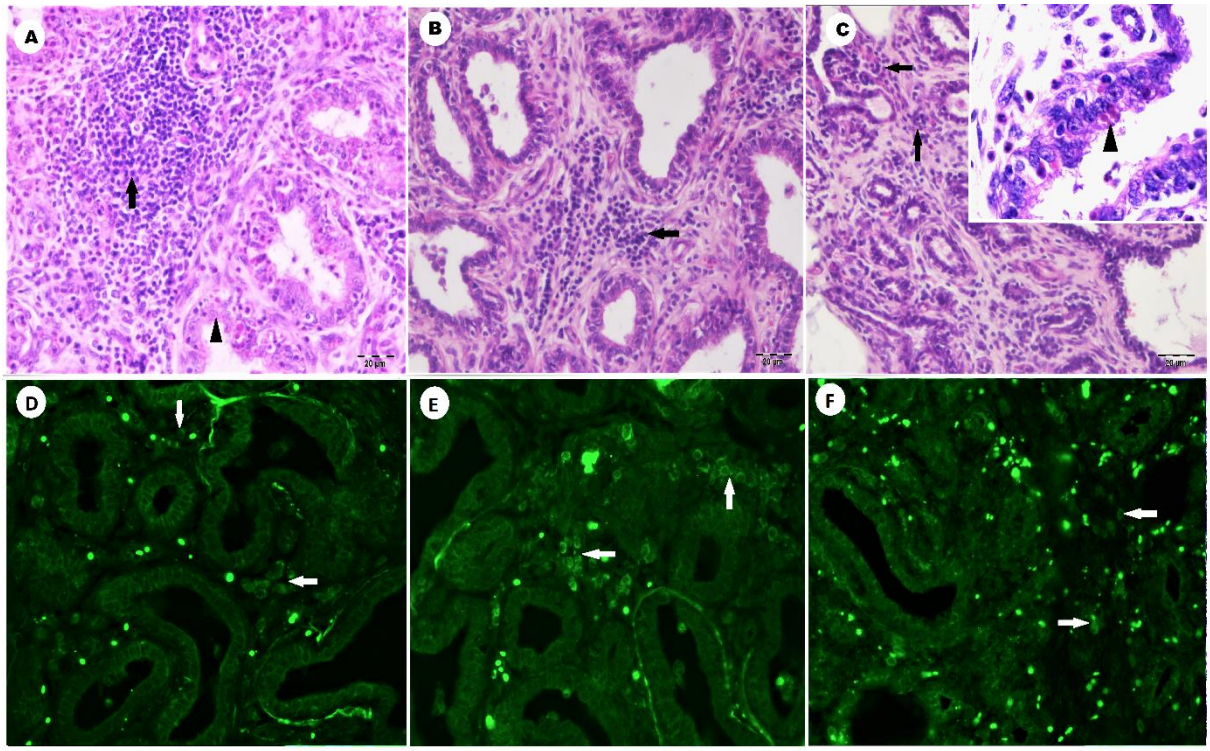


Figure 1. Histopathological and Immunofluorescence staining results A) Mononuclear cell infiltrations in the interstitial space (arrow), Degeneration and necrosis in the gland epithelium (arrowhead). BPIV-3. H & E. 20 µm. B) Mononuclear cell infiltrations (arrow), BPV-1. H & E. 20 µm. C) Plasma cell accumulation (arrows), intracytoplasmic eosinophilic inclusion bodies (arrowhead). BHV-1. H & E. 20 µm. D) Positivity in mononuclear cells of BPIV-3 (arrows). IF. 20 µm E) Positivity (arrows) in mononuclear cells of BPV-1 agent, IF. 20 µm. F) Positive reaction for BHV-1 agent (arrows) in mononuclear cells. IF. 20 µm.

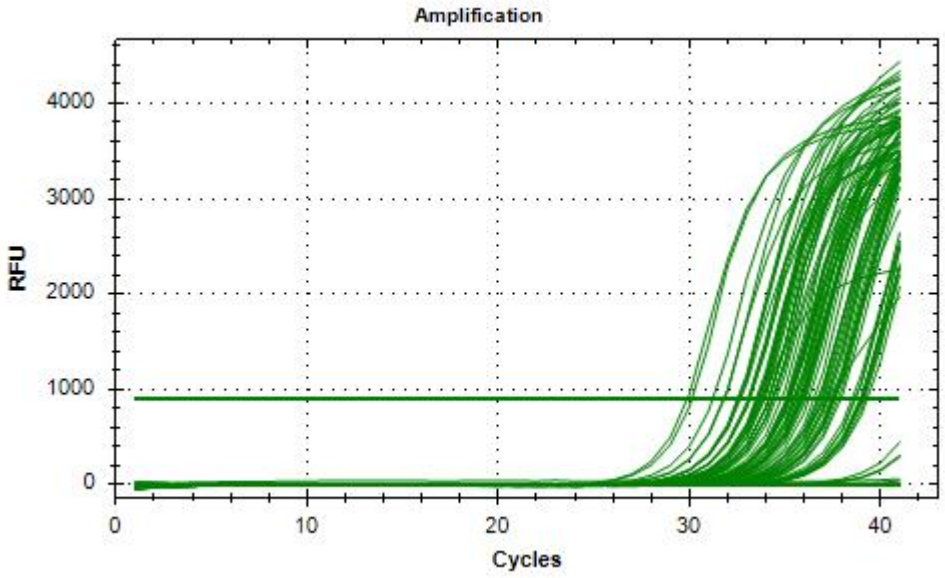


Figure 2. β-actin signals for all tissues

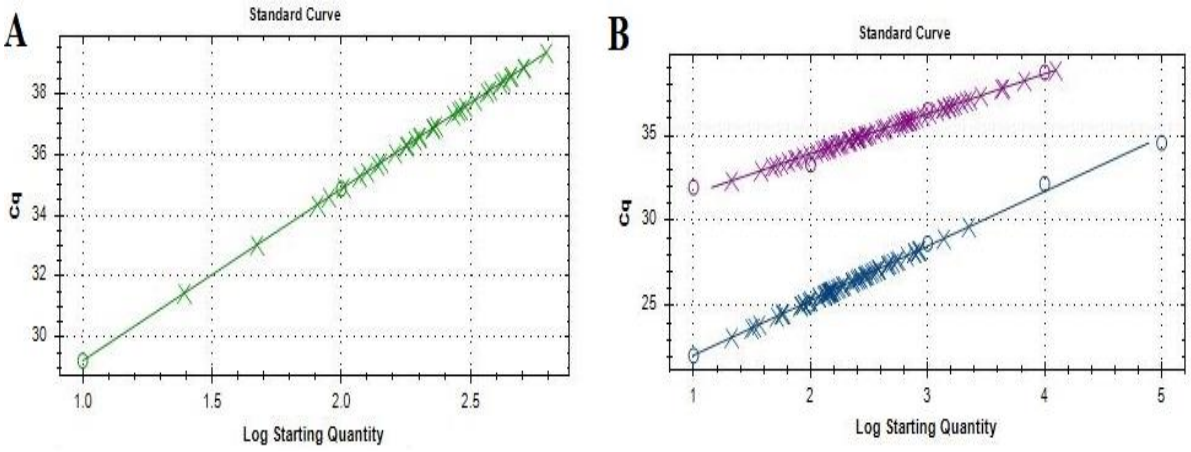


Figure 3. The standard curves for each virus. A) Standard curves of BPIV-3. B) Standard curves of BHV-1 and BPV-1 (purple; BHV-1, dark blue; BPV-1).

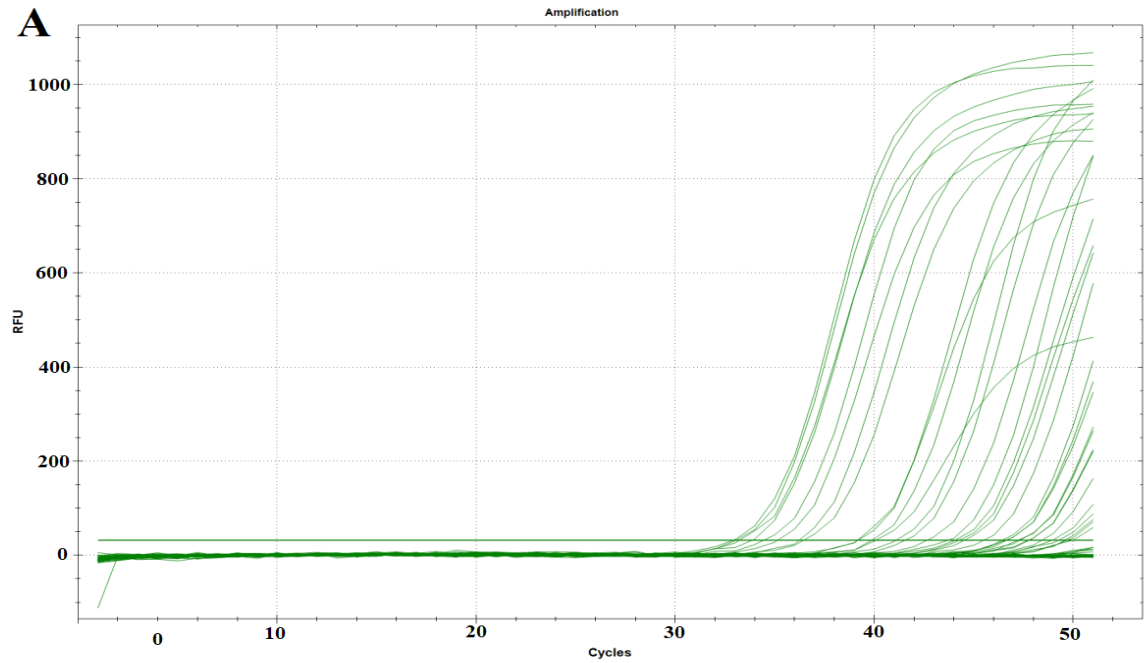


Figure 4. BPIV-3, BHV-1, BPV-1 and BPV-2 nucleic acid signals. A) BPIV-3**.**

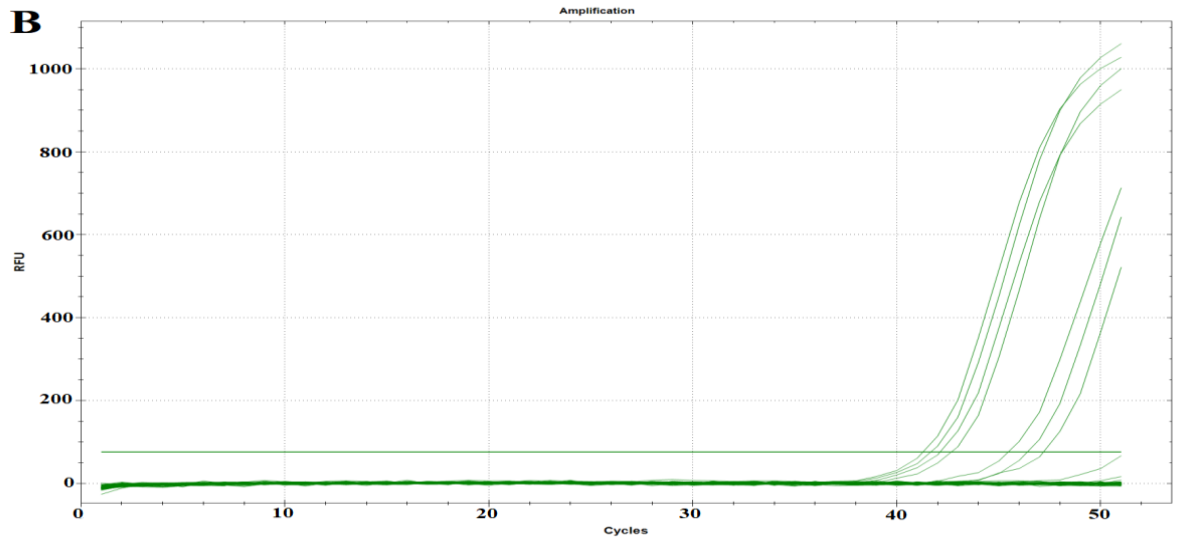


Figure 4. BPIV-3, BHV-1, BPV-1 and BPV-2 nucleic acid signals. B) BPV-1.

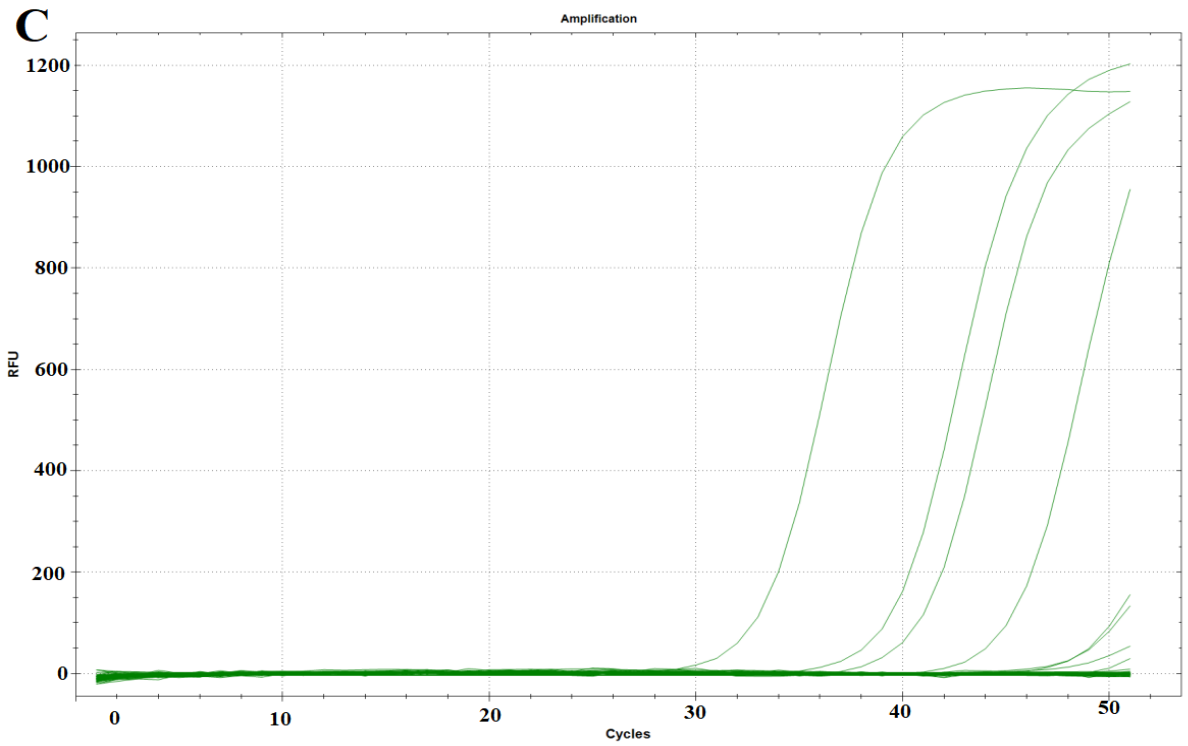


Figure 4. BPIV-3, BHV-1, BPV-1 and BPV-2 nucleic acid signals. C) BPV-2**.**

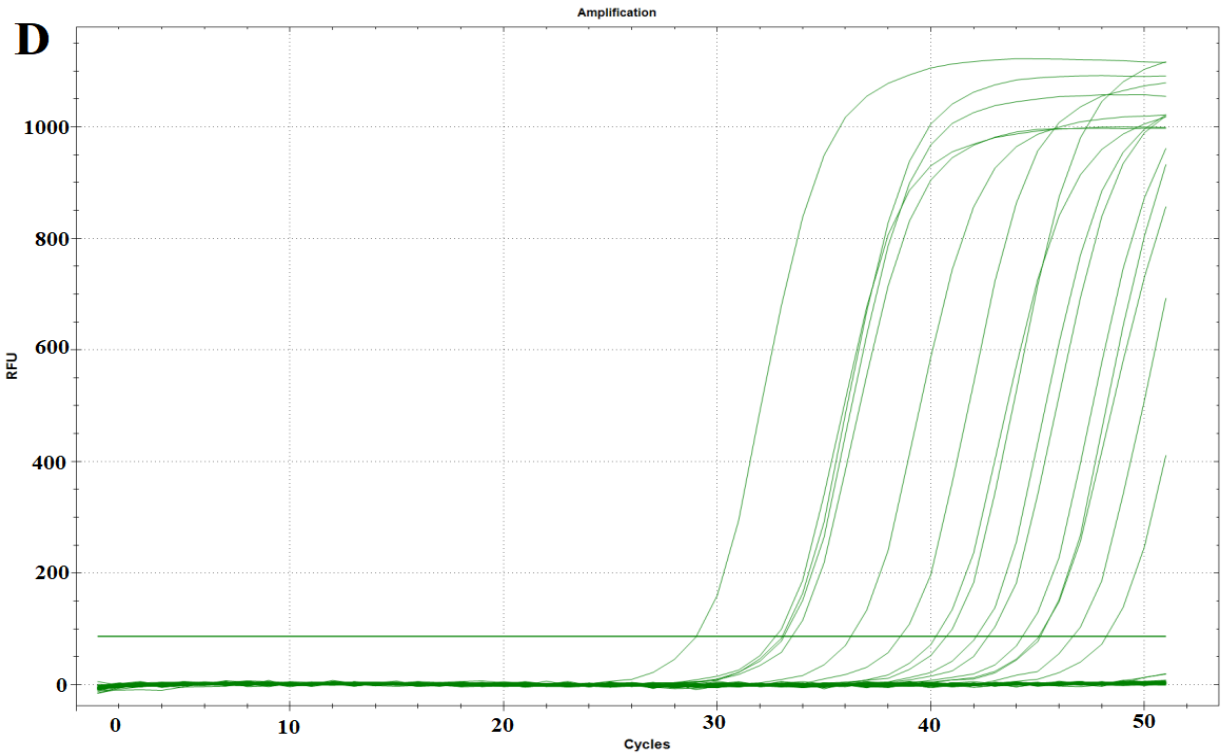


Figure 4. BPIV-3, BHV-1, BPV-1 and BPV-2 nucleic acid signals. D) BHV-1

Table 1. Primer sequences of BPIV-3, β-actin, BPV-1, BPV-2, and BHV1

Gen Name	Primer sequences	Reaction Conditions	References
BPIV-3	F:TGATTGGATGTTCGGGAGTGA R:AGAATCCTTTCCTCAATCCTGATATACT	94 ^{0}C for 15 s, 58 ^{0}C 30 s / 72 ^{0}C 30 s. (40 cycle)	Thonur et al. 2012
β-actin	F: GACAGGATGCAGAARGAGATCAC R: TCCACATCTGCTGGAAGGTG	94 ^{0}C for 15 s, 55 ^{0}C 30 s / 72 ^{0}C 30 s. (40 cycle)	Thonur et al. 2012
BPV-1	F: GGA GCG CCT GCT AAC TAT AGG A R: ATC TGT TGT TTG GGT GGT GAC	94 ^{0}C for 15 s, 57 ^{0}C 30 s / 72 ^{0}C 30 s. (40 cycle)	Pangty et al. 2010
BPV-2	F: GTT ATA CCA CCC AAA GAA GAC CCT R: CTG GTT GCA ACA GCT CTC TTT CTC	94 ^{0}C for 15 s, 57 ^{0}C 30 s / 72 ^{0}C 30 s. (40 cycle)	Pangty et al. 2010
BHV-1	F: TGTGGACCTAAACCTCACGGT R: GTAGTCGAGCAGACCCGTGTC	94 ^{0}C for 15 s, 59 ^{0}C 30 s / 72 ^{0}C 30 s. (40 cycle)	Thonur et al. 2012

Table 2. Real time RT-PCR results for 120 samples

Material	BPIV-3	BHV-1	BPV-1	BPV-2	BPIV-3 and BHV-1	BPIV-3 and BPV-1	BHV-1 and BPV-1
Mammary tissue	26	16	7	8	5	3	2
Percentage (%)	21.6%	13.3%	5.8%	6.6%	4.16%	2.5%	1.6%

DISCUSSION

Bovine mastitis disease causes important economic losses all over the World. Although, the number of studies on the presence and role of viral agents in mastitis cases is very low. BPIV-3, BHV-1, BPV-1, and BPV-2 are the most important agents among viral pathogens shown to be responsible for mastitis cases. Besides the severity of disease increases by various factors like stress, environmental conditions, immunodeficiency (Wellenberg et al. 2002, Underwood WJ et al. 2015).

Mastitis cases are among the common herd health problems in our country as in all over the world. Mastitis has been reported to occur in many mammalian species, especially in domestic dairy cows (Petrovski et al. 2006, Gomes and Henriques 2016). In the previous studies, the presence and the role of bacterial agents in mastitis cases are emphasized (Bogni et al. 2011, Gomes and Henriques 2016). However, no detailed studies have been found in the presence of viral agents that play an important role in diseases such as other respiratory system diseases.

Foot and Mouth Disease Virus (FMDV), Bovine Herpesvirus 4 (BHV-4), Bovine Herpesvirus 2 (BHV-2), cattle Pox Virus, Bovine Viral Diarrhea Virus (BVDV), Vesicular Stomatitis, Bovine Herpes Virus 1 (IBR / IPV), Bovine Herpesvirus-3 (BHV-3), Coryza Gangrenosa Bovum (CGB), Bovine Enterovirus (BEV), Bovine Parainfluenza 3 (BPIV-3), Bovine leukosis were reported as examples of viral infections that cause mastitis in cattle or that are involved in mastitis (Molen et al. 1985, Reid et al. 2006). In our country, except for some seroprevalence reports (Alkan et al. 1997, Yesilbag and Güngör 2008), no study was found in this scope.

In the previous study by Kawakami et al. (1966a), BPIV-3 was detected in the nasal fluid of Japanese cows with respiratory diseases and milk and mammary tissues of mastitis cows. In cases of mastitis caused by BPIV-3, there is fever and weight loss in cows. In addition, BPIV-3 is seen in both clinical and subclinical mastitis (Kawakami et al. 1966b, Wellenberg et al. 2002). In the studies, it has been shown that this virus causes mastitis in the cows with the bactericidal factors that cause mastitis (William et al. 1992, Wellenberg et al. 2002).

BHV-1 usually infects mammary tissue in young cows during the first lactation period. This infection may be sub-clinical or clinical. BHV-1 infections are particularly damaging to the ductus papillaris and sinus lectoris (Senft and Neudecker 1991). This situation makes the mastitis cases caused by bacteria more harmful. BHV-1 can be found in the milk of cows with mastitis (Wellenberg et al. 2002, Kubis et al. 2013). Intramammary inoculation of BHV-1 induce the signs of clinical mastitis. Besides, a significant decrease in milk production was recorded, and milk samples showed intense consistency after the intramammary inoculation of cows with the BHV-1 (Greig and Bannister 1965).

In an experimental study based on an outbreak of BHV-1 for 98 lactating animals was induced by injecting three seropositive cows with dexamethasone. They have reported that a significant decrease in milk production in initially-seronegative

cows that became infected but not in seropositive cows (Hage JJ et al. 1998)

Turner et al. (1976) reported that they isolated BHV-2 from vesicular fluid and swab samples from the mammary of 31 dairy cows. In herpes mammillitis cases in Canada, it was determined that the samples collected from the lesions were determined by virus isolation and electron microscopy and mastitis rate increased in the herd. As a result, BHV-2 infections are thought to increase the sensitivity of the mammary due to damage to the natural defense mechanism and to increase the sensitivity to bacterial infections (Martin et al. 1987).

In the present study, BPIV-3 agent was detected by the qRT-PCR in 26/120 samples, BHV-1 agent in 16/120 samples, BPV-1 agent in 7/120 samples, and BPV-2 agent in 8/120 samples. Coinfection with BPIV-3 and BHV-1 was detected in 5/120 samples, BPIV-3, and BPV-1 in 3/120 samples, BHV-1 and BPV-1 in 2/120 samples. Degeneration and necrosis of alveolar epithelial layer, desquamation of gland epithelium were observed as common findings. Nonspecific chronic inflammatory cells such as lymphocyte and macrophage infiltrations were detected in histopathological examination of BPIV-3 and BPV positive samples. infiltration of mononuclear cells, inclusion bodies in the cytoplasm of epithelial cells were observed in BHV-1 positive tissues. In the immunofluorescence staining, positive reactions for viral agents were observed in mammary gland epithelium, especially in mononuclear cells in the region. According to the results, the fluorescent positive reactions were detected 26/120 for BPIV-3, 16/120 for BHV-1 and 8/120 for BPV-2.

In accordance with the information in the literature, similar to the findings of nonspecific histopathological examinations observed in other tissues and organs infected with viral agents, histopathological examination of the mammary tissues revealed no specific findings other than the infiltration of chronic inflammatory cell formation. Especially the subclinical form of mastitis is high in clinical mastitis form, subclinical mastitis cannot be noticed by the breeders, the disease is a herd problem, the disease resistance to drug treatment reveals the importance of the disease (Sharma et al. 2006, Sandev et al. 2004).

According the literature, viruses induce reduction of the natural defense mechanisms of the udder and immune system deficiency like by causing teat lesions (Francis 1984, Wellenberg et al. 2002, Underwood WJ et al. 2015). Our results demonstrate that BPIV-3, BPV, and BHV-1 neither cause mastitis directly nor play a pivotal role in clinical mastitis, because of isolation of bacterial agent was not performed. But obtained findings like intracytoplasmic inclusion bodies in epithelial cells enfected with BHV-1 and positive immune signs of viral agents in the cytoplasm of the inflammatory cells in mammary tissues, made us think that viral agents may be responsible for clinical mastitis turn into subclinical mastitis or prolonged the inflammation after or before of the bacterial infection of the mammary glands occurred.

CONCLUSION

In conclusion, our findings demonstrate a high prevalence of viral agents in cattle mammary tissue in the east of Turkey. Suitable environment conditions for the animals waiting to be able to create an infectious disease in the normal flora and subclinical infected cows of the factors shows that the continuity of this threat. There is a critical need for preventative strategies in the farm for viral pathogens like rapid elimination of subclinically virus carrier animals from herd via periodically screening for viral disease in terms of subclinical mastitis.

ACKNOWLEDGEMENT

This study was supported by the Scientific Research Projects Coordination Unit of Atatürk University (PRJ2016 / 87)

REFERENCES

Alkan F, Ozkul A, Karaoğlu MT, Bilge S, Akça Y, Burgu I, Yesilbag K, Oguzoglu TC. Sığırlarda viral nedenli solunum sistemi enfeksiyonlarının epidemiyolojisi. Ankara Univ. Vet. Fak. Derg. 1997; 44: 1-8.

Bilge S. Detection of antibodies of IBR-IPV infection in blood and milk by serum neutralization test and virus isolation from milk samples in dairy cows. Ankara Univ. Vet. Fak. Derg. 1998; 45: 313–321.

Bocaneti F, Altamura G, Corteggio A, Velescu E, Roperto F, Borzacchiello G. Bovine papillomavirus: New insights into an old disease. Transbound Emerg Dis. 2016; 63(1): 14–23.

Bogni C, Odierno L, Raspanti C, Giraudo J, Larriestra A, Reinoso, E, Lasagno M, Ferrari M, Ducro´s E, Frigerio C, Bettera S, Pellegrino, M, Frola I, Dieser S, Vissio C. War against mastitis: current concepts on controlling bovine mastitis Pathogens. In: Me´ndez-Vilas A (ed) Science against microbial pathogens: communicating current research and technological advances. World Scientific, Singapore. 2011; 483–494.

Espinasse J, Gilbert Y, Saurat P. Features of bovine rhinotracheitis in a dairy herd in south-western France. Rev. Med. Vet. 1974; 125: 1441–1452.

Francis PG. Teat skin lesions and mastitis. Br. Vet. J. 1984; 140: 430–436.

Gomes F, Henriques M. Control of Bovine Mastitis: Old and Recent Therapeutic Approaches", Curr Microbiol. 2016;72(4): 377-82.

Gourlay RN, Stott EJ, Espinasse J, Barle C. Isolation of Mycoplasma agalactiae var. bovis and infectious bovine rhinotracheitis virus from an outbreak of mastitis in France. Vet. Rec. 1974; 95: 534–535.

Greig AS, Bannister GL. Infection of the bovine udder with bovine herpesvirus. Can. J. Comp. Med. Vet. Sci. 1965; 29: pp. 57-62.

Hage JJ, Schukkenb YH, Dijkstraa TH, Barkemaa HW, van Valkengoedb PHR, Wentink GH. Milk production and reproduction during a subclinical bovine herpesvirus 1 infection on a dairy farm. Prev Vet Med. 1998; 34: 97-106.

Hamad MA, Al-Shammari AM, Odisho SM, Yaseen NY. Molecular and Phylogenetic Analysis of Bovine Papillomavirus Type 1: First Report in Iraqi Cattle. Adv Virol. 2016; 2143024.

Kálmán D, Jánosi S, Egyed L. Role of bovine herpesvirus 4 in bacterial bovine mastitis. Microb Pathog. 2004; 37: 125-129.

Kawakami Y, Kaji T, Kume T, Omuro M, Hiramune T, Murase N, Matumoto M. Infection of cattle with parainfluenza 3 virus with special reference to udder infection. I. Virus isolation from milk. Jpn. J. Microbiol. 1966a; 10: 159–169.

Kawakami Y, Kaji T, Omuro M, Maruyama Y, Hiramune T, Murase N, Matumoto M. Infection of cattle with parainfluenza 3 virus with special reference to udder infection. II. Pathology of the virus to cattle, with particular reference to the mammary gland. Jpn. J. Microbiol. 1966b; 10: 171–182.

Kubis P, Materniak M, Kuzmak J. Comparison of nested PCR and qPCR for the detection and quantitation of BoHV6 DNA. J Virol Methods. 2013; 194: 94–101.

Lunardi M, Alfieri AA, Otonel RAA, de Alcântara BK, Rodrigues WB, de Miranda AB. Genetic characterisation of a novel bovine papillomavirus member of the Deltapapillomavirus genus. Vet Microbiol. 2013; 162(1): 207–213.

Martin JR, Harvey D, Montpetit C. Bovine herpetic mammillitis in Quebec. Can Vet. J. 1987; 28: 529-523.

Molen EJ, Vecht U, Houwers DJ. A chronic indurative mastitis in sheep, associated with maedi/visna virus infection. Vet Q. 1985; 7:(2) 112-119.

Olson C. Papillomaviruses. In: Dinter, Z., Morein, B. (Eds.), Virus Infections of Ruminants. Elsevier, Amsterdam. 1990; 189–200.

Pangty K, Singh S, Goswami R, Saikumar G, Somvanshi R. Detection of BPV-1 and -2 and Quantification of BPV-1 by Real-Time PCR in Cutaneous Warts in Cattle and Buffaloes. Transbound Emerg Dis. 2010; 57: 185–196.

Petrovski KR, Trajcev M, Buneski G. A review of the factors affecting the costs of bovine mastitis", J South Afr Vet Assoc. 2006; 77: 52–60.

Presnell J, Schreibman MP. Animal Tissue Techniques. 5th ed. London. The Johns Hopkins University Press Ltd, 1997; 269-271.

Reid SM, Parida S, King DP, Hutchings GH, Shaw AE, Ferris NP, Zhang Z, Hillerton JE, Paton DJ. Utility of automated real-time RT-PCR for the detection of foot-and-mouth disease virus excreted in milk. Vet Res. 2006; 37: 121-32.

Roberts AW, Carter GR, Carter FA. Infectious bovine rhinotracheitis virus recovered from milk of a cow with mastitis. J. Am. Vet. Med. Assoc. 1974; 164: 413.

Sandev N, Koleva M, Bınev R, Ilieva D. Influence of enzootic leukosis virus upon the incidence of subclinical mastitis in cows at a different stage of infection. Vet. Arhiv. 2004; 74: 411-416.

Senft B, Neudecker J. Abwehrmechanismen der bovinen Milchdru¨se. Tiera¨rztl. Praxis. 1991; 19: 357–363.

Siegler HH, Marschang F, Morscher H. Beobachtungen u¨ber Zusammenha¨nge zwischen Virusinfectionen und boviner Mastitis. Tiera¨rztl. Umschau. 1984; 39: 602–604.

Sharma A, Dhingra P, Pander BL, Kumar R. Bovine subclinical mastitis: prevalence and treatment with homeopathic medicine. Intl. J. Cow Sci. 2006; 2: 40-44.

Turner AJ, Morgan IR, Sykes WE, Nicholls WA. Bovine herpes mammillitis of dairy cattle in Victoria. Aust Vet J. 1976 Apr; 52(4):170-3.

Thonur L, Maley M, Gilray J, Crook T, Laming E, Turnbull D, Nath M, Willoughby K. One-step multiplex real time RT-PCR for the detection of bovine respiratory syncytial virus, bovine herpesvirus 1 and bovine parainfluenza virus 3. BMC Vet Res. 2012; 8: 37.

Underwood WJ, Blauwiekel R, Schoell A. Biology and Diseases of Ruminants (Sheep, Goats, and Cattle), In: Laboratory Animal Medicine. Third Edition, 2015 Elsevier Inc. 2015; pp. 623-694.

Yesilbag K, Güngör B. Seroprevalance of bovine respiratoty viruse in orth-Western Turkey. Trop. Anim. Health. Prod. 2008; 40: 55-60.

Wellenberg GJ, van der Poelb WHM, Van Oirschota JT. Viral infections and bovine mastitis: a review. Vet Microbiol. 2002; 88: 27–45.

William JB, Kirubaharan JJ, Uthuman KM, Kumanan K, Balachandran S. Survey on incidence and complications of bovine cutaneous papillomatosis. Indian Vet. J. 1992; 69: 843–844.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):144-149
DOI: 10.30607/kvj.505685

RESEARCH ARTICLE

Antibiotic Resistance To *Campylobacter* spp. Isolated from The Livers of Slaughtered Ruminants and Aborted Ovine Fetuses

Beytullah KENAR[1]*, Mustafa GÖKÇE[2]

[1]Afyon Kocatepe Üniversitesi, Veteriner Fakültesi, Mikrobiyoloji Anabilim Dalı, ANS Kampüsü.
[2]Kütahya İl Gıda Tarım ve Hayvancılık Müdürlüğü, Tavşanlı İlçe Müdürlüğü

ABSTRACT

The presence of *Campylobacter* spp. obtained from clinically healthy 50 sheep, goats and cattle slaughtered in slaughterhouses in Afyonkarahisar and Kutahya provinces and 44 aborted ovine fetuses obtained from the same region was investigated in this study. The subtypes were isolated by culture methods and identified by API Campy (Biomerieux, France) test kits. *Campylobacter* spp. was isolated from 7 samples (15.91%) out of 44 aborted ovine fetuses. After identification of *Campylobacter* spp., it was determined that 5 samples (71.43%) out of 7 were *C. fetus subsp. fetus* (71.43%) and 2 samples were *C. jejuni* (28.57%). Out of the 50 liver samples, *Campylobacter* spp. was isolated from 3 ovine livers (6%) and 1 goat liver (2%). Three of the isolates were identified as *C. jejuni* (75%) and 1 isolate as *C. coli* (25%). *Campylobacter* spp. was not isolated from cattle livers. Resistance rates for ciprofloxacin and tetracycline were 45.5% and 27.3% for erythromycin and 9.1% for ampicillin. No resistance was determined against gentamycin, chloramphenicol and streptomycin. The rate of susceptibility to antibiotics used was 72.7% for ampicillin, 54.5% for erythromycin, 90.9% for gentamycin, 100% for chloramphenicol, 72.7% for streptomycin and 27.3 % for ciprofloxacin and tetracycline.

Keywords: Antibiotic resistance, *Campylobacter* spp., Ovine fetus, Ruminant liver.

***

Mezbahalarda Kesilen Ruminant Karaciğerlerinden ve Koyun Abortuslarından İzole Edilen Campylobacter Türlerine Karşı Antibiyotik Dirençliliği

ÖZ

Bu çalışmada, Afyonkarahisar ve Kütahya illerinden mezbahalarda sağlıklı olarak kesilen koyun, keçi ve sığırlardan alınan 50'şer adet karaciğer örneği ile yine aynı bölgeden temin edilen 44 aborte koyun fetüsünde, *Campylobacter* spp. varlığı arandı. Kültür tekniğiyle izole edilen suşlar, API Campy (Biomerieux, France) test kitleriyle identifiye edildi. İncelenen 44 aborte koyun fetüsünden, 7 adet (%15,91) *Campylobacter* spp. izole edildi. İdentifikasyonları yapıldığında, örneklerin 5'inin *C. fetus subsp. fetus* (%71,43), 2'sinin *C. jejuni* (%28,57) olduğu tespit edildi. Ellişer karaciğer örneğinden, 3 koyun karaciğerinde (%6) ve 1 keçi karaciğerinde (%2) *Campylobacter* spp. izole edildi. İzolatların 3'ünün *C. jejuni* (%75), 1'inin *C. coli* (%25) olduğu tespit edildi. Sığır karaciğerlerinden ise *Campylobacter* spp. izole edilemedi. Elde edilen 11 adet *Campylobacter* izolatının analizinde siprofloksasin ve tetrasikline %45,5, eritromisine %27,3, ampisiline %9,1 oranında dirençlilik tespit edildi. Gentamisin, kloramfenikol ve streptomisine karşı ise dirençlilik gözlenmedi. Kullanılan antibiyotikler için tespit edilen duyarlılık oranları, ampisiline %72,7, eritromisine %54,5, gentamisine %90,9, kloramfenikole %100, streptomisine %72.7, siprofloksasin ve tetrasikline %27,3 olarak bulundu.

Anahtar Kelimeler: Antibiyotik direnci, *Campylobacter* spp., Koyun fetüs, Ruminant karaciğer

To cite this article: Kenar B. Gökçe M. Antibiotic Resistance To Campylobacter spp. Isolated from The Livers of Slaughtered Ruminants and Aborted Ovine Fetuses. Kocatepe Vet J. (2019) 12(2):144-149.

Submission: 31.12.2018 **Accepted**: 30.04.2019 **Published Online**: 15.05.2019
ORCID ID; BK: 0000-0001-6573-680X, MG: 0000-0001-8299-4325
*Corresponding author e-mail: bkenar@aku.edu.tr

INTRODUCTION

Campylobacter species are pathogenic microorganisms for animals and humans which can be commensal in the intestinal flora of various domestic and wild animals and can cause gastrointestinal and genital infections (Songer and Post, 2005).

Generally, the optimal growing temperature is 37°C. However, while thermophilic ones reproduce optimally at 42-43°C, they can also reproduce at 37°C. *C. jejuni, C. coli, C. lari* species are defined as thermophilic species. *Campylobacter* species are microaerophilic. Media containing 5% O_2, 10% CO_2 and 85% N_2 (Moore et al. 2005) is required for optimal reproduction.

Heat-sensitive *Campylobacter* species are inactivated at 60°C in solid and liquid foods in a short time. It has been reported that the *Campylobacter* species is inactivated under standard conditions in chlorinated drinking water and pasteurized milk (Obiri-Danso et al. 2001).

It was observed that *C. jejuni* which has been in the water for a long time passes into the coccoid form and enters into the stagnation phase and that it was not possible to cultivate it by using non-selective media in this phase. It was reported that during this time it is alive but transposed into the coccoid form that cannot be cultured and this form is described as viable but non-culturable (VBNC) (Rolling and Colwell, 1986, Jones et al. 1991, Purdy et al. 1999).
All domestic animals, humans and mammals as well as poultry are hosts. They have no specificity according to animal species. The most common form of a *Campylobacter* infection is enteritis. The course of the disease may range from a mild short course of enteritis to bloody ulcerative colitis. (Aydın et al. 2006).

Campylobacter species are generally susceptible to macrolides and quinolone group antibiotics and have been reported to be resistant to trimethoprim with cephalosporin group antibiotics such as cephalothin, cefoperazone, cefazolin. *C. lari* strains are resistant to nalidixic acid and are used to diagnose and differentiate it from other thermophilic species (Winn et al. 2006).

The antibiotic resistance observed in *Campylobacter* species develops through mutations in chromosomes and from other bacteria through plasmid or transposon transfer (Taylor and Courvalin, 1988).

Campylobacter species is a zoonosis and therefore it is epidemiologically closely related to food and public health and animal health. *Campylobacter fetus subsp. fetus* is epidemic in sheep abortus, causes sporadic abortion in cattle and sporadic infections in other animals. The agent can be active in up to 50% of the intestines and gall bladders of healthy cattle and sheep. During abortion cases, the rate of the agent in stool increases. Feces of infected animals spread into the environment through aborted fetuses and genital discharge (Aydın et al. 2006). Venereal infection is observed in cattle. Following the infection, sheep gain immunity that lasts 2-3 years. Epidemics show a recurrent periodic temporal distribution every 2-3 years (Ülgen 2002).

Campylobacter fetus subsp. venerealis causes abortion and infertility with venereal infection specific to cattle. The agent can be isolated from the distal urethra and glans penis of infected bulls. Accumulation in the female genital tract results in the ascending colonization towards the fallopian tubes. This usually occurs in the form of infertility and abortus occurs in less than 10% of infected cows (Songer and Post, 2005).

Infectious diseases causing abortion in sheep; Brucellosis, Campylobacteriosis, Salmonellosis, Chlamydiosis, Listeriosis, Leptospirosis, Toxoplasmosis and Aspergillosis (Kenar et al. 1990).

Ovine campylobacteriosis is a disease, which can cause severe economic losses due to abortions (Diker and Istanbulluoglu, 1986). The Epidemiology of sheep abortion due to Campylobacter species is similar to other sheep abortion infections (Ülgen 2002, Aydın et al. 2006, Batmaz 2013).

More precise and faster techniques have been developed in order to identify and determine the genetic characteristics of the *Campylobacter* species. In addition to biochemical tests, techniques such as Polymerase Chain Reaction (PCR), Immunomagnetic Separation (IMS), API Campy Test Kit, Polymorphic DNA (RAPD), Pulsed-Field Gel Electrophoresis (PFGE) are being used. Thus, in addition to time savings, identifications can be made more precisely (Lamoureux et al. 1997, Shih 2000, Madigan et al. 2003).

The objective of this study was to investigate the presence of *Campylobacter* species which is an important pathogen in terms of animal and public health, in aborted fetuses and sheep, goat and bovine livers and determine the antibiotic resistance of the obtained strains.

MATERIAL and METHOD

The study was carried out on the livers of sheep, goats, cattle slaughtered in slaughterhouses in Afyonkarahisar and Kütahya provinces in Midwest Anatolia and the fetuses from aborted sheep. 10 ml of the abomasum content of 44 sheep fetuses freshly supplied from abortus cases incurring in April 2015 -

May 2016 in the said environment was obtained by means of a sterile injector from each fetus. In the period of January 2016 - May 2016, 50 sheep, goat and bovine livers from healthy animals slaughtered in slaughterhouses were collected by transferring them to sterile specimen containers with the help of a sterile scalpel and scissors. The samples were transferred to the laboratory under a cold chain. Forty-four fetuses were collected from 38 sheep farms and a total of 194 samples including 50 liver samples from sheep, goats and cattle were used.

The isolation of *Campylobacter* species was carried out by using the following media and supplements: Nutrient Broth No. 2 (Oxoid CM 0067), Selective Supplement (Oxoid SR 048). Charcoal Cefaperazone Deoxycholate Agar (CCDA), (Oxoid CM 0739), CCDA Selective Supplement (Oxoid SR0155E).

The identification of *Campylobacter* species was carried out by using the following tests: Oxidase Test, Oxidase Identification Sticks (Oxoid BR 064), Catalase Test, 3% hydrogen peroxide (Merck 8597) and hippurate hydrolysis test (Merck-8.20648.0025), API Campy Test.

The tests were carried out according to the manufacturer's instructions. Isolated *Campylobacter* spp were cultivated on 5-7% sheep blood Mueller – Hinton Agar (Oxoid CM0337) media and disks with Ampicillin (Oxoid CT003B) 10 μg, Erythromycin (Oxoid CT0020B) 15 μg, Gentamicin (Oxoid CT0024B) 10 μg, Chloramphenicol (Oxoid CT0013B) 30 μg, Streptomycin (Oxoid CT0047B) 10 μg, Ciprofloxacin (Oxoid CT0425B) 5 μg, Tetracycline (Oxoid CT0054B) 30 μg were placed on the agar (Arda et al. 1987).

The samples were taken in sterile containers to the laboratory under a cold chain and isolation and identification procedures were started on their delivery date. The medium containing 5% O2, 10% CO2 and 85% N2 was kept at 37°C for 3-7 days. Their proliferation was checked. The *Campylobacter* species were left to incubate at 37°C for 48-72 hours in a microaerophilic medium.

Antibiotic susceptibility was assessed according to Clinical and Laboratory Standards Institute (CLSI) Performance standards for antimicrobial susceptibility testing (2012).

RESULTS

Campylobacter spp. was determined in 11 (5.67%) out of the total 194 samples. *Campylobacter* spp. was determined in 7 of the examined 44 lamb abomasum (15.91%), in 3 of the examined 50 sheep livers (6%) and 1 (2%) of the 50 goat livers. No isolation was made from bovine livers.

63.64% (7) of the samples which revealed the presence of *Campylobacter* spp. were isolated from sheep fetuses, 27.27% (3) from sheep livers and 9.09% (1) from goat liver samples.

5 out of the 11 isolates were identified as *Campylobacter fetus ssp. fetus* (45.45%), 5 were identified as *Campylobacter jejuni* (45.45%) and 1 was identified as *Campylobacter coli* (9.09%).

5 out of the 7 fetus samples found positive were identified with *Campylobacter fetus ssp. fetus* (71.43%), 2 samples with (28.57%) *Campylobacter jejuni,* 2 out of 3 sheep liver samples revealed (66.67%) *Campylobacter jejuni,* 1 had *Campylobacter coli* while 1 goat liver sample was identified with *Campylobacter jejuni.* The distribution of the identified *Campylobacter* species according to the samples is shown in Table 1.

Resistance to 7 different antibiotics for the identified 11 isolates was determined by the disc diffusion method. According to the test results the highest resistance was manifested against ciprofloxacin and tetracycline (45.45%) while the highest sensitivity was against gentamicin (90.91%) and chloramphenicol (100%). Resistance and sensitivity data against determined antibiotics and strains is shown in Table 2.

Table 1. Distribution of the identified *Campylobacter* species according to the samples

Agent	Sheep				Goat liver		Bovine liver		Total samples	
	Fetus n=44		Liver n=50		n=50		n=50			
	x	%	x	%	x	%	x	%	194	%
C. fetus subsp. fetus	5	11.36	-	0.00	-	-	-	-	5	2.58
C. jejuni	2	4.55	2	4.00	1	2.00	-	-	5	2.58
C. coli	-	-	1	2.00	-	-	-	-	1	0.52
Total	7	15.91	3	6.00	1	2.00	0	0	11	5.67

(x: number of positive samples, n: number of examined samples)

Table 2. *Campylobacter* spp. Antibiogram Results

Antibiotic	S		I		R	
	n	%	n	%	n	%
Ampicillin	8	72.73	2	18.18	1	9.09
Erythromycin	6	54.55	2	18.18	3	27.27
Gentamicin	10	90.91	1	9.09	-	-
Chloramphenicol	11	100.00	-	-	-	-
Streptomycin	8	72.73	3	27.27	-	-
Ciprofloxacin	3	27.27	3	27.27	5	45.45
Tetracycline	3	27.27	3	27.27	5	45.45

(n: Number of strains, S: sensitive, I: medium sensitive, R: resistant)

DISCUSSION

The study revealed that 11 (5.67%) samples were positive for Campylobacter spp. Resistance was manifested against ciprofloxacin and tetracycline in the identified isolates while sensitivity to gentamicin and chloramphenicol was determined. Campylobacteriosis is a common zoonotic disease throughout the world and observed in Latin America, Asia, Africa, North America, Europe and New Zealand (Gard 2016). The incidence of infections caused by *Campylobacter* spp. is constantly increasing. Campylobacteriosis is a zoonosis infection. *Campylobacter* spp. causes hundreds of millions of infections around the world every year (Kashoma et al 2015). The disease is also common in our country. Kenar and Erganiş (1994) carried out a study in Samsun and its periphery regarding *Campylobacter* induced abortions and reported that they had isolated *Campylobacter* subsp in 8 out of 35 aborted lamb fetuses (22.9%) and 5 of them were identified as *C. fetus subsp. fetus* (62.5%), 2 as (25.0%) *C. jejuni* and 1 as Aerotolerant campylobacter. Similarly, in a study carried out by Kenar et al. (1990) in the region of Konya they reported that they had isolated *Campylobacter* spp. at a rate of 7.5% in aborted fetuses. Küçükayan et al. (2003-2007) carried out a study and reported that they had isolated 6 of the examined 463 fetuses (1.29%) with *Campylobacter* spp. and all of them were diagnosed with *C. fetus subsp. fetus*. In a study carried out by Sağlam et al. (1998) in Northeast Anatolia they reported that 5 out of 119 (4.2%) aborted sheep fetuses were identified with *C. fetus subsp. fetus* ssp. In a previous study in the same region (Aydın et al. 1994), the isolation rate was reported as 31.25%. Likewise, in a study carried out by Arda et al. (1987) in the Central Anatolian Region, they reported that they had isolated and identified *C. fetus subsp. fetus* in 13 out of 173 aborted fetuses (7.5%). In this study, *Campylobacter* spp. was isolated in 7 (15.91%) out of 44 aborted sheep fetuses. The values were evaluated as compatible with other studies.

In a study carried out with sheep and goat liver surface swabs (Lazou et al. 2014a) it was reported that 44 (44%) of 100 samples were isolated with *Campylobacter* spp. In another study (Lazou et al. 2014b) by the same researchers they reported that 78,2% of the liver surfaces collected from lambs, sheep, goats and kids slaughtered in slaughterhouses had been determined as contaminated with *Campylobacter* agents. *Campylobacter* spp. was isolated in 3 (6%) sheep livers and 1 (2%) goat liver in the current study. The researchers think that the difference between the results of this study and the results of the others can be attributed to the difference in the course of the disease in the regions where the studies were carried out, the fact that the samples were collected when the infection was peaking or the difference in diagnostic techniques. Furthermore, the difference in the results could be attributed to the fact that the reported studies had used lambs and kids while we used sheep and goats in our study. *Campylobacter* spp. agents are more common in young animals. *Campylobacter* spp. could not be isolated from the bovine liver samples in the present study. In our country, Elmalı (2004) has reported isolation of 8% in liver samples taken from cattle and Açık (2006) has reported isolation of 6.5%. Enokimoto et al. (2007) reported that 5% of 108 cattle liver samples revealed *Campylobacter* spp., yet the isolation was 45% from bile samples taken from the same cattle. Matsumoto et al. (2008) reported that they had isolated *C. jejuni* at a rate of 1.4% in their study. Unlike the reported studies, the fact that *Campylobacter* could not be isolated in the current study can be attributed to the good hygienic conditions of the slaughterhouses where the samples were taken. This can be attributed to the influence of the more effective inspection of the production of food of animal origin initiated in our country by the Ministry of Food, Agriculture and Livestock in recent years, the noticeable increase of technical and hygienic standards in the abattoirs during the study as well as the reduction of cross contamination of the

carcass and internal organs caused by bile and stool contamination.

It was observed that the *Campylobacter* species isolated in our study were susceptible to chloramphenicol, gentamicin, ampicillin and streptomycin and less sensitive to erythromycin. The isolated *Campylobacter* species were resistant to ciprofloxacin and tetracycline. Hakkinen et al. (2007) reported resistance to ampicillin and tetracycline, but no resistance to erythromycin in their study. In their study on *Campylobacter* species isolated from cattle livers, Aneesa and Fakr (2013) reported sensitivity against erythromycin, gentamicin, chloramphenicol, streptomycin and medium level sensitivity against ampicillin, ciprofloxacin and tetracycline. Karikari et al. (2017) reported that the *Campylobacter* species they had isolated from cattle carcasses were resistant to erythromycin, ampicillin, chloramphenicol, sensitive to gentamicin and moderately sensitive to ciprofloxacin and tetracycline. The reason for the differences between the results of the studies mentioned above and the results presented in this study can be attributed to the differences in the usage rate and duration of the antibiotics in the investigated countries.

It has been concluded that the isolation rates of *Campylobacter* species may vary according to the regions where the study is conducted and that the antibiotic sensitivities may be different according to the regions and furthermore, that the improvement in the technical and hygienic conditions in slaughterhouses in our country in recent years may be effective in the isolation of *Campylobacter*.

ACKNOWLEDGEMENT

Mustafa Gökçe (thesis), Afyon Kocatepe University, Institute of Health Sciences "Afyonkarahisar and Kütahya provinces of slaughtered sheep, goats, beef liver and sheep aborted fetuses of the isolation and identification of the identification of antibiotic resistance of the Campylobacter species" titled with thesis: 2017-015 and 11.07.2017 Graduation It is summarized from the thesis dated.

This study has been presented as an oral paper presentation at the International Conference on Agriculture, forest, food sciences and Technologies (ICAFOF) Cappadocia/Turkey held on 15-17 May 2017.

AFYON KOCATEPE UNIVERSITY, ANIMAL TESTS ETHICS COMMITTEE (AKUHADYEK)
The research project AKUHADYEK-438-15-Reference with the date of 26/03/2015 Date and Number 27 has been approved and approved according to the AKUHADYEK regulation and universal ethical principles.

REFERENCES

Açık MN, Sığır ve koyun orijinli *Campylobacter jejun*i ve *Campylobacter coli*'nin moleküler, tiplendirilmesi, Doktora Tezi, Fırat Üniversitesi, Sağlık Bilimleri Enstitüsü. Mikrobiyoloji Anabilim Dalı, Elazığ, 2006.

Arda M, İstanbulluoğlu E, Diker S, Bisping W, Akay Ö, Aydın N, İzgür M, Karaer Z, Orta Anadolu Bölgesi Koyunlarında Abortus Olgularının Etiyolojisi ve Serolojisi Üzerinde Bir Çalışma, A Ü Vet Fak Derg. 1987; 34 (2): 195-206.

Aydın N, İzgür M, Diker KS, Yardımcı H, Esendal Ö, Paracıkoğlu J, Akan M, Veteriner Mikrobiyoloji, Bölüm: Diker KS, *Campylobacter*, *Arcobacter* ve *Helicobacter* İnfeksiyonları, sy: 237-258. İlke-Emek Yayınları, 2006, Ankara.

Aydın F, Leloğlu N, Şahin M, Otlu S, Kars yöresinde sığır ve koyunlarda görülen abortların bakteriyolojik yönden araştırması. I. Ulusal Veteriner Mikrobiyoloji Kongresi, 27-29 Ekim 1994, Ankara.

Aytuğ CN, Alaçam E, Özkoç Ü, Yalçın BC, Gökçen H, Türker H, Koyun Keçi Hastalıkları ve Yetiştiriciliği Tüm Vet. Hayvancılık Hizmetleri. 1990; 2: 161-163.

Batmaz H, Koyun ve Keçilerin İç Hastalıkları Nobel Tıp Kitabevleri Ltd. Şti. 2013; 240.

Buck GE, Kelly T, Effect of moisture content of the medium on colony morphology of *Campylobacter fetus subsp. jejuni*. J Clin Microbiol. 1981; 14 (5): 585-586.

Corry Jel, Post De, Colin P, Laisney MJ, Culture media for the isolation of Campylobacters. Int J Food Microbiol. 1995; 26: 43-76.

Diker KS, Istanbulluoglu E, Ovine abortion associated with Campylobacter jejuni. Vet. Rec. 1986; 118, 307.

Elmalı M, Sığırların Bakteriyolojik Et Muavenesi Örneklerinde Termofilik *Campvlobacter* Türlerinin İzolasvon ve İdentifikasvonu, Vet Bil Derg. 2004; 20, 1: 39-44.

Enokimoto M, Kubo M, Bozono Y, Mieno Y, Misawa N, Enumeration and identification of *Campylobacter* species in the liver and bile of slaughtered cattle. Int J Food Microbiol. 7 Aug. 2007; 118 (3): 259-63.

Gard J, Bovine Genital Campylobacteriosis-A Review., Int Vet Sci Res. 2016; 2(1): 029-031.

Gençer B, Zarakolu P, Kılıç S, Yurdakök K, Güvener E, *Campylobacter jejuni* izolasyonunda kullanılan üç selektif besiyerinin karşılaştırılması", Mikrobiol Bült. 2001; 35: 45-52.

Hakkinen M, Heiska H, and Hanninen ML, Prevalence of *Campylobacter* spp. in Cattle in Finland and Antimicrobial Susceptibilities of Bovine *Campylobacter jejuni* Strains. Microbıology. May 2007; 73(10): 3232–3238.

Hazıroğlu, R., Milli Ü, H, Veteriner Patoloji Cilt II. Tamer Matbaacılık, Yayıncılık, 1998; 2: 83-484.

Jones DM, Sutcliffe EM, Curry A, Recovery of viable but non culturable *Campylobacter jejuni*. J Gen Microbiol. 1991; 137 (10): 2477-82.

Karikari AB, Obiri-Danso K, Frimpong EH, Krogfelt KA, Antibiotic Resistance of *Campylobacter* Recovered from Faeces and Carcasses of Healthy Livestock, BioMed Research International. 2017; Article ID 4091856, 9 pages, https://doi.org/10.1155/2017/4091856

Kashoma IP, Kassem II, Kumar A, Kessy BM, Gebreyes W, Kazwala RR, Rajashekara G, Antimicrobial Resistance

and Genotypic Diversity of Campylobacter Isolated from Pigs, Dairy, and Beef Cattle in Tanzania. Front Microbiol. 2015; 6, 1240.

Kenar B, Erganiş O, Orta Karadeniz Bölgesinde Atık Yapan Koyunlardan *Campylobacter* Türlerinin İzolasyon ve İdentifikasyonu ile Antibiyotiklere Duyarlılıkları üzerinde Çalışmalar, Veterinarium. 1994; 5 (1-2), 4-12.

Kenar B, Erganiş O, Kaya O, Güler E, Konya bölgesinde koyunlarda atıklara sebep olan *Brucella*, *Campylobacter, Salmonella* ve *Chlamydia*'ların bakteriyolojik ve serolojik incelenmesi. Veterinarium. 1990; 1: 17-20.

Küçükayan U, Dakman A, Ülker U, Müştak K, Koyun kan serumları ve fetuslarının bakteriyel atık etkenleri yönünden incelenmesi. Etlik Vet Mikrobiyol Derg. 2007; 18: 11-16.

Lamoureux M, Mackay A, Messier, S, Fliss I, Blais BW, Holley RA, Simard RE, Detection of *Campylobacter jejuni* in food and poultry viscera using immunomagnetic separation and microtitre hybridization. J Appl Microbiol. 1997; 83: 641-651.

Lazou T, Dovas C, Houf K, Soultos N, Iossifidou E, Diversity of *Campylobacter* in retail meat and liver of lambs and goat kids. Foodborne Pathog Dis. April 2014a; 11 (4): 320-328.

Lazou T, Houf K, Soultos N, Dovas C, Iossifidou E, *Campylobacter* in small ruminants at slaughter: prevalence, pulsotypes and antibiotic resistance. Int J Food Microbiol. 3 March 2014b; 173: 54-61.

Matsumoto N, Tanıwakı T, Kınuta M, Murase T, Isolation of *Campylobacter jejuni* and Coliform Bacilli from Bile and Liver Obtained from Slaughter Cattle in Western Japan, J Food Prot. 2008; 71(6), 1228–1231.

Madigan, M. T., Martinko, J. M., Parker, J, Amplifying the DNA: The Polymerase Chain Reaction. Biology of Microorganisms. Pearson Education Inc. Prentice Hall, Upper Saddle River, NJ07458. USA. 2003, pp. 312-314.

Moore JE, Corcoan D, Dooley JS, Fanning S, Lucey B, Matsuda M, Mcdowell DA, Megraud F, Miller BC, O'mahoney R,O'rıordan L, O'rourke M, Rao JR, Rooney, PJ, Sails A, Whyte P, *Campylobacter*. Vet Res. 2005; 36: 351-382.

Nachamkin, I. *Campylobacter jejuni*. In: Food Microbiology: Fundamentals and Frontiers, 3rd Eds. Doyle MP, Beuchat LR, ASM Press, Washington DC. 2007; 237-248.

Clinical and Laboratory Standards Institute(CLSI), Performance standards for antimicrobial susceptibility testing; Twenty-Second Informational Supplement (January 2012). M100-S22. 950 West Valley RoadSuite 2500. Wayne, PA 19087 USA.

Obiri-Danso K, Paul N, Jones K, The effects of UVB and temperature on the survival of natural populations and pure cultures of *Campylobacter jejuni, Camp. coli, Camp. lari* and urease-positive thermophilic campylobacters (UPTC) in surface waters. J Appl Microbiol. 2001; 90 (2): 256-267.

Purdy D, Cawthraw S, Dickinson JH, Newell DG, Park SF, Generation of a superoxide dismutase-deficient mutant of *Campylobacter coli*: evidence for the significance of SOD in Campylobacter survival and colonization. Appl Environ Microbiol. 1999; 65: 2540-2546.

Rollins DM, Colwell RR, Viable but non-culturable stage of *Campylobacter jejuni* and its role in the natural aquatic environment. Appl Environ Microbiol. 1986; 52: 531-538.

Sağlam YS, Türkütanıt SS, Taştan R, Bozoğlu H, Otlu S, Kuzeydoğu Anadolu Bölgesi'nde Görülen Bakteriyel Sığır ve Koyun Abortlarının Etiyolojik ve Patolojik Yönden İncelenmesi. Vet Bil Derg. 1998; 14 (2): 133-145.

Shih DY, Isolation and identification of enteropathogenic *Campylobacter* spp. from chicken samples in Taipei. J Food Protect. 2000; 63 (3): 304-308.

Smibert RM, Genus *Campylobacter*. In: Bergey's Manual of Systematic Bacteriology. Eds: K Krieg NR, Holt JG, Williams & Wilkins, Baltimore, London. 1984; pp. 111-118.

Songer JG, Post KW, Veteriner Hekimlik Mikrobiyolojisi. *Campylobacter, Helicobacter* ve *Arcobacter* Cinsleri. Çev. Ed. **Ang Ö, Özgür NY,** Nobel Tıp Kitabevleri. 2005; 223-228.

Taylor D, Courvalin P, Mechanism of antibiotic resistance in *Campylobacter* species. Antimicrob. Agents Chemother. 1988; 32: 1107-1112.

Turgut K, Ok M, Veteriner Gastroenteroloji. Mikro Dizgi. Konya. 1997; 155-156.

Ülgen M, Özel Mikrobiyoloji Uludağ Üniv. Veteriner Fakültesi Yayınları: 2002-2; 51-58.

Winn W, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P, Woods G, Curved Gram negative bacilli and oxidase positive fermenters: Campylobacteraceae and Vibrionaceae. In: Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott-Raven Publishers, Philadelphia, 2006; pp. 321-361.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):150-157

DOI: 10.30607/kvj.526068

RESEARCH ARTICLE

Investigation of The Microbiological Quality of Sea Breeding (*Sparus aurata*) and Sea Bass (*Dicentrarchus labrax*) Fishes in Afyonkarahisar Province

Şebnem PAMUK*, Yağmur Nil DEMİREL, Zeki GÜRLER

Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Turkey, Afyonkarahisar

ABSTRACT

The aim of this study is to determine the microbiological quality levels of fish (*Sparus aurata* and *Dicentrarchus labrax*) sold in fishermen in Afyonkarahisar. In this study, 82 fish samples randomly selected and purchased from 14 different retail points were microbiologically analyzed. According to this; the microorganism ranges isolated from 43 *Sparus aurata* and 39 *Dicentrarchus labr*ax were; 5.0-7.88/4.90-7.34 log10 mL^{-1} TVBC; 3.30-7.17/ 3.90-7.54 log10 mL^{-1} psychrophilic microorganism; 1.30-5.89/ 1.30-4.53 log10 mL^{-1} staphylococcus / micrococcus, only in *Sparus aurata* 2.30-4.14 log10 mL^{-1} *S. aureus*; 1.30-6.49/1.30-6.11 log10 m^{-1} lactic acid bacteria (LAB); 1.30-6.55/ 1.30-7.14 log10 mL^{-1} enterobacteria, 1.30-6.62/1.30-6.60 log10 mL^{-1} total coliform and 1.30-4.30/1.30-4.47 log10 mL^{-1} levels *E. coli* and 1.2% of the samples (from *Sparus aurata) Salmonella* spp., were identified respectively.

Keywords: Fish, Microbial contamination, Microbiological quality, Seafood

***

Afyonkarahisar İli'nde Satışa Sunulan Çupra (*Sparus aurata*) ve Levrek (*Dicentrarchus labrax*) Balıklarının Mikrobiyolojik Kalitelerinin Araştırılması

ÖZ

Bu çalışmanın amacı, Afyonkarahisar'da balıkçılarda satışa sunulan balıkların (çupra ve levrek) mikrobiyolojik kalite düzeylerinin belirlenmesidir. Çalışmada, 14 farklı balıkçıdan rastgele seçilen 82 adet (43 çupra, 39 levrek) balık satın alınarak, mikrobiyolojik analizleri yapılmıştır. Buna göre; çupra ve levrek balıklarından izole edilen mikroorganizma aralıkları sırasıyla; 5.0-7.88/4.90-7.34 log_{10} mL^{-1} AMGC; 3.30-7.17/3.90-7.54 log_{10} mL^{-1} psikrofil mikroorganizma; 1.30-5.89/1.30-4.53 log_{10} mL^{-1} stafilokok/mikrokok; sadece çupra balığında 2.30-4.14 log_{10} mL^{-1} *S. aureus* ; 1.30-6.49/1.30-6.11 log_{10} mL^{-1} laktik asit bakterisi (LAB); 1.30-6.55/1.30-7.14 log_{10} mL^{-1} enterobakteri, 1.30-6.62/1.30-6.60 log_{10} mL^{-1} total koliform ve 1.30-4.30/1.30-4.47 log_{10} mL^{-1} düzeylerinde *E. coli* izole edilmiştir. Örneklerin %1.2'sinden (çupra balığı) *Salmonella* spp. identifiye edilmiştir.

Anahtar Kelimeler: Balık, Deniz ürünü, Mikrobiyal kontaminasyon, Mikrobiyolojik kalite

To cite this article: Pamuk Ş. Demirel Y.N. Gürler Z. Investigation of The Microbiological Quality of Sea Breeding (Sparus aurata) and Sea Bass (Dicentrarchus labrax) Fishes in Afyonkarahisar Province. Kocatepe Vet J. (2019) 12(2):150-157.

Submission: 12.02.2019 **Accepted**: 07.05.2019 **Published Online**: 15.05.2019

ORCID ID; ŞP: 0000-0001-7227-3364, YND: 0000-0002-1309-0936, ZG: 0000-0002-9037-2945

*Corresponding author e-mail: spamuk@aku.edu.tr

GİRİŞ

Balık, denize kıyısı olan ülkelerde toplam hayvansal protein alımının %30-80'ini karşılayan önemli bir hayvansal gıdadır (Adenike 2014). Balık eti, yüksek oranda doymamış yağ asidi, vitamin, mineral, oldukça az miktarda doymuş yağ asidi ile karbonhidrat içermekte ve beslenmede önemli yer tutmaktadır (Boulares ve ark. 2011, Nelson 2016). Bununla birlikte, deniz ürünleri avlandıktan sonra, transport sırasında ya da satışa sunuldukları yerlerde (Budiati ve ark. 2015, Mol ve Tosun 2011) kontamine olabilmekte ve mikroorganizma gelişimiyle birlikte bozulma süreci başlamaktadır (Goja 2013). Bu sürecin sonunda, mikrobiyel aktivite nedeniyle balıkların %30'u bozulmaktadır (Ghaly ve ark. 2010). Balıkların mikroorganizma ile kontamine olması ya avlandıkları akuatik ortamın kirli olduğunu ya da avlandıktan sonraki sürecin doğru işlemediğini işaret etmektedir (Adeyemo 2003). Taze balığın bozulma sürecine, balığın yakalandığı suyun bakteriyolojik kalitesi, deniz suyu sıcaklığı, suyun tuz oranı gibi çeşitli çevresel faktörler etkili olmaktadır (Boulares ve ark. 2013). İlk mikrobiyal düzey (Boulares ve ark. 2011, Ligia ve ark. 2008, Tripathy ve ark. 2007), avlanmanın nerede yapıldığına, yerleşim bölgelerine yakınlığına, mevsime (Bojanic ve ark. 2009), işleme prosedürlerine (örn., baş ayırma, iç çıkarma, kesme) hasata, (Attouchi ve Sadok 2010, Özden ve ark. 2007, Poli ve ark. 2001) ve depolama sıcaklığına (Gram ve Dalgaard 2002, Hernandez ve ark. 2009, Koutsoumanis ve ark. 2002) bağlıdır. Bu nedenle, soğuk muhafaza sıcaklığında aerob ortamda depolanan taze balık ve deniz ürünlerinin ana bozulma florasını, *Pseudomonas*, *Photobacterium*, *Flavobacterium* (Chytiri ve ark. 2004, Diop ve ark. 2010, Gram ve Huss 2000, Matamoros ve ark. 2006) dahil olmak üzere ağırlıklı olarak psikrotrofik Gram negatif türler olan *Aeromonas*, *Shewanella*, *Acinetobacter* ve *Moraxella*'nın (Cardinal ve ark. 2004, Franzetti ve ark. 2003, Joffraud ve ark. 2001, Lyhs ve ark. 2001) yer aldığı bildirilmiştir. Bu psikrotrofik bakteriler, kokusuz ve tatsız metabolitleri üretmek için balıktaki çeşitli maddelere hücum ederler (Diop ve ark. 2010, Galvez ve ark. 2010, Ligia ve ark. 2008). Özellikle salamura fermente balık ürünlerinde histamin birikiminin başlıca sorumlularından biri olan ve yüksek ozmotik basınç şartlarında canlı kalabilen halofilik laktik asit bakterileri balıklardan sıklıkla izole edilen bakterilerdir (Satomi 2018). Bozulma sürecinin, balıkların başlangıçta sahip olduğu toplam mikroorganizma sayısı ile direkt ilişkisi bulunmaktadır (Gram ve Huss 1996).

Dünyanın farklı bölgelerinde deniz ürünlerinin güvenliği ve kalitesinin araştırıldığı çalışmalar mevcut olmakla birlikte, bu çalışmalar sağlık risklerinin bilinmesi açısından da önem teşkil etmektedir (Begum ve ark. 2010, Boulares ve ark. 2011, Boulares ve ark. 2013, Goja 2013, Kapute ve ark. 2012, Odu ve Imaku 2013, Popovic ve ark. 2010, Rahal ve ark. 1981). Bir yarımada olan Türkiye, birçok iç su kaynağına da sahiptir. Resmi verilere göre; 2017 yılında su ürünleri üretimi 630.820 ton, iç tüketim miktarı 441.573 ton, çipura ve levrek üretim miktarı ise 61.090 ile 99.971 ton olarak bildirilmiştir (Anon 2018).

Bu çalışmanın amacı, balıkçı tezgahlarında satışa sunulan çupra ve levrek balıklarının kalite ve gıda güvenliği ile doğrudan ilişkili olan mikrobiyolojik kalitelerinin araştırılmasıdır.

MATERYAL ve METOT

Çalışmada, 2018 yılının Ekim-Kasım ve Ocak aylarında 10 farklı balıkçıdan satın alınan 43 adet çupra ve 39 adet levrek balığı mikrobiyolojik açıdan değerlendirildi. İncelenen toplam 82 adet balık örneği Aerob Mezofil Genel Canlı (AMGC), psikrofil mikroorganizma (PM), mikrokok/stafilokok (M/S), enterobakteri, total koliform, *E. coli* ve *Salmonella* spp. ve laktik asit bakterileri (LAB) yönünden analiz edildi. İşletmelerden satın alınan balık örnekleri soğuk zincir altında laboratuvara getirilerek 1-2 saat içerisinde analize alındı. Balıkların örneklenmesinde; her bir balık steril poşetlere alınarak, 225 ml Buffer Pepton Water (BPW, CM 0509) ile 2 dk. yıkandıktan sonra seri dilüsyonları yapılarak ilgili besi yerlerine yapıldı.

Mikrobiyolojik Analizler

Aerob Mezofil Genel Canlı (AMGC)

AMGC sayısının belirlemesinde Plate Count Agar (PCA-Oxoid CM0325) kullanıldı. Petriler 30±1^{0}C 48 saat inkübasyona bırakıldı. İnkübasyon sonunda gelişen tüm koloniler sayılarak düzeyleri belirlendi (ISO 2003).

Psikrofil Mikroorganizma (PM)

PM sayısını belirlemek için Plate Count Agar (PCA-Oxoid CM0325) kullanılarak, petriler 4^{0}C 72 saat inkübasyona bırakılarak beyaz renkli koloniler sayıldı (Cousin ve ark. 2001, ISO 2001).

Mikrokok/Stafilokok (M/S)

Örneklerinin M/S sayısı, BP Agar-Base (Oxoid CM0275, Egg Yolk Tellurite Emulsion Oxoid: SR0054) besi yerine ekim yapılarak 37^{0}C 'de 24-48 saat inkübasyondan sonra üreyen siyah kolonilerin sayılmasıyla belirlendi (Bennett and Lancette 1992). Baird-Parker besi yerinde üreyen tipik ve atipik koloniler koagulaz test kiti kullanılarak doğrulandı (Staphytect test kit OXOID).

Enterobakteri, Total koliform ve E. coli

Bu bakteri türlerini tespit etmek amacıyla, Chromocult Coliform Agar (Merck, 1.10426) kullanılarak, 35-37^{0}C'de 24 saat inkubasyon sonucunda tipik somon-kırmızı renk koloniler koliform grubu bakteri, koyu mavi-mor menekşe renginde olan koloniler *E. coli* ve renksiz koloniler ise

enterobakter olarak değerlendirildi (Blood ve Curtis 1995, De Boer 1998).

Salmonella spp.
Salmonella spp. izolasyonu için, ISO 6579 (ISO 2002) yöntemi uygulandı. Buffer Pepton Water'da (BPW, CM 0509), 2 dk. süreyle yıkanan balıkların, yıkama sıvıları 37^0C'de 18±2 saat ön zenginleştirmeye kaldırıldı.

İnkübasyonun ardından herbir zenginleştirme sıvısından 0.1 mL alınarak, içerisinde 10 mL of Rappaport Vassiliadis (RV) enrichment broth (CM 0866), bulunan tüplere geçilerek 41.5°C'de 24 saat inkübasyona kaldırıldı. Daha sonra, herbir kültür Xylose Lysine Deoxycholate (XLD, CM 0469) Agar'a çizme plak yöntemiyle ekilerek 37°C'de 48 saat inkübe edildi (ISO, 2002). İnkübasyonun ardından, siyah merkezli beş şüpheli koloni seçilerek, Nutrient Agar'a (CM 0003) subkültüre edildi. Kolonilere, Gram boyamanın ardından standart biyokimyasal testler uygulandı (triple sugar iron agar-CM 277; lysine iron agar-CM 381; urease test-CM 53; Simmons citrate-CM 155, ONPG-disc- DD13 ONPG, MR-VP test- CM 0043). Doğrulama testi, *Salmonella* antiserumu (O and H-Vi polyvalent antiserum, Difco 2264-47-2) (Flowers ve ark. 1992) ile gerçekleştirildi.

Laktik Asit Bakterileri
LAB için, MRS Agar'a (Oxoid CM0361) ekim yapılarak, petriler 37^0C'de 72 saat anaerob (AnaeroGen Oxoid ANOO35A) ortamda inkübasyona kaldırıldı. İnkübasyonun ardından boz/beyaz renkte üreyen kolonilerin sayımları yapıldı (ISO 1998).

BULGULAR

Balık örneklerinin tamamında (%100) aerob mezofil genel canlı ve psikrofil mikroorganizma, 39'unda (%47.5) M/S ve 33'ünde (%40.2) LAB izole edilirken, 66'sında (%80.4) enterobakteri, 58'inde (%70.7) koliform, 6'sında (%7.31) *E. coli* ve 10'unda (%12.1) *S. aureus* tespit edilmiştir. Sadece bir çupra balığı örneğinden (%1.2) *Salmonella* spp. identifiye edilmiştir. Balık türlerine göre; çupra balıklarının 32'sinden >6 $\log_{10}$ mL^{-1} (%74.4) AMGC, 42'sinden >2 $\log_{10}$ mL^{-1} (%97.6) psikrofil mikroorganizma, 20'sinden >2 $\log_{10}$ mL^{-1} (%46.5) S/M, 24'ünden >2 $\log_{10}$ mL^{-1} (%55.8) LAB; levrek balıklarının ise; 26'sından >6 $\log_{10}$ mL^{-1} (%66.6) AMGC, 38'inden >2 $\log_{10}$ mL^{-1} (%97.4) psikrofil mikroorganizma, 15'inden >2 $\log_{10}$ mL^{-1} (%38.4) S/M ve 15'inden >2 $\log_{10}$ mL^{-1} (%38.4) düzeylerinde LAB izole edilmiştir (Tablo 1). Enterobekteri, total koliform, *E. coli* ve *S. aureus* oranları ise çupra balıklarının sırasıyla; 37'sinde >4 $\log_{10}$ mL^{-1} (%86), 28'inde >4 $\log_{10}$ mL^{-1} (%65.1), 2'sinde >4 $\log_{10}$ mL^{-1} (%4.65), 9'undan >2 $\log_{10}$ mL^{-1} (%20.9) olarak; levrek balıklarında ise sırasıyla; 36'sından >4 $\log_{10}$ mL^{-1} (%92.3), 28'inde >4 $\log_{10}$ mL^{-1} (%71.7), 1'inde >4 $\log_{10}$ mL^{-1} (%2.56) düzeylerinde seyrederken, levrek balıklarından *S. aureus* identifiye edilmemiştir (Tablo 2).

TARTIŞMA

Yetiştiricilikle balık üretimi küresel boyutta 2008 yılında 52.5 milyon tona ulaşarak, dünya gıda balık tüketiminin% 45.6'sından sorumlu sektör durumuna gelmiştir (FAO 2012). Üretimi bu derece fazla olan bu gıdanın, avlanmadan sonraki aşamalarda uygun olmayan şartlarda muhafazası ve işlenmesi sebebiyle, gıda kaynaklı salgınların büyük bölümünden sorumlu olduğu belirtilmiştir (Fagan ve ark. 2011, Jain ve ark. 2008, Piérard ve ark. 1999, Shao ve ark. 2011). *Vibrio spp., Salmonella spp., Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Clostridium perfringens*, *Listeria monocytogenes* ve *Shigella* spp. gibi gıda patojenleri; gıda üretim zinciri sırasındaki kontaminasyon neticesinde balık dokusunda bulunabilmektedir (Roberts ve ark. 2005). Söz konusu patojenler, çevre şartlarında varlıklarını sürdürmekte ve balık tüketime hazır olana kadar dokularında canlı kalabilmektedir (Pillay 1992, Suhalima ve ark. 2008). Kirlenmiş balık havuzları (örn. dölleme, insan ve hayvan atık suları nedeniyle yüksek bakteriyel yük içeren) patojenlerin balık dokusuna (örn. sindirim sistemi, solungaçlar, kaslar, böbrekler ve karaciğer) nüfuz etmesini sağlamaktadır (Junior ve ark. 2014).

Birçok çalışmada, *E. coli*, *Salmonella* spp. ve *S. aureus*'un sudaki konsantrasyonunun, balık organ ve dokusundan elde edilen bakteri konsantrasyonu ile orantılı olduğu saptamıştır (Buras ve ark. 1987, El-Guzmán ve ark. 2004, Pal ve Gupta 1992, Shafai ve ark. 2004). Bu organizmalar, balıkların doğal mikrobiyotaları olmamakla birlikte, varlıkları balık besin ağı, konak-mikrobiyota etkileşimleri ve çevre belirleyicileri ile ilişkilendirilmektedir (Kostic ve ark. 2013). Örneğin, gıda kaynaklı hastalıkların nedenleri olarak rapor edilen *Salmonella* ve *S. aureus* gibi yaygın gıda kaynaklı patojenler, tipik çevresel kirleticiler olmamakla birlikte, genellikle gıda hazırlama veya işleme süreçlerinde gıdaları kontamine etmektedirler (Kasai ve ark. 2010, Vollaard ve ark. 2004). Nitekim; Kocatepe ve ark. (2011), balık tezgahlarının mikrobiyolojik kalitesini inceledikleri çalışmada, koliform sayısını $1.8x10^2$-$2.2x10^2$ kob/cm^2, AMGC sayısını $8.5x10^2$-$0.6x10^2$ kob/cm^2, Rahal ve ark. (1981) ise;, tezgahlardan AMGC ve *S. aureus* düzeylerini sırasıyla 4.39±0.17, 1.69±0.12 log kob/g aralığında saptadıklarını bilidirilmişlerdir.

Ülkemizde farklı deniz ürünlerinde yapılan çalışmalarda, AMGC ve psikrofil sayısı sırasıyla; 2.30±1.30 ile 3.36±0.87 log kob/g (İnanlı ve ark. 2011), AMGC, psikrotrofik mikroorganizma, *Enterobacteriaceae* sayıları 5.65±1.70 log kob/g, 6.90±1.60 log kob/g, 4.24±1.30 log kob/g, 3.61±0.72 log kob/g (Alak ve ark. 2010), AMGC, psikrotrofik

mikroorganizma, koliform sayıları 2.8-4.3 log kob/g, 2.6-4.1 log kob/g, <3-3.8 EMS/mL (Alparslan ve ark. 2017), AMGC, total koliform ve fekal koliform sayıları 3.25±1.26-5.42±0.91 log kob/g, 0.68±0.48-2.08±1.34 log kob/g, 0.54±0.16- 0.49±00.4 log kob/g (Mol ve Tosun 2011) düzeylerinde bulunmuştur. Benzer şekilde farklı balık türlerinde yapılan bir başka çalışmada, 50'şer adet sazan ve tirsi balığındaki *S. aureus*, *V. parahaemoliticus* ve *E. coli* oranları sırasıyla; %12, %4, %8 ve %4, %8, %4 olarak belirlenmiştir (Tavakoli ve ark. 2012). Junior ve ark. (2014), 40 balık örneğinden >1100 MPN/g termotolerant koliform ve $<1.0x10^2$-$1.2x10^6$kob/g düzeylerinde *Staphylococcus* spp. saptadıklarını ve örneklerin %55'inin termotolerant koliform ile kontamine olduğunu bildirmişlerdir Nilla ve ark. (2012), balık örneklerindeki AMGC, toplam koliform, *E. coli* düzeylerini sırasıyla; 1.8±0.25- 6.5±0.75 log kob/g, 8.0±0.55-6.1±0.40 log kob/g, 1.4±0.10-4.8±0.45 log kob/g olarak belirlediklerini ve örneklerin %83'ünün *Staphylococcus* spp. ile kontamine olduğunu vurgulamışlardır. Yapılan diğer çalışmalarda, balıklardan $2.8x10^3$-$9.8x10^4$ kob/g stafilokok (Goja 2013), <100 kob/g *S. aureus* (Popovic ve ark. 2010), $4.4x10^3$-$9.7x10^7$ kob/g stafilokok, $5.9x10^4$-$1.5x10^8$ kob/g mikrokok, örneklerin (n=18) %46'sından *S. aureus* (Odu ve Imaku 2013) belirlenmiştir. Ayrıca, 15-120/3-95 MPN/100 g fekal koloform ve %13 oranında *Enterobacteriaceae* (Goja 2013), 0.9-240 MPN/g total koliform, 110 MPN/g fekal koliform (Begum ve ark. 2010), <10 kob/g *E. coli*, $>10^2$ kob/g *Enterobacteriaceae* (Popovic ve ark. 2010), 5.02-5.51 log kob/g kolifom, $2.2x10^4$-$9.5x10^7$ kob/g *E. coli,* örneklerin (n=18) %13.3'ünden *E. coli* (Odu ve Imaku 2013), 2.52-3.73 log kob/g koliform, 0.94-1.11 log kob/g fekal koliform (Budiati ve ark. 2015) izole edilmiştir. AMGC düzeyleri ise; $1.9x10^8$-$2.0x10^4$ kob/g, $1.2x10^6$-$5.0x10^6$ kob/g ile 6.29-6.78 log kob/g, örneklerin (n=15) %26.6'sında $>10^5$ kob/g (Odu ve Imaku 2013), 5.30-6.84 log kob/g (Budiati ve ark. 2015) olarak belirlenirken, örneklerin (n=15) %6'sında $>10^3$ kob/g psikrofil mikroorganizma, 5.07-6.15 log kob/g psikrotrofik mikroorganizma (Odu ve Imaku 2013) izole edilmiştir. LAB ise; 4.51-5.28 log kob/g düzeylerinde saptanmıştır (Odu ve Imaku 2013). Bu çalışmada elde edilen veriler literatürle uyumlu bulunmuştur.

Türk Gıda Kodeksi Mikrobiyolojik Kriterler Tebliği'nde (Anon 2011) taze soğutulmuş balıkların mikrobiyolojik değerlerine ilişkin herhangi bir limit değere rastlanmamakla birlikte, sadece histamin düzeyine ilişkin limit değer verilmiştir.

Balıkların yüksek oranda koliform ve E. coli ile kontamine olması, çoğunlukla suyun kontaminasyon derecesi, balıkların beslenmesi gibi balık yetiştiriciliği ile ilgili çevresel faktörlerle ilişkilendirilmektedir. Koliformların sayısı ve gelişimi ortam sıcaklığına da bağlanmaktadır (Del Rio-Rodriguez ve ark. 1997). Balık derisi oldukça yüksek konsantrasyonda mikroorganizma içerebilmektedir. Bu durum, işleme, balıkların muhafaza edildiği buzun kalitesi ile ilişkili olup, balık ve deniz ürünlerinin soğutulmasında kullanılan buz, halk sağlığı için potansiyel bir tehlikeye dönüşebilmektedir (Gerokomou ve ark. 2011). Falcao ve ark. (2002), balık marketlerinde kullanılan buzun $4.0x10^2$-$5.3x10^2$ MPN/100 mL düzeyinde koliform, ayrıca *E. coli'* nin farklı serotipleri ile de kontamine olduğunu belirlemiştir.

Bu çalışmanın aksine, Fattal ve ark. (1992), Hejkal et al. (1983), Suhalima ve ark. (2008) ve El-Shafai ve ark. (2004), derideki koliform düzeyinin oldukça düşük olduğunu rapor etmişlerdir. Bu durum balıkların sudan alındıktan sonra oldukça hızlı bir şekilde analize alınması ile açıklanmaktadır. Balık derisi, birçok hayati fonksiyona sahip olmakla birlikte, patojen ve çevresel ksenobiyotiklere karşı savunma sisteminin ilk basamağıdır. *Staphylococcus* ve *E. coli*'ye karşı (Kasai ve ark. 2010) antibakteriyel aktivite gösteren pek çok antibiyotik ve biyoaktif madde (peptit, lizozim, lektin ve proteaz) balık deri mukusundan identifiye edilmiştir (Alvarez-Pellitero 2008, Long ve ark. 2013, Rakers ve ark. 2010). Ayrıca, koliform ve stafilokokların düşük düzeyleri, balık kesim işlemi ve raflardaki yerini alana kadar geçen süredeki oldukça düşük muhafaza sıcaklıkları ile açıklanabilir (Junior ve ark. 2014). Aerob mezofil ve psikrofil bakteriler, *Enterobacteriaceae*, sülfit indirgeyen clostridialar, *L. monocytogenes*, *Vibrio cholerae* ve *Vibrio parahaemolyticus* gibi bakteriler balık kalitesi hakkında fikir veren önemli gruplardır. Popovic ve ark. (2010) tarafından Hırvatistan'da yapılan bir çalışmada taze balık örneklerinin %66'sının mikrobiyolojik kriterler bakımından yasal limitlere uymadığını ve örneklerin %40'ında *Enterobacteriaceae* düzeylerinin kabul edilebilir sınırlar içerisinde bulunmadığı belirlenmiştir. Balıklardaki mikrobiyal bulaşmanın, çoğunlukla fileto işlemi sırasında, özellikle termostabil toksin üretimi nedeniyle gastroenterit vakaların en çok bilinen etkeni olan *S. aureus*'un rezervuarı olan insan tarafından çapraz kontaminasyona uğraması sonucu şekillendiği bildirilmektedir

Balıklardaki mikrobiyal bulaşmanın, çoğunlukla fileto işlemi sırasında, özellikle termostabil toksin üretimi nedeniyle gastroenterit vakaların en çok bilinen etkeni olan *S. aureus*'un rezervuarı olan insan tarafından çapraz olarak kontamine edilmesi sonucu şekillendiği bildirilmektedir (Junior ve ark. 2014). Deniz ürünü ve balıklardan enteretoksijenik *E. coli* ve *S. aureus* (Ayulo ve ark. 1994), çalışanların ellerinden ve nazal mukozalarından izole edildiği bildirilmektedir (Acco ve ark. 2003). Almanya'da bir gıda çalışanının, balık hazırlama aşamasında ürünü *E. coli* (STEC) *O104:H4* ile kontamine etmesi sonucu, 23 kişinin infeksiyonundan sorumlu olduğu rapor edilmiştir (Diercke ve ark. 2014).

Amerika Birleşik Devletleri'nde (ABD) shigellozis vakalarının %23'ünün infekte gıda çalışanlarından, %24'ünün ise çiğ gıda tüketilmesi sonucu şekillendiği kaydedilmiştir (Nygren ve ark. 2013). Mikroorganizmalar balıklara birçok yoldan bulaşmakla birlikte, uygun olmayan, sağlıksız işlem ve muhafaza şartları ve çapraz kontaminasyon öne çıkan etkenlerdir (Huang ve ark. 2001, Jablonski ve Bohach 2001). Tüketicilerin yetersiz hijyen alışkanlıkları da gıda hazırlama aşamasında meydana gelen çapraz kontaminasyon ve mikrobiyal gelişim ile direkt ilişkilidir (Redmond ve Griffith 2009).

Tablo 1. Balık Örneklerindeki AMGC, PS, M/S ve LAB Düzeyleri
Tablo 1. Levels of AMGC, PS, M/S and LAB in Fish Samples

Örnek	AMGC (>6 log kob/g)		*PM* (>2 log kob/g)		*M/S* (>2 log kob/g)		LAB (>2 log kob/g)	
	n	(%) b	n	(%) b	n	(%) b	n	(%) b
Çupra	32	74.4	42	97.6	20	46.5	24	55.8
Levrek	26	66.6	38	97.4	15	38.4	15	38.4

n: >6 log kob/g b: pozitf örnek oranı (%)

Tablo 2. Balık Örneklerindeki Enterobakteri, Total Koliform, *E. coli* ve *Salmonella* spp. Düzeyleri
Tablo 2. Levels of Enterobacter, Total of Coliforms, *E. coli* and *Salmonella* spp. in Fish Samples

Örnek	*Enterobakteri* (>4 log kob/g)		Total koliform (>4 log kob/g)		*E. coli* (>4 log kob/g)		*S. aureus* (>2 log kob/g)		*Salmonella* spp.	
	n	(%) b	n	(%) b	n	(%) b	n	(%) b	n	(%) b
Çupra	37	86	28	65.1	2	4.65	9	20.9	1	1.2
Levrek	36	92.3	28	71.7	1	2.56	-	-	-	-

n: pozitif örnek sayısı b: pozitf örnek oranı (%)

SONUÇ

Avrupa Birliği'nde (AB) 2010 yılından itibaren, balıkların toptan alımı ve depolanması aşamasında mikrobiyolojik tehlikeleri kontrol etmeye yönelik olarak, alım aşamasında hammadde için "menşe belgesi" ve "sertifika alma" olarak adlandırılan yeni bir AB düzenlemesi uygulanmaktadır (EC, 2008; EC, 2010). Kalite kontrol/güvenlik personeli veya alımdan sorumlu diğer personeller hammaddenin (balık) güvenli bir alandan (çiftlik veya toplama alanı) gelip gelmediğini kontrol etmekle yükümlüdür. Balık ve diğer malzemelerle birlikte paketleme malzemeleri için de benzer bir tutum uygulanmaktadır. Gelen materyal güvenilir olmayan bir kaynaktan temin edildiyse, personel ürünü reddetme hakkına sahiptir. Her ne kadar gelen materyaller için gıda güvenliği açısından gerekli sertifikaları kontrol etmek önleyici tedbirler için yeterli olsa da, HACCP (Hazard Analysis Critical Control Point-Tehlike Analizi Kritik Kontrol Noktası) planının bir parçası olan doğrulama prosedürleri için zaman zaman numunelerin akredite bir laboratuvara gönderilmesi önerilmektedir.

Balık, üretimden ya da avlanmadan itibaren tüketim noktalarına ulaşana kadar dağıtım, işleme ve muhafaza aşamalarında oldukça hızlı ve kolay bozulabilen bir gıdadır. Taze balığın ve deniz ürünlerinin bozulmasında kompleks bir mikroflora rol oynamaktadır. Taze balıkların, balıkçılarda işlem öncesi faaliyetlere bağlı olarak bozulmaya başladığı ve bu balıkların dondurulduktan sonraki aşamalarda yetersiz ve uygun olmayan satış koşullarında bulunması, bozulma sürecini hızlandıran önemli faktörler arasında değerlendirilmektedir. Balıkların genel mikrobiyolojik kalitesi ve raf ömrü üzerine etkili olan aerob mezofil ve psikrofil mikroorganizmalar saprofit nitelikte olduklarından balıklardaki düzeyleri bozulma sürecine direkt etki etmektedir. Spesifik patojenler ile indikatör mikroorganizmaların üründeki varlığı, özellikle az pişmiş ya da çiğ tüketim durumlarında ciddi halk sağlığı tehlikeleri ile ilişkilendirilmektedir. Yerel balıkçılarda, sağlıksız depolama tesisleri ve uygun olmayan muhafaza sıcaklıklarına bağlı olarak şekillenen hızlı bakteriyel çoğalma, özellikle Afyonkarahisar gibi deniz balıklarının uzun transport süreçlerinden sonra geldiği şehirlerde, önemli bir sorun olarak karşımıza çıkmaktadır.

Bu çalışmanın sonuçlarına dayanarak, tüm taze balık satış noktalarında (örn., taze balık satıcılar, toptancılar, marketler) düzenli denetimlerin yapılmasının, kontaminasyon kaynaklarının en aza indirilebilmesi için hijyen eğitimlerinin verilmesinin, taze balığa ilişkin mikrobiyal limit değerlerin belirlenmesinin halk sağlığının korunması noktasında gerekli olduğu düşünülmektedir.

KAYNAKLAR

Acco M, Ferreira FS, Henriques JAP, Tondo EC. Identification of multiple strains of *Staphylococcus aureus* colonizing nasal mucosa of food handlers. Food Microbiology. 2003; 20(5): 489-493.

Adenike OMojisola. The effect of different processing methods on the nutritional quality and microbiological status of cat fish (Clarias lezera). Food Processing and Technology. 2014; 5:6.

Adeyemo OK. Consequences of Pollution and Degradation of Nigerian Aquatic environment on Fisheries Resources. The Environmentalist 2003; 23(4): 297-306.

Alak G, Hisar SA, Hisar O, Kaban G, Kaya M. Microbiological and chemical properties of bonito fish (*Sarda sarda*) fillets packaged with chitosan film, modified atmosphere and vacuum. Kafkas Universitesi Veteriner Fakültesi Dergisi. 2010; 16: 73-80.

Alparslan Y, Metin C, Hasanhocaoğlu Yapıcı H, Baygar T. Köyceğiz lagününde avlanan kefal türlerinin duyusal, kimyasal ve mikrobiyolojik kalitesinin belirlenmesi. Journal of Aquaculture Engineering and Fisheries Research. 2017; 3(4): 160-170.

Alvarez Pellitero P. Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. Veterinary Immunology and Immunopathology. 2008; 126: 171-198.

Anon. Türk Gıda Kodeksi Mikrobiyolojik Kriterler Yönetmeliği, http://www.resmigazete.gov.tr/eskiler/2011/12/20111229m3-6.htm. 2011; Ankara.

Anon. Gıda Tarım ve Hayvancılık Bakanlığı, Balıkçılık ve Su Ürünleri Genel Müdürlüğü, Su Ürünleri İstatistikleri.www.tarimorman.gov.tr/sgb/Belgeler/SagMenuVeriler/BSGM.pdfhttp://www.tarim.gov.tr/BSGM. 2018; Ankara.

Attouchi M, Sadok S. The effect of powdered thyme sprinkling on quality changes of wild and farmed gilthead sea bream fillets stored in ice. Food Chemistry. 2010; 119: 1527-1534.

Ayulo AMR, Machado RA, Scussel VM. Enterotoxigenic *Escherichia coli* and *Staphylococcus aureus* in fish and seafood from the southern region of Brazil. International Journal of Food Microbiology. 1994; 24(1-2): 171-178.

Begum M, Abu Ahmed AT, Das M, Parveen S. Acomparative microbiological assesment of five types of selected fishes collected from two different market. Advances in Biological Research. 2010; 4(5): 259-265.

Bennett RW, Lancette GA: *Staphylococus aureus*. **In,** Bacteriological Analytical Manual. 7th ed. 1992; 161-164, AOAC, Arlington.

Blood RM, Curtis GDW. Media for 'total' enterobacteriaceae, coliforms and *Escherichia coli*. International Journal of Food Microbiology. 1995; 26(1): 93-115.

Bojanic KL, Kozacinski I, Filipovic Z, Cvrtila N, Zdolec NB. Quality of sea bass meat during storage on ice. MESO. 2009; 11: 70-74.

Boulares M, Mejri L, Hassouna M. Study of the microbial ecology of wild and aquacultured Tunisian fresh fish. Journal of Food Protection. 2011; 74(10): 1762-1768.

Boulares M, Mankai M, Aoudhi C, Moussa Olfa B, Hassouna M. Characterisation and identification of spoilage psychotrophic Gram-negative bacteria originating from Tunisian fresh fish. Annual of Microbiology. 2013; 63: 733-744.

Budiati T, Rusul G, Wan-Abdulla WN, Ahmad R, Mat Arip Y. Microbiological Quality of Catfish (Clarias Gariepinus) and Tilapia (Tilapia Mossambica) Obtained from Wet Markets and Ponds in Malaysia. Aquaculture Research and Development. 2015; 6: 1.

Buras N, Duek L, Niv S, Hepher B, Sandbank E. Microbiological aspects of fish grown in treated wastewater. Water Research. 1987; 21(1): 1-10.

Cardinal M, Gunnlaugsdottir H, Bjoernevik M, Ouisse A, Vallet JL. Sensory characteristics of cold-smoked Atlantic salmon (Salmo salar) from European market and relationships with chemical, physical and microbiological measurements. Food Research International. 2004; 37: 181-193.

Chytiri S, Chouliara I, Savvaidis IN, Kontominas MG. Microbiological, chemical and sensory assessment of iced whole and filleted aquacultured rainbow trout. Food Microbiology. 2004; 21: 157-165.

Cousin MA, Jay JM, Vasavada PC. Psychrotrophic microorganisms, In: Downes FP., Ito K. (Eds.), Compendium of methods fort he microbiological examination of foods, 4th ed. American Public Health Association, Washington, D.C. 2001; 159-166.

De Boer E. Update on media for isolation of enterobacteriaceae from foods. International Journal of Food Microbiology. 1998; 45(1): 43-53.

Del Rio-Rodriguez, Inglis V, Millar SD. Survival of *Escherichia coli* in the intestine of fish. Aquaculture Research.]997; 28: 257-264.

Diercke M, Kirchner M, Claussen K, Mayr E. Transmission of shiga toxin-producing *Escherichia coli* O104:H4 at a family party possibly due to contamination by a food handler, Germany 2011. Epidemiology and Infection. 2014; 142(1): 99-106.

Diop MB, Destain J, Tine E, Thonart P. Les produits de la mer au sénégal et le potentiel des bactéries lactiques et des bactériocines pour la conservation. Biotechnology Agronomy Society and Environment. 2010; 14: 341-350.

EC. Establishing a community system to prevent, deter and eliminate illegal, unreported and unregulated fishing, amending regulations (EEC) No 2847/93, (EC) No 1936/2001 and (EC) No 601/2004 and repealing regulations (EC) No 1093/94 and (EC) No 1447/1999. 2008; http://eur-lex.europa.eu/LexUriServ /Lex UriServ.do?uri=OJ :L: 2008:286:0001:0032.

EC. Summary record of the meeting of working group 3 (markets and trade policy) of the advisory committee on fisheries and aquaculture. 2010; http://ec.europa.eu/fisheries/dialog/acfa120608_en.pdf

El-Shafai SA, Gijzen HJ, Nasr FA, El-Gohary FA. Microbial quality of tilapia reared in fecal-contaminated ponds. Environmental Research. 2004; 95(2): 231-238.

Fagan RP, McLaughlin JB, Castrodale LJ, Gessner BD, Jenkerson SA,2 Funk EA, Hennessy TW, Middaugh JP, Butler JC. Endemic Foodborne Botulism among Alaska Native Persons-Alaska, 1947–2007. Clinical Infectious Diseases. 2011; 52(5): 585–592.

FAO. Food and Drug Administration. Report of the global conference an Aquaculture 2010. Farming the waters for people and food. FAO Fisheries and Aquaculture. 2012; 84, Rome.

Fattal B, Dotan A, Tchorsh Y. Rates of experimental microbiological contamination of fish exposed to polluted water. Water Research. 1992; 26(12): 1621-1627.

Flowers RS, D'aoust JY, Andrews WH, Bailcy JS. *Salmonella.* **In,** Compendium of the Methods for the Microbiological Examination of Foods. 1992; 685.

Franzetti L, Scarpellini M, Mora D, Galli A. *Carnobacteriu*m spp. in seafood packaged in modified atmosphere. Annual of Microbiology. 2003; 53: 189-198.

Galvez A, Abriouel H, Ben Omar N, Lucas R. Microbial antagonists to food-borne pathogens and biocontrol. Current Opinion Biotechnology. 2010; 21: 142-148.

Gerokomou V, Voidarou C, Vatopoulos A, Velonakis E, Rozos G, Alexopoulos A, Plessas S, Stavropoulou E, Bezirtzoglou E, Demertzis PG, Akrida-Demertzi K. Physical, chemical and microbiological quality of ice used to cool drinks and foods in Greece and its public health implications.

Anaerobe. 2011; 17(6): 351-353.

Ghaly AE, Dave D, Budge S, Brooks MS. Fish spoilage mechanism and preservation techniques review. American Journal of Applied Sciences. 2010; 7(7): 859-877.

Gram L, Huss HH. Microbiological spoilage of fish and fish products. International Journal of Food Microbiology. 1996; 33 121-137.

Goja AM. Microbiological assessment of three types of fresh fish (Tilapia niloticus, Labeo niloticus and Hydrocynus spp.) sold in Ed Dueim, Sudan. New York Science Journal. 2013; 6(4): 49-54.

Gram L, Dalgaard P. Fish spoilage bacteria – problems and solutions. Current Opinion Biotechnology. 2002; 13: 262-266.

Gram L, Huss HH. Fresh and processed fish and shellfish. In: Lund BM, Baird-Parker AC, Gould GW (eds) The microbiological safety and quality of foods, 1st edn. Chapman and Hall, London. 2000; 472-506.

Guzman MC, Bistoni MA, Tamagni LM, Gonzalez RD. Recovery of *Escherichia coli* in fresh water fish, Jenynsia multidentata and Bryconamericus iheringi. Water Research. 2004; 38(9): 2368-2374.

Hernandez MD, Lopez MB, Alvarez A, Ferrandini E, Garcia GB, Garrido MD. Sensory, physical, chemical and microbiological changes in aquacultured meagre (*Argyrosomus regius*) fillets during ice storage. Food Chemistry. 2009; 114: 237-245.

Huang SL, Weng,YM, Chiou RYY. Survival of *Staphylococcus aureus* and *Escherichia coli* as Affected by Ethanol and NaCl. Journal of Food Protection. 2001; 64(4): 546-550.

International Organization for Standardization (ISO 15214). Microbiology of food and animal feding stuffs-Horizontal methods for the enumeration of mesophilic lactic acid bacteria. Colony-count technique at 30°C. 1998; Geneva, Switzerland.

International Organization for Standardization (ISO/DIS 17410). Microbiology of the food chain-Horizontal method for the enumeration of psychrotrophic microorganisms. 2001; Geneva, Switzerland.

International Organization for Standardization (ISO 6579). Microbiology of food and animal feeding stuffs-horizontal method for the detection of *Salmonell*a spp. 2002; Geneva, Switzerland.

International Organization for Standardization (ISO 4833). Microbiology of food and animal feeding stuffs-horizontal methods for the enumeration of microorganisms. Colony-count technique at 30°C. 2003; Geneva, Switzerland.

İnanlı AG, Karaton N, Çoban ÖE. Sensoral, chemical and microbiological quality of anchovy cake. African Journal of Biotechnology. 2011; 10(48): 98-70-9874.

Jain S, Chen L, Dechet A, Hertz AT, Brus DL, Hanley K, Wilson B, Frank J, Greene KD, Parsons M, Bopp CA, Todd R, Hoekstra M, Mintz ED, Ram PK. An Outbreak of Enterotoxigenic Escherichia coli Associated with Sushi Restaurants in Nevada, 2004. Clinical Infectious Diseases 2008; 47:1-7.

Jablonski LM, Bohach G. *Staphylococcus aureus*. In: Doyle MP, Beuchat LR, Montville TJ. Food Microbiology. Fundamentals and Frontiers. 2001; ASM Press, Wahington, DC.

Jacobsen C, Bekaert K, Saebo A and Oehlenschläger J. Seafood Research from Fish to Dish - Quality, Safety & Processing of Wild and Farmed Seafood. Wageningen Academic Publishers. 2006; Netherlands.

Joffraud JJ, Leroi F, Roy C, Berdagué JL. Characterization of volatile compounds produced by bacteria isolated from the spoilage flora of cold-smoked salmon. International Journal of Food Microbiology. 2001; 66: 175-184.

Junior PG, Assunçao AWA, Baldin JC, Amaral LA. Microbiological quality of whole and filleted shelf-tilapia. Aquaculture. 2014; 433: 196-200.

Kocatepe D, Taşkaya G, Kaya Y, Turan H, Erkoyuncu İ. Farklı balıkçı tezgahlarının mikrobiyolojik yönden incelenmesi. Biyoloji Bilimleri Araştırma Dergisi. 2011; 4(2): 73-77.

Kapute F, Likongwe J, Kang'ombe J, Kiiyukia C, Mpeketula P. Quality assessment of fresh lake *Malawi Tilapia* (Chambo) collected from selected local and super markets in Malawi. Internet Journal of Food Safety. 2012; 14: 112-120.

Kasai K, Ishikawa T, Komata T, Fukuchi K, Chiba M, Nozaka H, Nakamura T, Sato T, Miura T. Novel L-amino acid oxidase with antibacterial activity against methicillin-resistant *aureus Staphylococcus* isolated from epidermal mucus of the flounder *stellatus Platichthys*. The FEBS Journal. 2010; 277: 453-465.

Kostic AD, Howitt MR, Garrett WS. Exploring host–microbiota interactions in animal models and humans. Genes and Development. 2013; 27: 701-718.

Koutsoumanis K, Giannakourou MC, Taoukis PS, Nychas GJE. Application of shelf life decision system (SLDS) to

marine cultured fish quality. International Journal of Food Microbiology. 2002; 73: 375-382.

Ligia AS, Prinyawiwatkul W, King JM, Kyoon H, Bankston JD, Ge B. Effect of preservatives on microbial safety and quality of smoked blue catfish (Ictalurus furcatus) steaks during roomtemperature storage. Food Microbiology. 2008; 8: 958-963.

Long Y, Li Q, Zhou B, Song G, Li T, Cui Z. De novo assembly of mud loach (Misgurnus anguillicaudatus) skin transcriptome to identify putative genes involved in immunity and epidermal mucus secretion. Plos One. 2013; 8(2): 1-14.

Lyhs U, Korkeala H, Vandamme P, Bjorkroth J. *Lactobacillus alime*ntarius: a specific spoilage organism in marinated herring. International Journal of Food Microbiology. 2001; 64: 355-360.

Matamoros S, Pilet MF, Gigout F, Prevost H, Leroi F. Selection of psychotrophic bacteria active against spoilage and pathogenic micro-organisms relevant for seafood products. 2006; Wageningen Academic Publishers. 2006; Netherlands.

Mol S., Tosun ŞY. The quality of fish from retail markets in İstanbul, Turkey. Journal of Fisheries Sciences. 2011; 5(1): 16-25.

Nelson JS, Grande TC, Wilson MVH. Fishes of the World. John Wiley and Sons Pulishers. 2016; Canada.

Nilla SS, Khan AR, Khan MR, Ahsan DA, Mustafa G. Bacteriological quality of marketed mola fish, *Amblypharyngodon mola* from Dhaka metropolis. Bangladesh Journal of Zoology. 2012: 40(1): 77-88.

Nygren BL, Schilling KA, Blanton EM, Silk BJ. Foodborne outbreaks of shigellosis in the USA, 1998-2008. Epidemiology and Infection. 2013; 141(2): 233-241.

Odu NN, Imaku LN. Assesment of the microbiological quality of street-vended ready-to-eat bole (roasted plantain) fish (*Trachurus Trachurus*) in port harcourt metropolis, Nigeria. Researcher. 2013; 5(3): 9-18.

Ozden O, Inugur M, Erkan N. Effect of different dose gamma radiation and refrigeration on the chemical and sensory properties and microbiological status of aquacultured sea bass (Dicentrarchus labrax). Radiation Physics and Chemistry. 2007; 76: 1169-1178.

Pal D, Gupta CD. Microbial pollution in water and its effect on fish. Journal Aquatic Animal Health. 1992; 4: 32-39.

Pıérard D, Crowcroft N, De bock S, Potters D, Crabbe G, Van loock F, Lauwers S. A case-control study of sporadic infection with O157 and non-O157 verocytotoxin-producing *Escherichia coli.* Epidemiology and Infection. 1999; 122(3): 359-365.

Pillay TVR. Water and wastewater use. **In,** Pillay TVR. Aquaculture and the environment. Cambridge University Press. 1992; 49-55, England.

Poli MB, Parisi G, Zambacavallo G, Mecatti M, Lupi P, Gualtieri M, Franci O. Quality outline of European sea bass (Dicentrarchus labrax) reared in Italy: shelf life, edible yield, nutritional and dietetic traits. Aquaculture. 2001; 202: 303-313.

Popovic NT, Skukan AB, Dzıdara P, Coz-Rakovac R, Strunjak-Perovic I, Kozacınskı L, Jadan M, Brlek-Gorski D. Microbiological quality of marketed fresh and frozen seafood caught off the Adriatic coast of Croatia. Veterinarni Medicina. 2010; 55(5): 233-241.

Rakers S, Gebert M, Uppalapati S, Meyer W, Maderson P, Sell AF, Kruse C. Fish matters': the relevance of fish skin biology to investigative dermatology. Experimental Dermatology. 2010; 19(4): 313-324.

Rahal EG, Abd El Rahman H, Ismail SAS. Bacteriological Quality Assessment of Pagha fish (*Trachurus Indicus*) in Suez city fish markets. Department of Food Hygiene and Control, Faculty of Veterinary Medicine. Suez Canal University. 1981; 1-9.

Redmond EC, Griffith CJ. The importance of hygiene in the domestic kitchen; implications for preparation and storage of food and infant Formula. Perspective Public Health. 2009; 129: 69-76.

Roberts TA, Cordier JL, Gram L, Tompkin RB, Pitt JI, Gorris LGM, Swanson KMJ. Fish and fish products microorganisms in food. Springer. 2005; 174-249, USA.

Satomi M. Scombroid (histamine) poisoning associated with seafood. **In,** Bari L, Yamazaki K. Seafood Safety and Quality. 2018; Chapter 12, CRC Press, US. Shao D, Shi Z, Wei J , Ma Z. A brief review of foodborne zoonoses in China. Epidemiology and Infection. 2011; 139(10): 1497-1504.

Suhalima R, Huanga YW, Burtleb GJ. Survival of *Escherichia coli* O157:H7 in channel catfish pond and holding tank water. LWT. 2008; 41: 1116-1121.

Tavakoli HR, Soltani M, Bahonar A. Isolation of some human pathogens from fresh and smoked shad (*Alosa kessleri*) and silver carp (*Hypophthalmichthys molitrix*). Iranian Journal of Fisheries Sciences. 2012; 11(2): 424-429.

Tripathy S, Kumar N, Mohanty S, Samanta M, Mandal RN, Maiti NK. Characterisation of *Pseudomonas aeruginosa* isolated from freshwater culture systems. Microbiology Research. 2007; 162: 391-396.

Vollaard AM, Ali S, Van Asten HAGH , Ismid IS, Widjaja S, Visser LG, Surjadi Ch, Van Dissel JT. Risk factors for transmission of foodborne illness in restaurants and street vendors in Jakarta, Indonesia. Epidemiology and Infection. 2004; 132(5): 863-872.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):158-167
DOI: 10.30607/kvj.526779

RESEARCH ARTICLE

Comparative Examination of Commonly Used Some Fixatives with Routine Histochemical Staining's for The Optimal Histological Appearance in The Gill Tissue of Zebrafish

Aykut ULUCAN[1]*, Hayati YÜKSEL[2], Muhammed Bahaeddin DÖRTBUDAK[2], Seda YAKUT[3]

[1]Bingol University, Vocational School of Health Services, Department of Medical Services and Techniques, Turkey
[2]Bingol University, Faculty of Veterinary Medicine, Department of Pathology, Turkey
[3]Bingol University, Faculty of Veterinary Medicine, Department of Histology and Embryology, Turkey

ABSTRACT

Histopathological studies related to Zebrafish which are used as a model organism in researching the pathogenesis of many diseases increase day by day. Because of the increasing importance of zebrafish research, experiments on this animal model are more prominent and gill tissue is frequently examined in various disease models using zebrafish. As it is known, at the end of the experimental animal model studies, if the histopathological examination is to be done, the tissues should be fixed. The purpose of fixation is to keep cellular and extracellular components in vivo as much as possible. Therefore, the choice of fixation methods and fixatives has a significant effect on tissue processing. In this study, we aimed to optimize fixation techniques and staining protocols for producing ideal slides and histological images of gill tissue in zebrafish. In our study, to determine the optimal histology in the zebrafish-gill tissue, the tissues were fixed with Bouin's, Carnoy's, 10% neutral buffered formalin (NBF), Davidson's, and Dietrich's solutions. Following the routine tissue processing, the sections were stained with Hematoxylin and Eosin (H&E) and Masson's Trichrome (MT) stains. Consequently, tissue morphology was best preserved with Bouin's and NBF solutions. The best results in H&E stain were obtained from tissues fixed with NBF solution, while Dietrich's solution fixation was found to be ideal for MT staining.
Keywords: Fixatives, Hematoxylin and Eosin, Masson's Trichrome, Zebrafish, Gill

***

Zebra Balığı Solungaç Dokusunda Optimal Histolojik Görünüm için Rutin Histokimyasal Boyama ile Sık Kullanılan Fiksatiflerin Karşılaştırmalı Olarak İncelenmesi

ÖZ

Birçok hastalığın patogenezinin araştırılmasında model bir organizma olarak kullanılan Zebra balığı ile ilgili histopatolojik çalışmalar gün geçtikçe artmaktadır. Zebra balığı araştırmalarının artan önemi nedeniyle, bu hayvan modelindeki deneyler daha önemlidir ve solungaç dokusu zebra balığı kullanılan çeşitli hastalık modellerinde sıklıkla incelenir. Bilindiği üzere, deneysel hayvan modeli çalışmalarının sonunda, eğer histopatolojik inceleme yapılacaksa, dokular fikse edilmelidir. Fiksasyonun amacı, hücresel ve hücre dışı bileşenleri mümkün olduğunca in vivo halde korumaktır. Bu nedenle, fiksasyon yöntemlerinin ve fiksatiflerin seçiminin doku takibi üzerinde önemli bir etkisi vardır. Bu çalışmada, zebra balığı solungaç dokusundan ideal preparatlar ve histolojik görüntüler elde etmek için fiksasyon tekniklerini ve boyama protokollerini optimize etmek amaçlanmıştır. Çalışmamızda, zebra balığı-solungaç dokusundaki ideal histolojiyi belirlemek için, dokular, Bouin, Carnoy, %10'luk nötral tamponlu formaldehit, Davidson ve Dietrich solüsyonları ile fikse edilmiştir. Rutin doku işleminin ardından, kesitler Hematoksilen ve Eosin (H&E) ve Masson Trikrom (MT) boyaları ile boyandı. Sonuç olarak, doku morfolojisi en iyi Bouin ve NBF solüsyonları ile korunmuştur. H&E boyamasında en iyi sonuçlar NBF çözeltisi ile fikse edilmiş dokulardan elde edilirken, Dietrich solüsyonu fiksasyonunun MT boyaması için ideal olduğu bulundu.
Anahtar Kelimeler: Fiksatifler, Hematoxylin ve Eosin, Masson Trikrom, Zebra balığı, Solungaç

To cite this article: Ulucan A. Yüksel H. Dörtbudak M.B. Yakut S. Comparative Examination of Commonly Used Some Fixatives with Routine Histochemical Staining's for The Optimal Histological Appearance in The Gill Tissue of Zebrafish. Kocatepe Vet J. (2019) 12(2):158-167.

Submission: 13.02.2019 **Accepted**: 26.04.2019 **Published Online**: 15.05.2019
ORCID ID; AU: 0000-0001-8844-8237, HY: 0000-0002-1724-1770, MBD: 0000-0001-5777-964X, SY: 0000-0003-1673-5661
*Corresponding author e-mail: aulucan@bingol.edu.tr

INTRODUCTION

The importance of histological and pathological examinations is unquestionable when evaluating the results of screening studies with fish models (disease research, environmental pollutants, toxicological-carcinogenic-teratogenic research, etc.) (Pikarsky et al. 2004, Flores-Lopes and Thomaz 2011, Strzyzewska et al. 2016, Hadi and Alwan 2012, Huiting et al. 2015). In today's scientific studies, zebrafish is an important model organism in terms of having some advantages. These advantages are similar genomic structures, metabolic activities, embryonic developmental characteristics, immune systems, and histological structures in some tissues (Mohseny and Hogendoorn 2014, Morris 2009). In addition, features such as easy breeding, feeding, short generation, durability, and high fecundity have increased the importance of zebrafish in experimental researches (Muto and Kawakami 2013, Newman et al. 2014).

Gill tissue is examined in most of the toxic and infectious disease models made with zebrafish (Brundo and Salvaggio 2018, Souza et al. 2017, Progatzky et al. 2016, Chandrarathna et al. 2018, Christoffersen et al. 2017). Most of the teleosts use the gills as the main respiratory surface. Teleosts have four pairs of gill arches extending from the floor to the roof of the buccal cavity. Each gill arch contains a plurality of gill filaments, each consisting of two halves called hemibranchs. Each hemibranch has many thin subdivisions called gill lamellae. The purpose of these structures is to provide a large surface area that supports respiratory and excretory functions. Gill filaments have central cartilaginous support, afferent and efferent arterioles, and other anastomosing vessels comprising the central venous sinus. They are covered with a thin epithelium continuation of the covering the gill arches and the oral mucosa of the buccal cavity. Each lamella is best regarded as a thin cell envelope. The spaces between the pillar cells called lacunae connect afferent and efferent arterioles. The contractile pillar cells control the lacunar diameter, thereby regulating blood flow (Genten et al. 2010, Roberts 2012).

The aim of fixation is to keep cellular and extracellular components as close as possible to the tissue structure in vivo. In the fixation process with many fixatives, the existing microorganisms in the tissues die, autolysis stops and the macromolecular structure of the tissue is balanced by forming cross-links between the tissue components. Fixation without specimen distortion is difficult because all fixation methods produce artifacts such as shrinkage, swelling, hardening, and color change. Moreover, fixation and fixative selection may be of great importance in the tissue processing stages, mainly in cross-sectional sampling and staining procedures (Bancroft and Gamble 2019).

In this study is intended to be a resource for scientists interested in human and model organism histology, quality, consistency, sectioning, staining, and histological visualization. We aim to optimize fixation techniques and staining protocols to produce ideal slides and images to explain common difficulties associated with various aspects of zebrafish histology. In our study, the effect of fixation with Bouin's, Carnoy's, 10% neutral buffered formaldehyde (NBF), Davidson's and Dietrich's solutions on the zebrafish gill tissue histology as a result of hematoxylin and eosin (H & E) and Masson's Trichrome (MT) staining were examined. Also, we tried to develop the fixation methods with compared of Bouin's, Carnoy's, NBF, Davidson's and Dietrich's solutions and, adapted staining procedures with H&E and MT stains for gill tissue of fish.

Bouin's solution is suitable for a variety of tissue types and is frequently recommended for small biopsy samples. It is known that Bouin's solution lyses erythrocytes in part or completely. It has been reported that it causes swelling in collagen with the dissolution of iron and calcium stores, but it protects the glycogen and connective tissue well. It creates bright staining with cytoplasmic dyes and protects the nucleus extremely well with chromosomes. Since the basophilic character of the cytoplasm is neutralized with the picric acid in the content of Bouin's solution, it allows clear staining of the nucleus and cytoplasm (Carson and Hladik 1996, Culling 1983, Singhal et al. 2016, Roberts 2012, Bancroft and Gamble 2019).

Carnoy's solution is used in cases where fast and effective fixation is required and suitable for the fixation of all types of tissue. It is good advice for glycogen and plasma cells. Since it is a good nuclear fixative, it is also used in cases where nucleic acids need to be examined. It is also preferred for histochemical and specific staining of fibrous proteins and carbohydrate complexes. After fixation, the lysis of erythrocytes and soluble cell granules in acid can cause loss of pigments in tissues. It has been reported that it cannot protect the collagen tissue and does not stain the acid-fast bacilli well. The Carnoy's solution is expensive and also has the disadvantage of fixative, such as shrinkage in tissues (Ahmed et al. 2011, Pereira et al. 2015, Roberts 2012).

NBF is a buffered form of 10% formalin solution to pH 7.0 ± 0.2 with various phosphate salts. This buffering process eliminates the various disadvantages of formaldehyde, such as the lysis of erythrocytes and the formation of formalin pigment (especially blood vessels and bloody tissues), and provides longer-term use. In NBF fixation, aldehyde bonds are formed between proteins and therefore the in vivo relationships of tissue components remain

constant. Various tissue samples (surgical samples and biopsies) are routinely fixed in formaldehyde (Gnanapragasam 2010). Formalin fixation paralyzes cell metabolism and preserves tissue structures for accurate histopathological diagnosis (Nechifor-Boilă et al. 2015). Formaldehyde in the NBF form is the most commonly used fixative because it preserves a large range of tissues and tissue components and is also quite inexpensive (Maraschin et al. 2017). This fixative is useful in many special dyes and even immunohistochemical applications (Ahmed et al. 2011, Spaniol et al., 2016, Stefanović et al. 2017, Roberts 2012).

Davidson's solution is a good choice for the fixation of various tissues, such as bone marrow samples, gynecological materials, breast tissue, tumors, especially eyes and testicular tissues, lymph nodes, and biopsy materials. It provides fast and effective penetration to the tissues, so the morphological details are well preserved. One of the most important advantages of fixative is that does not cause shrinkage in tissues and minimal formalin pigmentation. This fixative can quickly penetrate into tissues and is easily removed from the tissues, so there is no need to rinse after long-term fixation. But, it may also lead to lysis of erythrocytes. Immunohistochemical staining results were also successful in fixation with this solution (Eltoum et al. 2001, Howard et al. 2004, Latendresse et al. 2002, Sahota et al. 2013, Telser, 2006).

Dietrich's solution is used in most experimental research, using many aquatic animals, zebrafish and small animal samples such as copepods (zooplankton), and one of the aquatic vertebrates like amphipods. The fixative has a synergistic interaction with the decalcification process. It breaks down electrostatic and hydrogen bonds and may lead to denaturation and degradation of nucleic acids with proteins in tissue samples. It is more effective in the fixation of nucleoproteins than cytoplasmic proteins (Dietrich and Krieger 2009, Feist et al. 2004, Berzins et al. 2011, Peterson et al. 2013, Khan and Thulin 1991, Uslu et al. 2017).

It has been reported that histochemical dyes such as H&E, MT, Periodic Acid Schiff (PAS), Paraldehyde Fuchsin (PAF), Toluidine blue, Alcian blue, Alizarin red, Oil red, Bodian Protargol and Von Kossa stains have been used in studies performed in zebrafish (Bensimon-Brito et al. 2012, Çakıcı and Üçüncü 2007, Nusslein-Volhard and Dahm 2002, Ung et al. 2010).
In our study, H&E and MT stains, the most common histological staining methods used in routine histopathology, were preferred. Hematoxylin stains nucleic acids in the nucleus and Eosin stains cytoplasm and extracellular proteinous structures. H&E provides light microscopic observation of tissues and intercellular structures; therefore, in a well-made tissue, intranuclear details can be distinguished. H&E staining is also used to provide adequate general information about cells and tissues and to diagnose major histopathological changes. It has advantages such as easy application, reliable and low cost (Kumar and Kiernan 2010, Bancroft and Gamble 2019).

Compared to H&E staining, it is preferred for a more specific diagnosis by demonstrating increased connective tissue and collagen fibers with MT staining. It allows the cells to be distinguished from collagen fibers around them. Through Wiegert's ferrous hematoxylin in MT, various granules and fibrillar structures, myelin sheath, muscle epithelium, and even spirochetes and cytoplasmic details are more clearly displayed. In MT staining, ponceau and acid fuchsin, together with aniline blue or methyl green, help to stain the connective tissue prominently in bluish or light green. With MT staining, the diagnosis of disorders such as collagen growth, tumors (especially muscle tissue and fibroblast origin), hematopathological, neuromuscular, and dermatological disorders and liver cirrhosis and kidney (pyelonephritis) diseases can be diagnosed. Bouin's, Zenker's, Helly, Formal sublimate, Formalin-zinc, and Picro-mercuric alcohol fixatives have been reported to be the other ideal fixatives for this staining method (Martinello et al. 2015, Carson and Hladik 1996, Mao et al. 2016, Cima et al. 2017, Rahunen et al. 2016).

MATERIALS and METHODS

In this study, 30 zebrafish (*Danio rerio*, wild type, 1-year-old) were divided into five groups with 6 fish in each group (n = 6). The study materials were taken from our earlier work (The specified study was carried out at Bingol University Animal Research Center with the permission of Bingol University Animal Experiments Local Ethics Committee dated 26.09.2014 and numbered 2014.09.02 and this study was partially supported by The Scientific and Technological Research Council of Turkey (TUBITAK) with 215Z467 numbered project.

Zebrafish care and maintenance: Established protocols for zebrafish care and feeding were applied (Westerfield 2000).

Preparation and application of fixatives: Fixatives used in the study (Bouin's, NBF, Carnoy's, Davidson's and Dietrich's fixatives), fixation times and post-fixation applications are given in Table 1. In addition, the chemicals and their amounts used in the preparation of fixatives are shown in the table state for each fixative (Table 2). Fixation was performed at room temperature (21°C) for all samples. After fixation, decalcification was performed for 5 days according to Versanate EDTA method (Mueller et al.

2017) and then tissue samples were taken for routine histopathological examination (Bancroft and Gamble 2019). We do not prefer to acidic decalcification because this destroys DNA and causes pale H&E staining.

Anesthesia, necropsy and histopathological examination: Euthanasia was performed by adding with 160 mg/L Tricaine-S pH 7.0 (tricaine methanesulfonate, Syndel, USA) in system water. It should be noted that the relative merits of Tricaine-S and/or hypothermal shock through immersion in ice water (2–4°C) have been recently debated (Matthews and Varga 2012, Wilson et al. 2009). To ensure fast euthanasia in fish, both methods (cold, Tricaine) were combined, allowing the fish do not react to the following immersion in fixatives steps. After the routine tissue processing, 5 micron thick sections were taken from the paraffin blocks with rotary microtome. The used fixative may influence staining method. For this reason, we have optimized staining procedures for both the fixative the tissues we are stained. In the present study, we preferred the H&E staining protocol, in which the study by Cooper et al. 2018 achieved optimal results for zebrafish (Copper et al. 2018). The samples were stained with MT according to previous studies (Carson and Hladik 1996, Alturkistani et al. 2016, Bancroft and Layton 2019) and MT kit (with aniline blue) was purchased commercially (Masson trichrome kit, Catalog number 04-010802; Bio-Optica, Italy).

Subsequently, the slides were evaluated under a light microscope and the results were scored. In the evaluation, general staining character of tissues with H&E and staining of collagen fibrils with MT were taken into consideration. According to this; the staining levels of tissues were classified as, no staining (0), mild staining (+1), moderate staining (+2) and severe staining (+3). In this evaluation, the scores of the group averages of samples prepared with different fixatives and stained with H&E and MT were presented graphically (Figures 1, 2).

Findings

Microscopic examination of gill tissues taken from fish in the experimental groups showed different staining densities in both H&E and MT staining. H&E and MT staining reaction scores of experimental groups obtained by histopathological examination of gill tissues are given in Figures 1 and 2.

Bouin's solution fixed tissue findings: In H&E staining, common lysis of erythrocytes, well preserved primary and secondary lamellar structure morphology and clearly differentiated pillar cells were observed. Eosinophilic and basophilic cellular structures were found to be adequately stained in a differential manner. Although collagen fibers were seen to be swollen, MT staining revealed adequate staining (Figure 3A-3B).

Carnoy's solution fixed tissue findings: In H&E staining, it was observed that widespread lysis in erythrocytes, primary and secondary lamellar structure morphology were not well protected, the lamellar epithelium was swollen and irregular, and pillar cells could not be clearly identified. Eosinophilic and basophilic cellular structures were not stained in a sufficient differential and generally, excessive staining was formed. However, it was revealed that the collagen fiber morphology was well preserved and sufficient staining was achieved with MT stain (Figure 3C-D).

NBF fixed tissue findings: In H&E staining, it was observed that the erythrocytes did not lysize and had normal histological morphology, the secondary and primary lamellar structure morphology was adequately protected, but there was a slight shrinkage in the lamellar epithelium and the pillar cells could be clearly selected. Eosinophilic and basophilic cellular structures were clearly stained in a differential manner and generally, there is ideal staining. The morphology of collagen fibers was well preserved and partially weakened by MT staining (Figure 3E-F).

Davidson's solution fixed tissue findings: In H&E staining, the presence of lysis in the erythrocytes, limited preservation of the primary and secondary lamellar structure morphology, and significant swelling and irregularity in the lamellar epithelium were detected. However, the pillar cells could be clearly selected. Eosinophilic and basophilic cellular structures were clearly stained in a differential manner and generally, there is ideal staining. It was observed that the collagen fiber morphology was well preserved and sufficient staining was achieved with MT stain (Figure 3G-H).

Dietrich's solution fixed tissue findings: In H&E staining, the presence of partial lysis of erythrocytes, primary and secondary lamellar structure morphology was partly preserved, the lamellar epithelium was slightly swollen, and the pillar cells were clearly distinguishable. Eosinophilic and basophilic cellular structures were stained differentially and in general, weak staining was determined. The morphology of the collagen fibers is well preserved and optimal staining is achieved with MT stain (Figure 3I-J).

Table 1. Fixatives, application times and post-fixation procedures.

		Fixatives				
		Bouin's	**Carnoy's**	**NBF**	**Davidson's**	**Dietrich's**
Fixation & other application times		12 h	2 h	48 h	24 h	24 h
Post-fixation applications	50% Ethanol	3 changes in 12 h	-----	-----	-----	-----
	70% Ethanol	3 changes in 12 h	-----	-----	-----	-----
	100% Ethanol	-----	12 h	-----	-----	-----

Table 2. Fixatives and their contents.

Contents	**Fixatives**				
	Bouin's	**Carnoy's**	**NBF**	**Davidson's**	**Dietrich's**
Formaldehyde	250 ml	-----	100 ml	250 ml	100 ml
Acetic acid	750 ml	100 ml	-----	125 ml	20 ml
Picric acid	50 ml	-----	-----	-----	-----
Chloroform	-----	30 ml	-----	-----	-----
Ethanol (95%)	-----	600 ml	-----	375 ml	300 ml
Distilled water	-----	-----	900 ml	375 ml	580 ml
Sodium dihydrogen phosphate, monohydrate ($NaH_2PO_4 * H_2O$)	-----	-----	4 g	-----	-----
Disodium hydrogen phosphate, anhydrous (Na_2HPO_4)	-----	-----	6,5 g	-----	-----

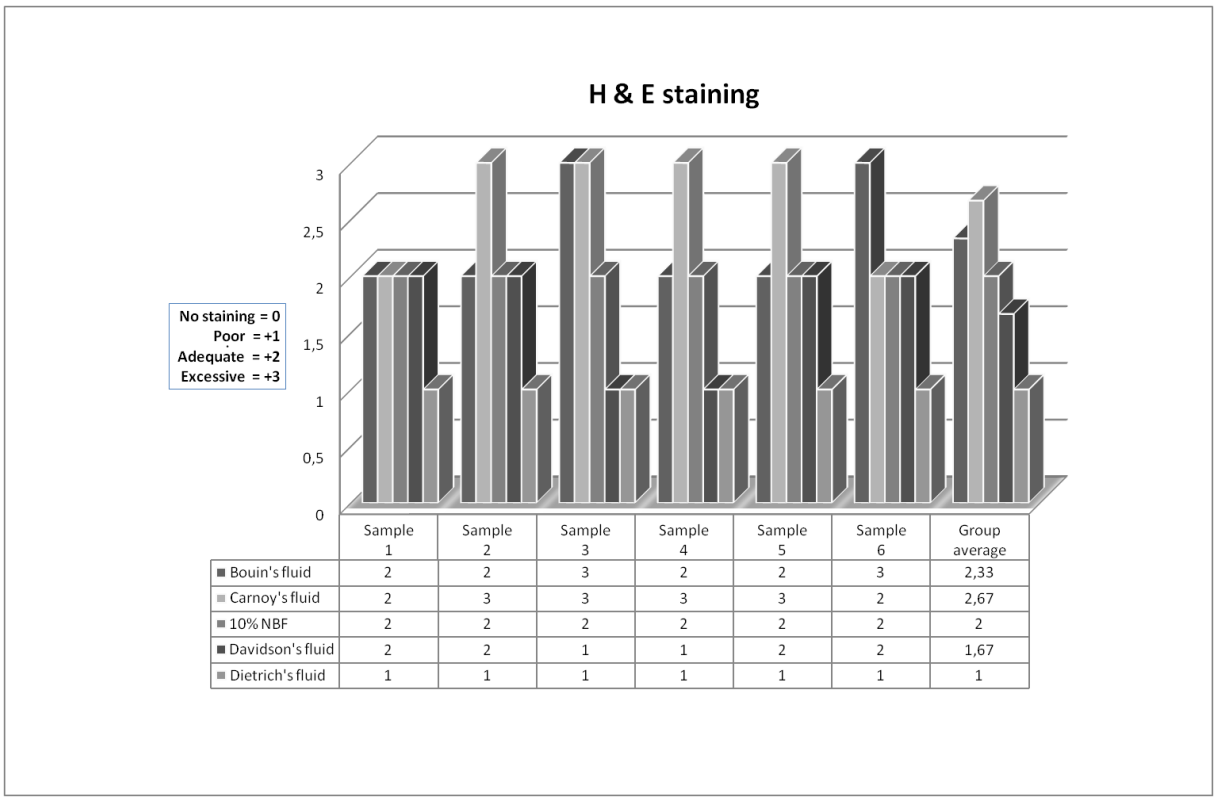
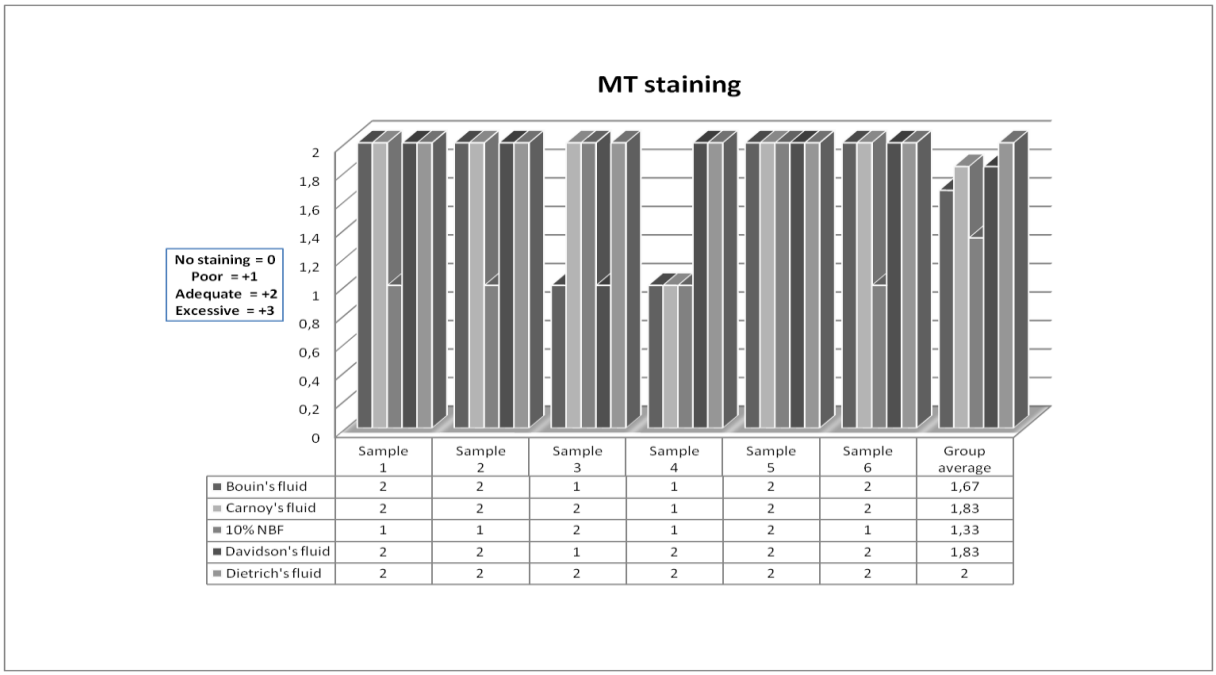


Figure 1. H&E staining scores with used different fixatives.



	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Group average
Bouin's fluid	2	2	1	1	2	2	1,67
Carnoy's fluid	2	2	2	1	2	2	1,83
10% NBF	1	1	2	1	2	1	1,33
Davidson's fluid	2	2	1	2	2	2	1,83
Dietrich's fluid	2	2	2	2	2	2	2

Figure 2. MT staining scores with used different fixatives.

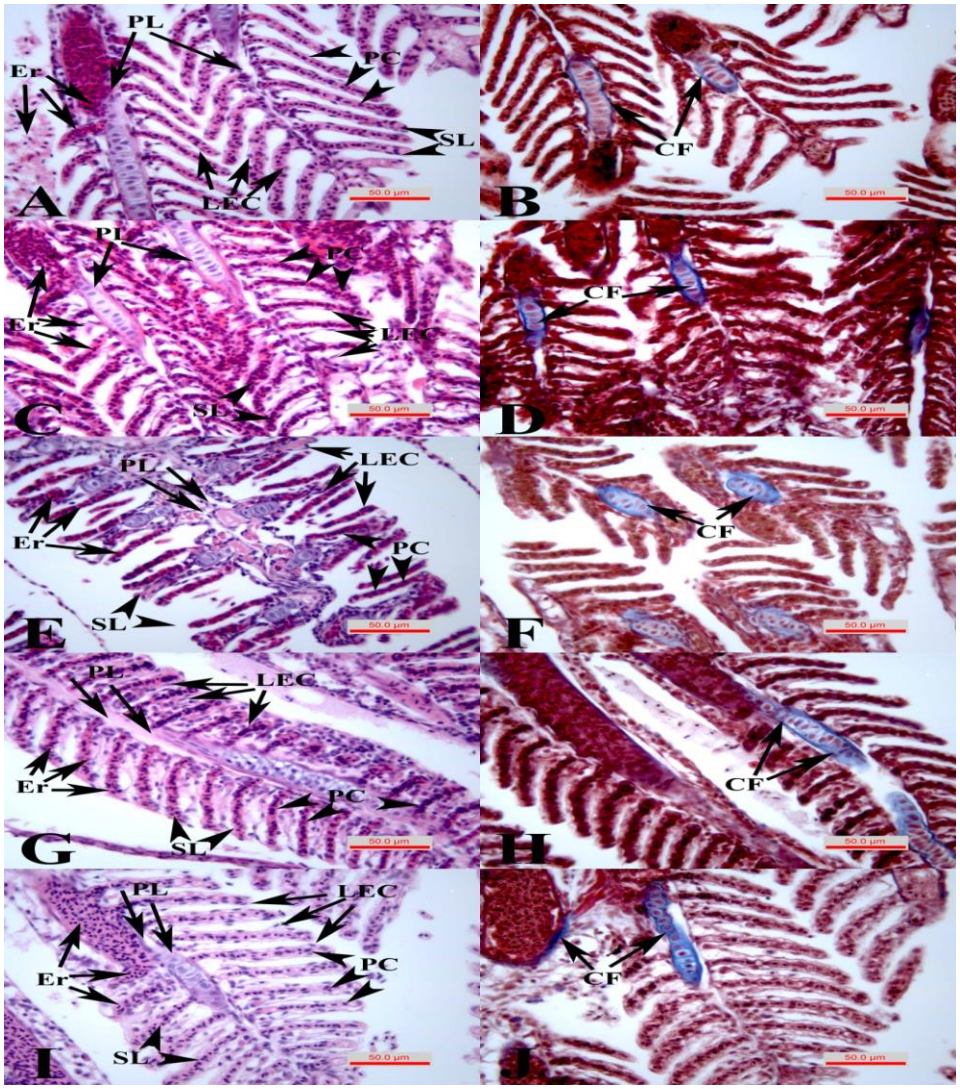


Figure 3. Histology of zebrafish gill tissues, X400 magnification. Bouin's solution fixed and H&E (A) and MT (B) stained tissue sections. Carnoy's solution fixed and H&E (C) and MT (D) stained tissue sections. NBF fixed and H&E (E) and MT (F) stained tissue sections. Davidson's solution fixed and H&E (G) and MT (H) stained tissue sections. Dietrich's solution fixed and H&E (I) and MT (J) stained tissue sections. **Er:** Er: erythrocytes, **PL:** primary gill lamellae, **SL:** secondary gill lamellae, **LEC:** Lamellar epithelial cells, **PC:** Pillar cells, **CF:** Collagen fibers.

DISCUSSION

Tissue and cellular structures can be examined anywhere on the entire slide. Ideally, any methods used to achieve this would minimize the occurrence of an artifact. Any artifact that may be formed in the tissues, by accidental or during tissue processing will lead to deviations in the histological appearance and inaccurate evaluations (Copper et al. 2018). For example, many investigators have reported similar histological findings in fish organs exposed to environmental pollutants or in fish exposed to waterborne diseases (Miki et al. 2018). In this sense, it can be more useful to use appropriate fixation and staining techniques to facilitate differential diagnosis. For this reason, it is necessary to idealize the staining quality by standardizing the fixation techniques with the tissue staining methods.

Histological preparation of gill tissue samples forms a fixed specimen that will slightly reduce over time and can be examined under the microscope and captured images. With this ideal in opinion, we aimed to improve and compare methods used in histological preparation of zebrafish gill tissue samples. In this study, it was observed satisfactory procedures for fixation and staining of gill tissues.

In large fishes, organ or tissues are dissected separately and immerses into the fixative. But, in small fishes such as himedaka or zebrafish, whole bodies of fish immersed directly into the fixative. It is well known that fish tissues are quickly reduced with autolysis after death, and therefore, the key to successful fixation is how rapidly they can inactivate endogenous enzymes than governs autolysis. Researchers (Speilberg et al. 1993, Doaa and Hanan 2013) have observed defective fixation of tissues such as the liver, intestine, and brain when whole bodies of fish are immersed in 10% formalin. Therefore, they recommended the use of rapidlyt permeable fixatives, such as NBF and Davidson's solutions, for fish fixation. Beyond the zebrafish researchers, we have observed a number of findings for the purpose of experiments that will extend to common model system researchers. Thus, as a result of the use of Bouin's, NBF, Carnoy's, Davidson's and Dietrich's fixatives, gill tissue morphology and histological staining findings were evaluated comparatively. All fixatives used in this study provided successful fixation, but showed different effects on histo-morphological structure and H&E and MT staining reactions.

It has been reported that gill filaments and gill lamellae with well-preserved structures were observed in fish fixed with only 20% formalin solution. On the other hand, it was observed that detached of the epithelium from the capillary, and artifactual spaces formed in the gill lamellae in fish fixed with the Bouin's or Davidson's solution only for 6 h. Also, it was noticed that the gill structure poorly separated and the gill filaments and lamellae were not seen so clearly in the tissue samples which were fixed only with Davidson's solution. It has been determined in the fish tissues fixed with Bouin's solution for 5 h after 20% formalin solution fixation for 1 h at 4°C, gill filaments and gill lamellae with almost well-preserved structures. Also, epithelium lifting has been prevented in fish fixed with Davidson's solution for 5 h after 20% formalin solution fixation for 1 h at 4°C, the gill filaments and lamellae was poorly separated (Miki et al. 2018).

It has been reported that various fixatives such as Bouin's, NBF, Carnoy's, Dietrich', Davidson's, Zenker and Alcohol derivatives are used in the fixation of some tissues of zebrafish and it was determined that different fixation applications resulted in different staining results in histological examinations (Bird et al. 2012, Fournie et al. 2000, Peterson et al. 2013, Grunow et al. 2015, Copper et al. 2018). Fixatives containing acetic acids such as Bouin's, Carnoy's, Davidson's, and Dietrich's have been reported to cause erythrocyte lysis but NBF inhibits the lysis of erythrocytes. Fixatives other than NBF have been reported to cause denaturation and clotting of proteins and nucleic acids in tissues due to dehydration and hydrogen bonding due to acetic acid and alcohol (chloroform in Carnoy's) (Peterson et al. 2013). It was reported that Davidson's solution penetrates rapidly and effectively, while Dietrich's solution leads to less shrinkage and deterioration (Dietrich and Krieger 2009, Peterson et al. 2013). In our study, except for NBF, there was lysis in erythrocytes in all of the fixatives used, and furthermore, this supported the well-preserved morphology in Bouin's solution and NBF-fixed gills.
In a research, some problems have been identified in the preparation of histological samples of zebrafish tissues. The first is the differential tissue shrinkage that follows the gaps between tissues that cannot be obtained in live fish. It was proposed that shrinkage may be associated with differences between the solution and tissue osmolality and the required experiments. In addition, in some juvenile and adult fish, it was observed that the internal organs were inadequate fixed and resulted in tissue degeneration (Copper et al. 2018). In our study, depending on the used fixative types, H&E and MT staining reaction scores showed some differences in samples in the same experimental groups. It can be said that these differences may be due to manual staining technique in histopathological examination of gill tissues. In H&E staining, Carnoy's solution has been found to provide a more severe nuclear staining, which is thought to be due to the agglutination of nucleic acids. In addition, it was found that the most ideal H&E staining was with NBF and the other fixatives produced different intensities. Bouin's, Carnoy's and

Davidson's solutions showed strong cytoplasmic staining with eosin. Dietrich's solution was inadequate for H&E staining and was generally weaker than other fixatives.

Although similar good staining results were obtained in the MT staining of tissues fixed with NBF and Carnoy's solution (Peterson et al. 2013), in our study, the most ideal MT staining density was observed in Dietrich's solution. Carnoy's and Davidson's solutions have been shown to provide an even density in MT staining. Bouin's solution has been reported to be the ideal fixative for MT staining (Singhal et al. 2016). However, in our study, Bouin's solution did not give the ideal results for MT staining in zebrafish gill tissue.

CONCLUSIONS

As a result, fixation applications using different fixatives made various effects on H&E and MT staining techniques in gill tissue of zebrafish. Gill tissue morphology was optimally protected by Bouin's solution and NBF solution, whereas H&E staining showed optimal results with NBF, also MT staining showed ideal results with Dietrich's solution fixation. Bouin fixative did not allow the shrinkage of the gill tissue, minimal formalin pigment formation, and enabled cellular morphological details to be seen even in the gill with complex morphology. Eosinophilic and basophilic cellular structures were clearly stained in a differential manner and generally ideal H&E staining was identified in NBF fixation. Furthermore, the structure of collagen fibers is well preserved and optimal staining is achieved by MT staining technique in Dietrich's solution fixation. Considering the preservation of gill tissue morphology and H & E and MT staining methods, other fixative solutions have several disadvantages.

ACKNOWLEDGMENTS

The author declares no conflict of interests. This study was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK, Project number: 215Z467).

Bingöl University Animal Experiments Local Ethics Board dated 26.09.2014 and numbered 2014.09.02.

REFERENCES

Ahmed, H. G., Mohammed, A. Hussein, M. A comparison study of histochemical staining of various tissues after Carnoy's and formalin fixation. Sudan Journal of Medical Sciences. 2011; 5(4).

Alturkistani, HA., Tashkandi, FM., Mohammedsaleh, ZM. Histological stains: a literature review and case study. Global journal of health science, 2016; 8(3): 72.

Bancroft, JD., Gamble, M. Theory and practice of histology techniques. In: Fixation of tissues, Ed; Layton C, Bancroft JD, Suvarna SK, 8th Ed., Churchill Livingstone Elsevier, 2019; pp. 40-63.

Bancroft, J. D. Layton, C. Theory and practice of histology techniques. In: Connective and other mesenchymal tissues with their stains, Ed; Layton C, Bancroft JD, 8th Ed., Churchill Livingstone Elsevier, 2019; pp. 153-175.

Bensimon-Brito, A., Cardeira, J., Cancela, ML., Huysseune, A. Witten, PE. Distinct patterns of notochord mineralization in zebrafish coincide with the localization of Osteocalcin isoform 1 during early vertebral centra formation. BMC developmental biology. 2012; 12(28).

B**ird, NC., Windner, SE. Devoto, SH.** Immunocytochemistry to study myogenesis in zebrafish. Myogenesis. Springer. 2012; pp.153-169.

Berzins, IK., Smolowitz, RM., Lewbart, GA., Diagnostic Techniques and Sample Handling. Invertebr. Med. Second Ed., 2011.

Brundo, MV., Salvaggio, A. Zebrafish or Danio rerio: A New Model in Nanotoxicology Study. In: Bozkurt Y, editor. Recent Adv. Zebrafish Res., Rijeka: IntechOpen; 2018.

Carson, FL., Hladik, C. Histotechnology—A Self Instructional Text. In: Connective and muscle tissue, Ed. Carson FL**.** Chicago, IL: American Society of Clinical Pathologists Press. 1996; pp. 134-140.

Chandrarathna, H., Nikapitiya, C., Dananjaya, S., Wijerathne, C., Wimalasena, S., Kwun, HJ., Heo, GJ., Lee, J., De Zoysa, M. Outcome of co-infection with opportunistic and multidrug resistant Aeromonas hydrophila and A. veronii in zebrafish: Identification, characterization, pathogenicity and immune responses. Fish & shellfish immunology. 2018; 80**:** 573-581.

Christoffersen, TB., Kania, PW., Von Gersdorff JL., Buchmann, K. Zebrafish Danio rerio as a model to study the immune response against infection with Ichthyophthirius multifiliis. Journal of fish diseases. 2017; 40: 847-852.

Cima, L., Riva, G., D'errico, A., Casartelli-Liviero, M., Capelli, P., Tomezzoli, A., Montin, U., Carraro, A., Scarpa, A., Ghimenton, C. Fast chromotrope aniline blue special stain is a useful tool to assess fibrosis on liver biopsy during transplantation. Transplantation proceedings, Elsevier. 2017; 667-670.

Copper, JE., Budgeon, LR., Foutz, CA., Van Rossum, DB., Vanselow, DJ., Hubley, M J., Clark, DP., Mandrell, DT., Cheng, KC. Comparative analysis of fixation and embedding techniques for optimized histological preparation of zebrafish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 2018; 208: 38-46.

Culling, CFA. Handbook of histopathological and histochemical techniques: In: museum techniques, Ed; Culling, CFA, 3rd Ed., Butterworth-Heinemann, 1983; pp. 529-557.

Çakici, Ö., Üçüncü, Sİ. Zebra balığında Danio rerio'da (Teleostei: Cyprinidae) oosit gelişimi. Ege Journal of Fisheries and Aquatic Sciences, 2007; 24 (1).

Dietrich, D., Krieger, HO. Histological analysis of endocrine disruptive effects in small laboratory fish, John Wiley & Sons, Inc., Hoboken, New Jersey, USA, 2009.

Doaa, MM., Hanan, H. Histological changes in selected organs of Oreochromis niloticus exposed to doses of lead acetate. J Life Sci Biomed. 2013; 3: 256-263.

Eltoum, I., Fredenburgh, J., Myers, RB., Grizzle, WE. Introduction to the theory and practice of fixation of tissues. Journal of Histotechnology. 2001; 24: 173-190.

Feist, SW., Lang, T., Stentiford, GD., Road, B., Nothe, T. Biological effects of contaminats: Use of liver pathology of the European flatfish dab (Limanda limanda L .) and flounder (Platichthys flesus L .) for monitoring. 2004.

Flores-Lopes, F., Thomaz, A. Histopathologic alterations observed in fish gills as a tool in environmental monitoring. Brazilian Journal of Biology. 2011; 71: 179-188.

Fournie, JW., Krol, RM., Hawkins, WE. Fixation of fish tissues. In: The laboratory fish. Academic Press. 2000. pp. 569-578.

Genten, F., Terwinghe, E., Danguy, A. Atlas of Fish Histology. Science Publishers, Enfield, NH, USA, 2010.

Gnanapragasam, VJ. Unlocking the molecular archive: the emerging use of formalin-fixed paraffin-embedded tissue for biomarker research in urological cancer. BJU international. 2010; 105: 274-278.

Grunow, B., Kirchhoff, T., Lange, T., Moritz, T., Harzsch, S. Histochemistry on vibratome sections of fish tissue: a comparison of fixation and embedding methods. Aquatic Biology. 2015; 23: 251-263.

Hadi, A., Alwan, S. Histopathological changes in gills, liver and kidney of fresh water fish, Tilapia zillii, exposed to aluminum. International Journal of Pharmacy & Life Sciences. 2012; 3 (11).

Howard, DW., Lewis, EJ., Keller, BJ., Smith, CS. Histological techniques for marine bivalve mollusks and crustaceans. 2004.

Huiting, L., Laroche, F., Feng, H. The zebrafish as a tool to cancer drug discovery. Austin journal of pharmacology and therapeutics. 2015; 3: 1069.

Khan, R., Thulin, J. Influence of pollution on parasites of aquatic animals. Advances in parasitology. Academic Press. 1991. pp. 201-238.

Kumar, GL., Kiernan, JA. Dako Education Guide. In: Special Stains and H & E | Pathology 2010. Dako North America.

Latendresse, JR., Warbrittion, AR., Jonassen, H., Creasy, DM. Fixation of testes and eyes using a modified Davidson's fluid: comparison with Bouin's fluid and conventional Davidson's fluid. Toxicologic pathology. 2002; 30: 524-533.

Mao, H., Su, P., Qiu, W., Huang, L., Yu, H., Wang, Y. The use of Masson's trichrome staining, second harmonic imaging and two-photon excited fluorescence of collagen in distinguishing intestinal tuberculosis from Crohn's disease. Colorectal Disease. 2016; 18: 1172-1178.

Maraschin, BJ., Silva, VPD., Rock, L., Sun, H., Visioli, F., Rados, PV., Rosin, MP. Optimizing Fixation Protocols to Improve Molecular Analysis from FFPE Tissues. Brazilian dental journal. 2017; 28: 82-84.

Martinello, T., Pascoli, F., Caporale, G., Perazzi, A., Iacopetti, I., Patruno, M. Might the Masson trichrome stain be considered a useful method for categorizing experimental tendon lesions? Histol Histopathol. 2015; 30: 963-969.

Matthews, M., Varga, ZM. Anesthesia and euthanasia in zebrafish. ILAR journal. 2012; 53: 192-204.

Miki, M., Ohishi, N., Nakamura, E., Furumi, A., Mizuhashi, F. Improved fixation of the whole bodies of fish by a double-fixation method with formalin solution and Bouin's fluid or Davidson's fluid. Journal of Toxicologic Pathology. 2018; 31(3): 201-206.

Mohseny, A., Hogendoorn, P. Zebrafish as a model for human osteosarcoma. Current Advances in Osteosarcoma. Springer, Cham. 2014; pp. 221-236.

Morris, JA. Zebrafish: a model system to examine the neurodevelopmental basis of schizophrenia. Progress in brain research. Elsevier. 2009; 179: 97-106.

Mueller, C., Harpole, MG., Espina, V. One-step preservation and decalcification of bony tissue for molecular profiling. Molecular Profiling. Springer. 2017; 85-102.

Muto, A., Kawakami, K. Prey capture in zebrafish larvae serves as a model to study cognitive functions. Frontiers in neural circuits. 2013; 7: 110.

Nechifor-Boilă, AC., Loghin, A., Vacariu, V., Halaţiu, VB., Borda, A. The storage period of the formalin-fixed paraffin-embedded tumor blocks does not influence the concentration and purity of the isolated DNA in a series of 83 renal and thyroid carcinomas. Rom J Morphol Embryol. 2015; 56: 759-763.

Newman, M., Ebrahimie, E., Lardelli, M. Using the zebrafish model for Alzheimer's disease research. Frontiers in genetics. 2014.; 5: 189.

Nusslein-Volhard, C. & Dahm, R. Zebrafish, Ed; Ralf. Oxford University Press. 2002.

Pereira, MA., Dias, AR., Faraj, SF., Cirqueira, CDS., Tomitao, MT., Carlos NS., Ribeiro JRU. & De Mello, ES. Carnoy's solution is an adequate tissue fixative for routine surgical pathology, preserving cell morphology and molecular integrity. Histopathology. 2015; 66: 388-397.

Peterson, TS., Kent, ML., Ferguson, JA., Watral, VG., Whipps, CM. Comparison of Fixatives, Fixation Time, and Severity of Infection on PCR Amplification and Detection of Mycobacterium marinum and Mycobacterium chelonae DNA in Paraffin-Embedded Zebrafish (Danio rerio). Diseases of aquatic organisms. 2013; 104: 113.

Pikarsky, E., Ronen, A., Abramowitz, J., Levavi-Sivan, B., Hutoran, M., Shapira, Y., Steinitz, M., Perelberg, A., Soffer, D., Kotler, M. Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. Journal of virology. 2004; 78: 9544-9551.

Progatzky, F., Cook, HT., Lamb, JR., Bugeon, L., Dallman, MJ. Mucosal inflammation at the respiratory interface: a zebrafish model. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2016; 310: 551-561.

Rahunen, K., Rieppo, L., Lehenkari, P., Finnilä, M. & Saarakkala, S. Evaluation of quantitativity of histological collagen stains in articular cartilage. Osteoarthritis and Cartilage. 2016; 24: 307-308.

Roberts, RJ. Fish pathology. In: Laboratory Methods, Ed; Roberts RJ, Smail DA, Munro ES, 4_{th} Ed., John Wiley & Sons. 2012; pp. 439-480.

Sahota, PS., Popp, JA., Hardisty, JF., Gopinath, C. Toxicologic pathology: nonclinical safety assessment. 1_{st} Ed., CRC press. 2013.

Singhal, P., Singh, NN., Sreedhar, G., Banerjee, S., Batra, M., Garg, A. Evaluation of histomorphometric changes in tissue architecture in relation to alteration in fixation protocol–an invitro study. Journal of clinical and diagnostic research: JCDR. 2016; 10: ZC28.

Souza, JP., Baretta, JF., Santos, F., Paino, IM. & Zucolotto, V. Toxicological effects of graphene oxide on adult zebrafish (Danio rerio). Aquatic toxicology. 2017; 186: 11-18.

Spaniol, K., Guthoff, R., Schrader, S., Borrelli, M.,, Kajasi, N., Schramm, M., et al. Diagnostik von Binde- und Hornhauterkrankungen. Klin Monbl Augenheilkd 2016;233:e17–28.

Speilberg, L., Evensen, Ø., Bratberg, B., Skjerve, E. Evaluation of five different immersion fixatives for light microscopic studies of liver tissue in Atlantic salmon Salmo salar. Diseases of aquatic organisms. 1993; 17: 47-55.

Stefanović, D., Samardžija, G., Redžek, A., Arnaut, M., Nikin, Z., Stefanović, M. Buffered Romanowsky-Giemsa method for formalin fixed, paraffin embedded sections: taming a traditional stain. Biotechnic & Histochemistry. 2017; 92: 299-308.

Strzyzewska, E., Szarek, J., Babinska, I. Morphologic evaluation of the gills as a tool in the diagnostics of pathological conditions in fish and pollution in the aquatic environment: a review. Veterinární Medicína. 2016; 61(3).

Telser, A. Histological and histochemical methods: theory and practice, 3rd edition. Shock 2006.

Ung, CY., Lam, SH., Hlaing, MM., Winata, CL., Korzh, S., Mathavan, S., Gong, Z. Mercury-induced hepatotoxicity in zebrafish: in vivo mechanistic insights from transcriptome analysis, phenotype anchoring and targeted gene expression validation. Bmc Genomics. 2010; 11: 212.

Uslu, B., Dioguardi, CC., Haynes, M., Miao, DQ., Kurus, M., Hoffman, G., Johnson, J. Quantifying growing versus non-growing ovarian follicles in the mouse. Journal of ovarian research. 2017; 10: (3).

Westerfield, M. The zebrafish book: a guide for the laboratory use of zebrafish. 2000; http://zfin.org/zf_info/zfbook/zfbk.html; Accessien date: 02.02.2019.

Wilson, JM., Bunte, RM., Carty, AJ. Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (Danio rerio). Journal of the American Association for Laboratory Animal Science. 2009; 48: 785-789.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):168-177

DOI: 10.30607/kvj.543261

RESEARCH ARTICLE

Prenatal and Neonatal Exposure to Glyphosate-Based Herbicide Reduces The Primordial to Primary Follicle Transition in The Newborn Rat Ovary: A Preliminary Study

Ruhi TÜRKMEN[1]*, Türkan TÜRKMEN[2]

[1]*Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Afyonkarahisar, Turkey*
[2]*Afyonkarahisar Health Sciences University, Faculty of Medicine, Department Of Medical Microbiology, Afyonkarahisar, Turkey*

ABSTRACT

This study investigated how a glyphosate-based herbicide (GBH) affects the proportional distribution of ovarian follicles that develop from the 18th day of the embryo period (E18) to the 7th postnatal day (PND7) in newborn female rats. A total of 6 pregnant rats that were used in the study were divided into two groups so that there would be 3 pregnant rats in the control group and 3 pregnant rats in the GBH group. Starting from E21 to E18 the pregnant rats in the experimental group were administered at 50 mg/kg/day GBH subcutaneously (s.c.) and the physiological saline was administered as vehicle to the control group. Subsequently, female pups received vehicle or 2 mg/kg GBH from PND1 to PND7. On PND8, all female offspring (neonatal period, 6 newborn female rats from each group) were sacrificed by light ether anesthesia. For the histological examination of the dissected ovaries, the primordial, primary, secondary and preantral follicle numbers were determined using Crossman's modified triple staining method and Periodic Acid-Shiff (PAS) staining methods. The percentage of primordial follicles was significantly higher in the ovaries of female rats in GBH exposed group compare to the control group. However, the percentage of primary, secondary and preantral follicles was lower. Thus, it was observed that prenatal and neonatal GBH exposure decreased the transition of primordial follicle to primary follicle.

Keywords: Endocrine disruptors, follicle composition, glyphosate-based herbicide, newborn rat, ovary

***

Glifosat-Bazlı Herbisite Prenatal ve Neonatal Dönemde Maruziyet Yenidoğan Rat Ovaryumunda Primordiyalden Primer Foliküle Geçişi Azaltır: Bir Ön Çalışma

ÖZ

Yapılan bu çalışmada glifosat bazlı herbisit (GBH)'in yenidoğan dişi sıçanlarda embriyo döneminin 18. gününden (E18) doğum sonrası 7.gün (PND7) arasında gelişmekte olan ovaryum foliküllerinin oransal dağılımını nasıl etkilediği araştırılmıştır. Çalışmada kullanılan toplam 6 gebe sıçan; 3 gebe sıçan kontrol grubunda, 3 gebe sıçan GBH grubunda olacak şekilde 2 gruba ayrıldı. Gebe sıçanlara E18'den başlayarak E21. güne kadar günlük subkutan olarak (s.c.) deney grubuna 50 mg/kg/gün GBH ve kontrol grubuna ise taşıt madde FTS (fizyolojik tuzlu su) uygulandı. Daha sonra yenidoğan dişi yavru sıçanlara PND1'den PND7'ye kadar 2 mg/kg dozunda GBH ve taşıt madde uygulamasına devam edildi. Son ilaç uygulamadan bir gün sonra PND8'de (neonatal periyot, 6 dişi yavru/her grupta) yavru dişi sıçanlar hafif eter anestezi ile sakrifiye edildi. Diseke edilen ovaryumların histolojik incelemesi için Crossman'ın modifiye üçlü boyama yöntemi ve Periodic Acid-Shiff (PAS) boyama yöntemleri kullanılarak primordial, primer, sekonder ve preantral folikül sayıları belirlendi. GBH'ye maruz kalan dişi yavru sıçanların ovaryumları kontrol grubuna göre karşılaştırıldığında primordial foliküllerin yüzdesi önemli derecede fazla bulundu. Bununla birlikte primer, sekonder ve preantral folikül yüzdesinin ise azlığı dikkat çekti. Sonuç olarak prenatal ve neonatal GBH maruziyetinin primordial-primer folikül geçişini azalttığı gözlenmiştir.

Anahtar Kelimeler: Endokrin bozucular, folikül kompozisyonu, glifosat-bazlı herbisit, yenidoğan sıçan, ovaryum

To cite this article: Türkmen R. Türkmen T. Prenatal and Neonatal Exposure to Glyphosate-Based Herbicide Reduces The Primordial to Primary Follicle Transition in The Newborn Rat Ovary: A Preliminary Study. Kocatepe Vet J. (2019) 12(2):168-177.

Submission: 22.03.2019 **Accepted**: 07.05.2019 **Published Online**: 15.05.2019
ORCID ID; RT: 0000-0003-4726-3900, TT: 0000-0003-0736-8927
*Corresponding author e-mail: ruhiturkmen@gmail.com

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a heterogeneous class of environmental pollutants that interfere with the endocrine system by mimicking natural hormones, working antagonistically with natural hormones or by blocking the metabolic pathways of natural hormones that might result temporary or permanent damage to endocrine system (Scognamiglio et al. 2016). Nowadays, it is suspected that more than 800 chemicals interfere with hormone receptors or hormone metabolism and pesticides constitute an important group of these toxic chemicals (Scognamiglio et al. 2016). The term pesticide covers a wide range of chemicals including insecticides, fungicides, herbicides, rodenticides, molluscicides, nematicides, plant growth regulators and others (Aktar et al. 2009). Among these, herbicides, particularly glyphosate-based herbicides (GBHs), have a wide range of use to destroy the weeds in both agricultural and non-agricultural areas (eg, horticulture). After the first introduction to the consumers in 1974, the use of the compound has increased dramatically in parallel to the prevalence of glyphosate-resistant genetically modified crops (Gillezeau et al. 2019). River and surface waters are polluted due to their excessive and unconscious use in agriculture, horticulture, and turfs and hence, humans and other mammals are exposed to this pollutant with food and water (Acquavella et al. 2004). Parvez et al. (2018) found that most of the pregnant women (93%) had glyphosate in their urine. In a recent study, the presence of glyphosate in maternal and umbilical cord serum of pregnant women in three regions of Thailand has been demonstrated (Kongtip et al. 2017). The authors showed that pregnant women working or living with families who are working in agriculture have higher rates of exposure to the herbicide glyphosate compared to pregnant women who are not working in agriculture or non-farmers.

For many years, the use of glyphosate is thought to play a role only in plant metabolic pathways. The herbicidal effect of glyphosate is attributed to the cleavage of the Shikimate pathway by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which is responsible for the biosynthesis of essential aromatic amino acids, thereby destroying the protein synthesis and ultimately leading the plant death (Mesnage et al. 2015). The Shikimate pathway is not present in humans but is found in bacteria living in human gut (Ratia et al. 2014). Many studies have shown that glyphosate-induced imbalances in intestinal bacteria may be associated with the emergence of different chronic diseases such as celiac disease (Samsel and Seneff 2013), diabetes (Samsel et al. 2013), and neurological disorder (Seneff et al. 2015). Similarly, the endocrine disrupting effects of glyphosate and GBHs are demonstrated in *in vitro* studies with the human cells (Gasnier et al. 2009) and different model species (Richard et al. 2005, Benachour et al. 2007, Benachour and Séralini 2009, Mesnage et al. 2013, Defarge et al. 2016).

Ovary is one of the most important organs for female fertility due to the production of gamete and biosynthesis of hormones. The development and maturation of ovarian follicles is called folliculogenesis (Cox and Takov 2018). The development of ovaries in rats begins before birth and the first and main change is the formation of follicles (Malekinejad et al. 2011). In the early stage of ovarian development, the ovulocytes are paused in the meiosis-I stage and wait for two key developmental processes: (1) primordial follicle formation, (2) transition from primordial to primary follicle (McGee and Hsueh 2000). This process involves (i) embryonic days of 18 through 21 (E18-21) in which the majority of the oocytes in the rats were in the oocyte places and the apoptosis was observed, (ii) peak days 0 to 3 (PND0-3) in which the apoptosis and primordial follicle formation is present, and (iii) the final PND4-7 stage where no oocytes are observed in ovarian sections since all oocyte cavities have been demolished and shaped as primordial follicles and primary follicle transition is present in ovarian sections (Hirshfield and DeSanti 1995, Pepling and Spradling 2001, Kezele and Skinner 2003). In the neonatal period (PND0-7), ovarian follicle development is independent of the pituitary gonadotropins (Luteinizing Hormone (LH) or Follicle Stimulating Hormone (FSH) and the follicles remain as a preantral follicle (Picut et al. 2015).

Various investigators have reported that GBHs cause ovarian damage and dysfunction in pregnant mice (Ren et al. 2018) and rats (Hamdaoui et al. 2018). However, it is not known whether embryonic and neonatal exposure to endocrine disrupters such as GBHs has an effect on ovarian follicle development in juvenile rats. In this study, we aimed to determine the effect of commercial endocrine disrupting chemical GBH on the proportional distribution of developing ovarian follicles from embryonic day 18 (E18) to postnatal 7th day (PND7) in newborn female rats.

MATERIALS and METHODS

Chemical substance

The glyphosate formulation used in this study was Knockdown 48 SL marketed by Safa Agriculture Inc. in Turkey. The active ingredient is a liquid-water soluble formulation containing 48% isopropylamine salt as excipients and inert ingredients. This GBH was selected based on the fact that it is one of the most widely used herbicide against weeds in Turkey and it is a representative of high glyphosate content

formulation that targets the weeds with difficult eradication.

Animals, Experimental Design and Treatment

This study was approved by Afyon Kocatepe University Animal Experiments Local Ethics Committee (No: 67; Date: 03 May, 2017). A total of six female Wistar Albino two months old rats obtained from the Experimental Animals Unit of Afyon Kocatepe University were used in the study. The rats were kept in propylene cages, in 12-hour dark/light cycle, 22±2 °C temperature and humidity (30-70%) was checked in the rooms. Clean tap water and standard rat feed ad libitum was given. The estrous cycle was followed by vaginal cytology of the rats. Three female rats determined to be in the proestus period and one male rat were placed in the same cage. The next day, rats with sperms in the vaginal smear were found to be pregnant and were recorded as 0 days (E0) of pregnancy. The estrus cycles were monitored for seven days and the pregnancy of the rats which were observed in the period of diestrous was confirmed. The experiment consisted of two parts. In the first part of the experiment, six pregnant rats were divided into the experimental and control groups, each including three pregnant rats. Pregnant rats were given 50 mg/kg/day GBH subcutaneously (s.c.) and control group received physiological saline as vehicle on E18 days. GBH was prepared by dissolving it in saline solution. Both vehicle and GBH were administered daily by subcutaneous injections until delivery. In the second part of the experiment, a total of twelve newborn female rats were divided into two groups of six, the experimental and control groups. After birth, an amount of 2 mg/kg/day vehicle (physiological saline) and GBH were administered to the newborn female rats every 48 hours of from PND1 to PND7 subcutaneously. The doses of GBH administered to pregnant and juvenile rats were selected based on the US Environmental Protection Agency, with reference to the level of no side effect of GBH (NOAEL) (USEPA 1993). After the last drug administration in PND8, the female offspring of both groups were sacrificed by light ether anesthesia and the ovarian-uterus-oviduct triad was collectively removed.

Histological Evaluation and Follicle Count

The ovaries from the organs were dissected under the stereo-microscope. The dissected ovaries were stained in Bouin solution for 3 hours at room temperature. After washing in 50% alcohol, dehydration was performed subsequently in 70%, 80%, 95%, and 100% alcohol. In order to clarify the samples prior to paraffin embedding, they were washed in xylene two times. Paraffin-embedded ovarian tissues were cut at 4-5 μm thickness with a microtome and taken on slides. The number and developmental stages of the follicles were determined using samples stained with Crossman's modified triple staining method and Periodic Acid Schiff (PAS). Two consecutive sections covering the largest cross-sectional area of the medulla and cortex sides of both the right and the left ovaries of the experimental and control groups were examined under light microscope to determine the number of follicles. The follicles classified as described in Pedersen and Peters (2007) and were averaged as reported by Nilsson et al. (2007). In this classification, granulosa cell shape and cell layer count are considered. According to this, the follicle that contains the flat single-row granulosa cells surrounding the oocyte is classified as the Primordial follicle (A), the follicle surrounded by single-row cubic granulosa cells as the primary follicle (B), the follicle surrounded by two or more ordered cubic granulosa cells was considered as secondary follicle (C). The follicle that has a diameter considerably larger than that of the other follicles along with small gaps between the granulosa cells and more than 3 granulosa cell lines was called the preantral follicle (D) (Table 1).

Statistical analysis

Chi-square test was used to compare the primordial, primary, secondary and preantral numbers between the two groups. All the analyses were performed with SPSS 22.0 program and $p<0.05$ was considered as statistically significant throughout the study.

RESULTS

The effect of GBH on the follicle composition

The difference of the primordial, primary, secondary, and preantral counts obtained from the two study groups were statistically significant. The percentage of primordial follicles was higher in the glyphosate applied group compared to the control group (***$p<0.01$) whereas the percentage of primary, secondary, and preantral follicles were lower (*$p<0.05$) (Table 2., Figure 1.)

The effects of GBH on follicle types and numbers

All follicle types were evaluated in both GBH and control group preparations. The general state of ovaries of the control group and in the experimental group were first compared. The size of ovary follicles in the experimental group exposed to GBH was smaller than the control group and the number of primordial follicles was higher (Figure 2.)

The ovarian sections of the control and GBH groups were stained with Crossman's modified triple staining method and Periodic Acid Schiff (PAS) staining. Primordial, primary, secondary and preantral follicle counts were performed in the preparations of the medulla and cortex regions of ovarian sections. It was observed that most of the follicles remained in the primordial follicle stage in the GBH group implying a significantly reduced transition from the primordial

follicle to the primary, secondary and preantral follicle (Figures 3, 4, 5, and 6).

Table 1. Classification of follicles

Follicle Classes	Name	Definition
A	Primordial	single layer flat granulosa cell (GC) layer
B	Primary	single layer cubic (GC) layer
C	Secondary	2-3 rows of cubic (GC) layers
D	Preantral	3≤ rows of GC with wide antral clearance, distinct monolayer

Table 2. Comparison of follicle numbers between GBH and control group

			Variable				Total	χ^2	p
			primordial	primary	secondary	preantral			
Variable	GBH	Number (f)	376	90	27	9	502	91.470	0.001
		Percentage(%)	74.9%	17.9%	5.4%	1.8%	100.0%		
	Control	Number (f)	79	40	42	21	182		
		Percentage(%)	43.4%	22.0%	23.1%	11.5%	100.0%		
	Total	Number (f)	455	130	69	30	684		
		Percentage(%)	66.5%	19.0%	10.1%	4.4%	100.0%		

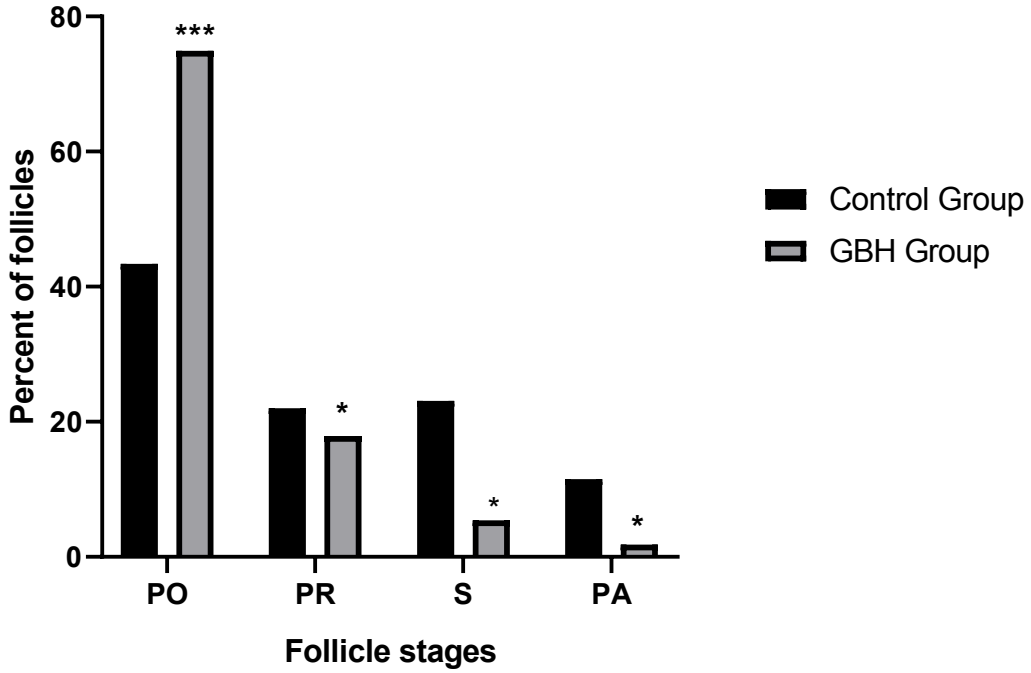


Figure 1. Effects of prenatal and neonatal exposure to GBH on follicle composition in newborn rat ovaries. The percentage of primordial follicles increased in GBH treated ovaries, while percentage of primary, secondary and preantral follicles decreased compared to controls. PO, primordial follicles; PR, primary follicles; S, secondary follicles; PA, preantral follicles. *$p < 0.05$, **$p < 0.001$, $n = 6$.

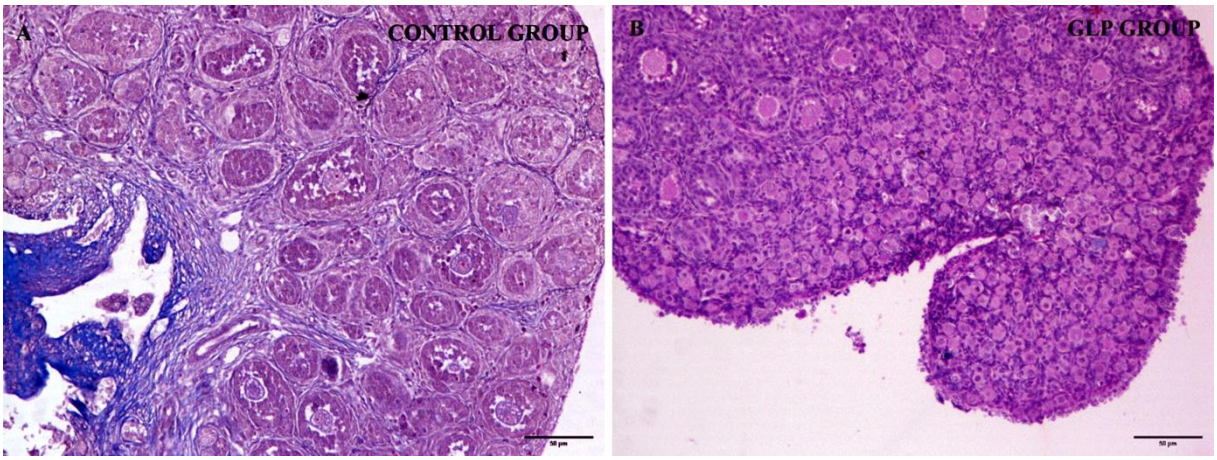


Figure 2. Representative photomicrograph of Crossman Modified Triple and Periodic acid–Schiff (PAS)-stained sections of ovary of control and GBH groups of rats. A. Control group, Crossman Modified Triple (20x), B. GBH group, PAS (20x), Bar = 50 μm.

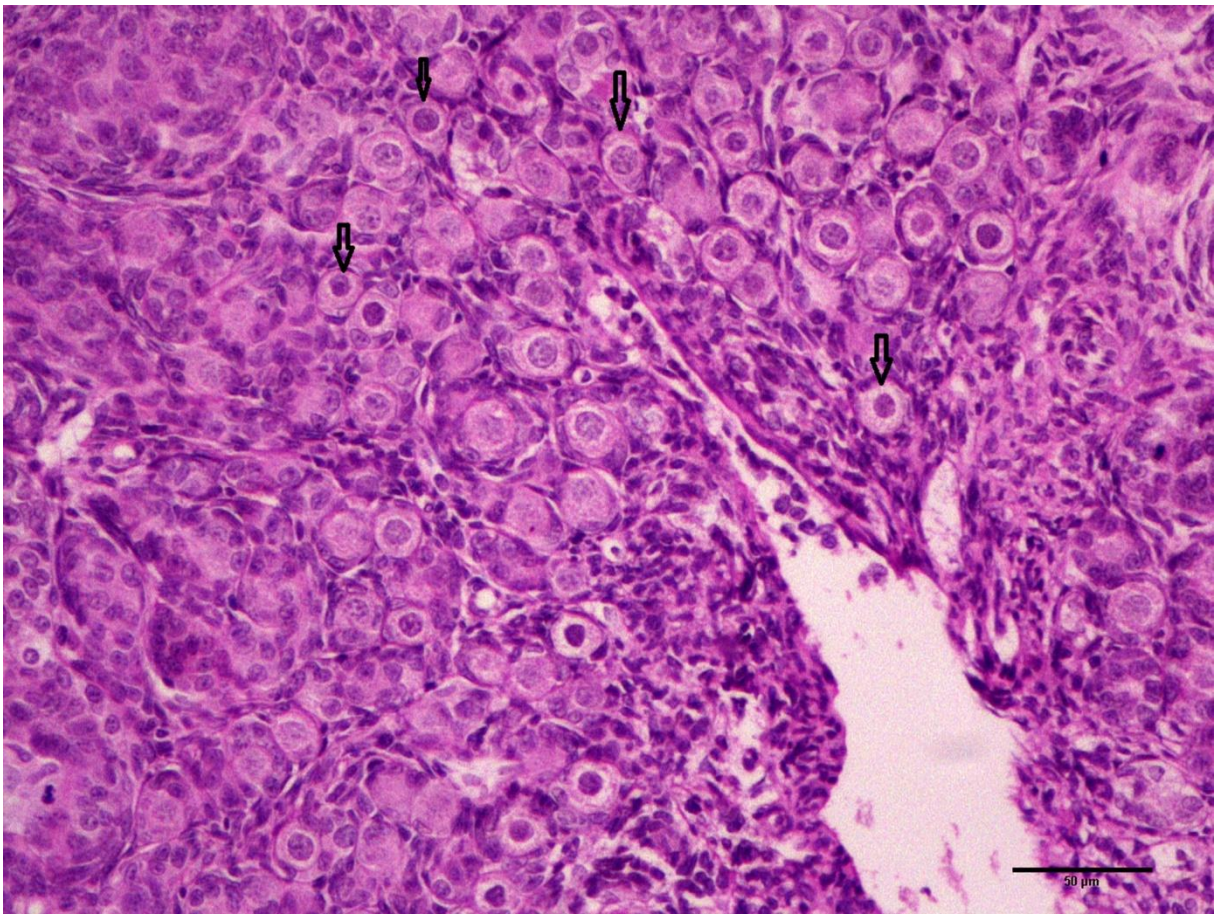


Figure 3. Primordial follicles in GBH group ovarian medulla. Arrow: primordial follicles. PAS (20x), Bar = 50 μm.

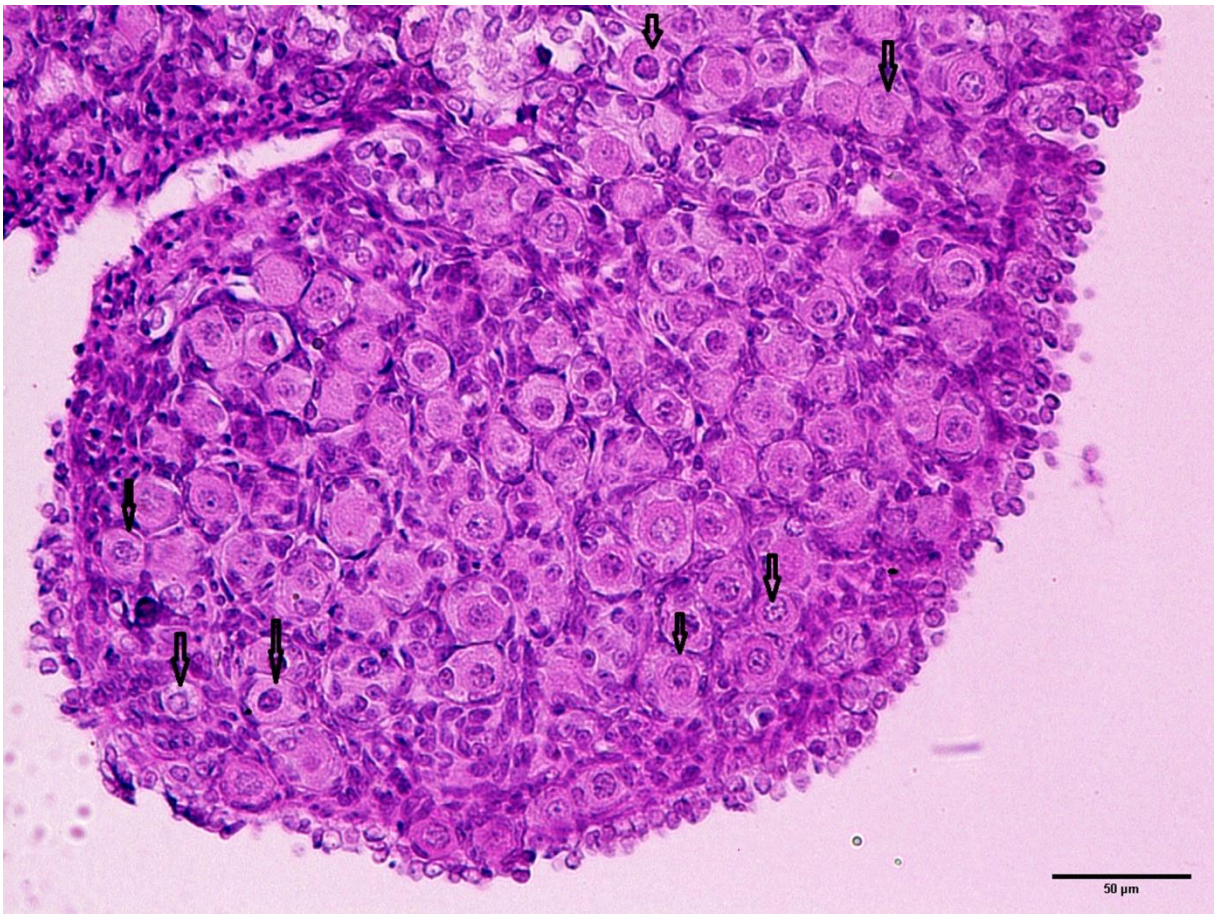


Figure 4. Primordial follicles in ovarian cortex of GBH group. Arrow: primordial follicles. PAS (20x), Bar = 50 µm.

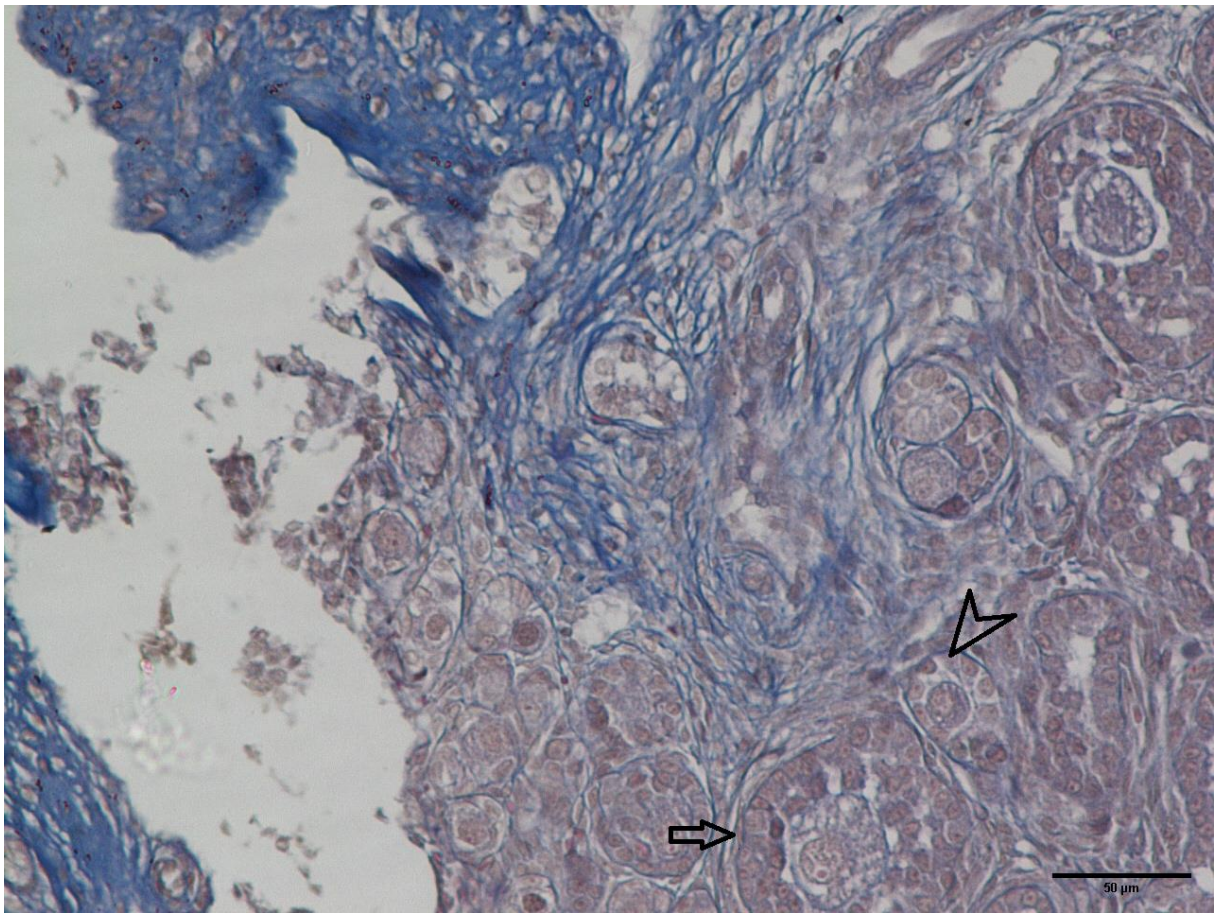


Figure 5. Primary and secondary follicle in the control group ovarian medulla. Arrowhead: primer follicul; Arrow: secondary follicul. Crossman Modified Triple (20x), Bar = 50 µm.

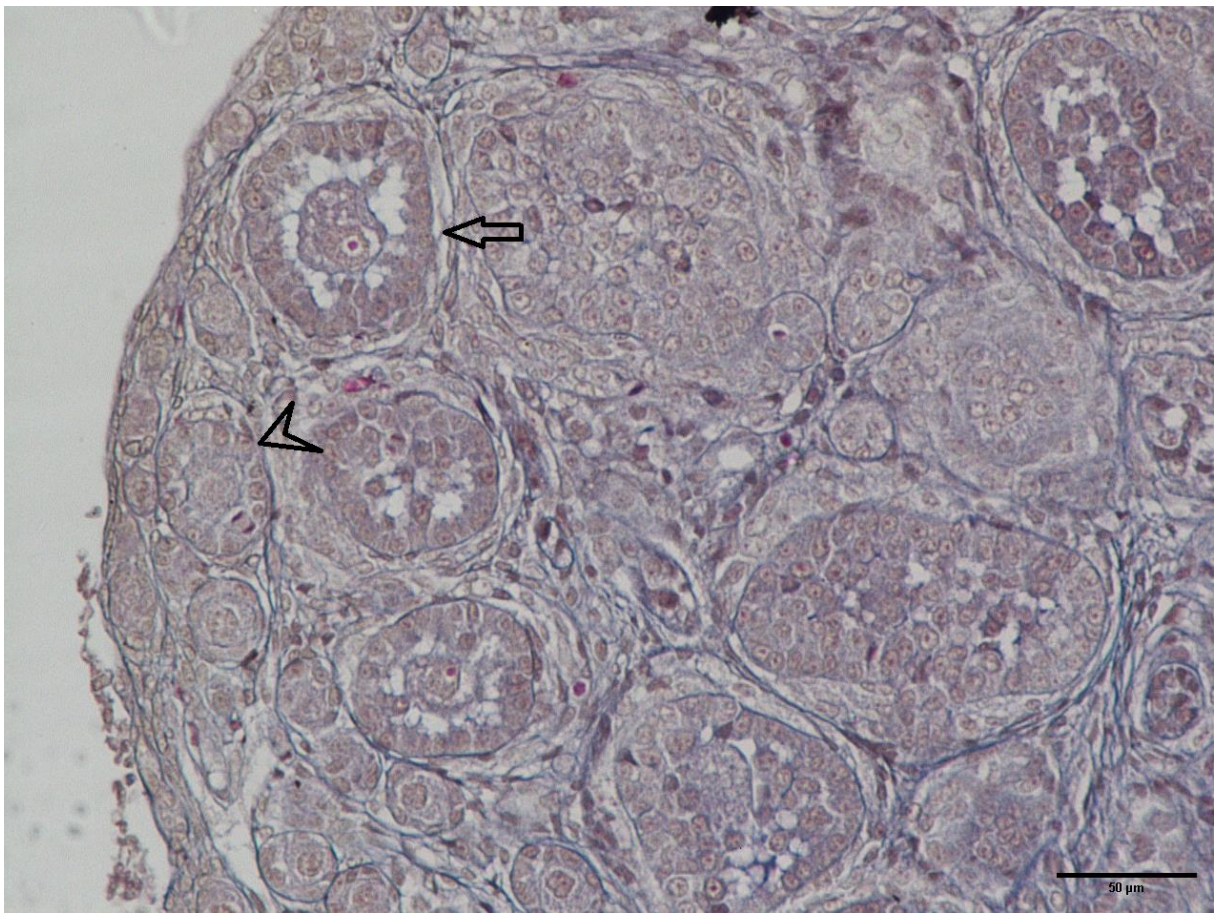


Figure 6. Primary and preantral follicles in the ovarian cortex of the control group. Arrowhead: primer follicul; Arrow: preantral follicul. Crossman Modified Triple (20x), Bar = 50 µm.

DISCUSSION

In contemporary agriculture, many technological advances are employed to increase the productivity of crops. Weeds in particular can cause large scale crop losses and consequently to a reduction in yield. Nowadays, herbicides are the most commonly used pesticide class in the fight against weeds. Today, commercial glyphosate product is used in over 140 countries in various chemical forms such as isopropylamine salt, ammonium salt, diammonium salt, dimethylammonium salt, and potassium salt (Landrigan and Belpoggi 2018). In Turkey, a total of 3208 products containing the active ingredient glyphosate has received a license from the Ministry of Agriculture and Forestry (T.C. Tarım ve Orman Bakanlığı 2019). It is still debatable whether glyphosate is harmful to human health, especially in relation to exposure levels. Albeit controversial, cancer is the forefront of concern regarding the glyphosate exposure. However, most regulatory authorities consider that glyphosate is not carcinogenic, as highlighted in a recent article by the US Environmental Protection Agency (USEPA) (USEPA 2017). Other claimed health problems include kidney diseases, pregnancy complications and reproductive dysfunction, but none have been empirically confirmed (Bai and Ogbourne 2016, Myers et al. 2016, Van Bruggen et al. 2018).

Many contaminants used in both agricultural and non-agricultural activities are known to have endocrine disrupting effects, and glyphosate-based herbicides are one of the most prominent contaminant (Drašar et al. 2018). In particular, a fetus may be exposed to glyphosate in the uterus by contamination of the mother. Regarding the effect of glyphosate on fetus in the uterus, Richard et al. (2005) and Benachour et al. (2007) reported that glyphosate has a toxic effect on human placental cells. Benachour et al. (2007) investigated the effects of glyphosate on human embryonic and placental cells and addressed how these effects are increased with the dose and exposure time and strongly concluded that exposure to glyphosate affect fetal development. Benachour and Séralini (Benachour and Séralini 2009) also show that even in low concentrations, GBH can induce apoptosis and necrosis (hence toxic effects) in human embryonic, navel and placenta cells. Since glyphosate can pass through the placenta as indicated by Poulsen et al. (2009), the baby may be exposed to glyphosate even in the uterus. This may lead to deterioration of the estrogen balance with endocrine disrupting activity of glyphosate that affects the development of testicular cells and testosterone production (Richard et al. 2005, Haverfield et al. 2011, Clair et al. 2012). In addition, the endocrine disrupting effects of GBHs in males were documented in several experimental studies (Dallegrave et al. 2007, Romano et al. 2012, Cassault-Meyer et al. 2014, Avdatek et al. 2018a, 2018b). The ovary is a female reproductive gland and is the main source of the female hormones estrogen and progesterone, as it shows a cyclic rhythm of germ cell maturation, including the proliferation, synthesis and accumulation of egg yolk in oocytes (Stefansdottir et al. 2014). Hamdaoui et al. (2018) reported that GBH caused ovarian damage and induced a decrease in absolute and relative ovarian organ weight. Although there are some studies examining the effects of GBH on female reproductive system, to date no studies have examined the effects on the ovarian follicular composition of the newborn female rats. Considering the fact that the ovarian follicles are formed in fetal and neonatal periods in rats, the nature of the effects of GBH exposure at E18 and PN7 days on the follicular activation (transition from primordial to primary follicle) in female ovary rat ovary was presented for the first time in this study.

In the present study, we found that GBH administration changed the follicular composition in the ovary. We also found that the transition from primordial follicle to primary follicle in the ovary of animals decreased with the GBH and therefore the number of secondary and preantral follicles was low. Ren et al. (2018) applied pure glyphosate and the trademark Roundup to pregnant mice from day 1 to day 19 (E1-E19) of the pregnancy and did not detect any change in primary and secondary follicle numbers compared to the control group. This may be attributed to the difference in dose and duration of glyphosate administration or the animals used in the study. Wistar race rats were used in our study were previously shown to be more sensitive to endocrine disruptors (Diel et al. 2004). Furthermore, GBH was applied between E18 and PND7 days including primordial and primary follicle development stages in embryonic and neonatal period in our study. In a study designed in a similar way to our study, an organic chlorinated insecticide, methoxychlor, was applied to Wistar breed rats and the results revealed that methoxychlorine reduced the number of primordial and primary follicles in the ovaries and increased the number of secondary follicles (Ozden-Akkaya et al. 2017). The findings of Ozden-Akkaya et al. (2017) are not in congruence with our results. This may be due to the fact that methoxychlor had prevented the formation of the primordial follicle in the fetal period by destroying the oocyte bases and induction of primary and secondary follicle from the primordial follicle. In addition, Ozden-Akkaya et al. (2017) collected ovaries between PND 50-60 days in their study whereas we collected and analyzed ovaries PND8 where follicle development reached to preantral follicle stage

CONCLUSION

The critical stage in ovarian biology is the transition from primordial follicle where development stopped to primary follicle where development persists. With the results of our study, changes the female rats' ovarian follicle composition and reduction of primary follicle transition from primordial follicle due to the exposure to GBH at the fetal and neonatal periods were reported for the first time in the literature. However, more specific advanced studies are needed to demonstrate the role of GBH in the ovarian follicles development processes. The findings will shed light on future research and more effective approaches.

ACKNOWLEDGEMENT

The study was supported by a grant from the Afyon Kocatepe University Scientific Research Projects Coordination Unit (Project no: 17.KARİYER.154).

The study was conducted at the Department of Histology and Embryology, Afyonkarahisar Health Sciences University, Faculty of Medicine, Afyonkarahisar, Turkey. The authors are grateful to Murat Tosun, Professor at the Department of Histology and Embryology, for gently provide the facilities and giving valueble scientific background. Also, a short summary of this study was orally presented at the 8th International Multidisciplinary Congress of Eurasia (IMCOFE), 24-26 April, 2019, in Antalya, Turkey.

Ruhi Turkmen and Turkan Turkmen contributed equally to this work.

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

This study was approved by Afyon Kocatepe University Animal Experiments Local Ethics Committee (No: 67; Date: 03 May, 2017).

REFERENCES

Acquavella JF, Alexander BH, Mandel JS, Gustin C, Baker B, Chapman P. Glyphosate biomonitoring for farmers and their families: Results from the farm family exposure study. Environ Health Perspect. 2004;112(3):321–6.

Aktar MW, Sengupta D, Chowdhury A. Impact of pesticides use in agriculture: their benefits and hazards. Interdiscip Toxicol. Slovak Toxicology Society; 2009;2(1):1–12.

Avdatek F, Birdane YO, Türkmen R, Demirel HH. Ameliorative effect of resveratrol on testicular oxidative stress, spermatological parameters and DNA damage in glyphosate-based herbicide-exposed rats. Andrologia. 2018a;50(7):e13036.

Avdatek F, Türkmen R, Demirel HH, Birdane YO. Glifosat Bazlı Herbisite Maruz Kalan Sıçanlarda N-Asetilsisteinin Testis Oksidatif Hasarı, Spermatolojik Parametreler ve DNA Hasarı Üzerindeki Koruyucu Etkisi. Kocatepe Vet J. 2018b;11(3):1–9.

Bai SH, Ogbourne SM. Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. Environ Sci Pollut Res. 2016;23(19):18988–9001.

Benachour N, Séralini G-E. Glyphosate Formulations Induce Apoptosis and Necrosis in Human Umbilical, Embryonic, and Placental Cells. Chem Res Toxicol. 2009;22(1):97–105.

Benachour N, Sipahutar H, Moslemi S, Gasnier C, Travert C, Séralini GE. Time- and Dose-Dependent Effects of Roundup on Human Embryonic and Placental Cells. Arch Environ Contam Toxicol. 2007;53(1):126–33.

Van Bruggen AHC, He MM, Shin K, Mai V, Jeong KC, Finckh MR, Morris JG. Environmental and health effects of the herbicide glyphosate. Sci Total Environ. Elsevier; 2018;616–617:255–68.

Cassault-Meyer E, Gress S, Séralini GÉ, Galeraud-Denis I. An acute exposure to glyphosate-based herbicide alters aromatase levels in testis and sperm nuclear quality. Environ Toxicol Pharmacol. Elsevier B.V.; 2014;38(1):131–40.

Clair É, Mesnage R, Travert C, Séralini G-É. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicol Vitr. 2012;26(2):269–79.

Cox E, Takov V. Embryology, Ovarian Follicle Development. StatPearls. StatPearls Publishing; 2018.

Dallegrave E, Mantese FD, Oliveira RT, Andrade AJM, Dalsenter PR, Langeloh A. Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. Arch Toxicol. 2007;81(9):665–73.

Defarge N, Takács E, Lozano VL, Mesnage R, de Vendômois JS, Séralini GE, Székács A. Co-formulants in glyphosate-based herbicides disrupt aromatase activity in human cells below toxic levels. Int J Environ Res Public Health. 2016;13(3).

Diel P, Schmidt S, Vollmer G, Janning P, Upmeier A, Michna H, Bolt HM, Degen GH. Comparative responses of three rat strains (DA/Han, Sprague-Dawley and Wistar) to treatment with environmental estrogens. Arch Toxicol. 2004;78(4):183–93.

Drašar P, Poc P, Stárka L. Glyphosate, an important endocrine disruptor. Diabetol Metab Endokrinol Vyziv. 2018;21(2):93–6.

Gasnier C, Dumont C, Benachour N, Clair E, Chagnon MC, Séralini GE. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology. 2009;262(3):184–91.

Gillezeau C, Van Gerwen M, Shaffer RM, Rana I, Zhang L, Sheppard L, Taioli E. The evidence of human exposure to glyphosate: A review. Environ. Heal. A Glob. Access Sci. Source. BioMed Central; 2019. p. 2.

Hamdaoui L, Naifar M, Rahmouni F, Harrabi B, Ayadi F, Sahnoun Z, Rebai T. Subchronic exposure to kalach 360 SL-induced endocrine disruption and ovary damage in female rats. Arch Physiol Biochem. 2018;124(1):27–34.

Haverfield JT, Ham S, Brown KA, Simpson ER, Meachem SJ. Teasing out the role of aromatase in the healthy and diseased testis. Spermatogenesis. Taylor & Francis; 2011;1(3):240–9.

Hirshfield AN, DeSanti AM. Patterns of ovarian cell

proliferation in rats during the embryonic period and the first three weeks postpartum. Biol Reprod. 1995;53(5):1208–21.

Kezele P, Skinner MK. Regulation of Ovarian Primordial Follicle Assembly and Development by Estrogen and Progesterone: Endocrine Model of Follicle Assembly. Endocrinology. 2003;144(8):3329–37.

Kongtip P, Nankongnab N, Phupancharoensuk R, Palarach C, Sujirarat D, Sangprasert S, Sermsuk M, Sawattrakool N, Woskie SR. Glyphosate and Paraquat in Maternal and Fetal Serums in Thai Women. J Agromedicine. Taylor & Francis; 2017;22(3):282–9.

Landrigan PJ, Belpoggi F. The need for independent research on the health effects of glyphosate-based herbicides. Environ Heal. 2018;17(1):51.

Malekinejad H, Hamidi M, Sadrkhanloo R-A, Ahmadi A. The Effect of Tamoxifen on the Fetal and Neonatal Ovarian Follicles Development in Rats. Iran. J. Basic Med. Sci. Mashhad University of Medical Sciences; 2011 May.

McGee EA, Hsueh AJW. Initial and cyclic recruitment of ovarian follicles. Endocr. Rev. 2000. p. 200–14.

Mesnage R, Bernay B, Séralini G-E. Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity. Toxicology. 2013;313(2–3):122–8.

Mesnage R, Defarge N, Spiroux de Vendômois J, Séralini GE. Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. Food Chem Toxicol. 2015;84:133–53.

Myers JP, Antoniou MN, Blumberg B, Carroll L, Colborn T, Everett LG, Hansen M, Landrigan PJ, Lanphear BP, Mesnage R, Vandenberg LN, vom Saal FS, Welshons W V., Benbrook CM. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. Environ Heal. 2016;15(1):19.

Nilsson E, Rogers N, Skinner MK. Actions of anti-Mullerian hormone on the ovarian transcriptome to inhibit primordial to primary follicle transition. Reproduction. 2007;134(2):209–21.

Ozden-Akkaya O, Altunbas K, Yagcı A. Effects of methoxychlor on IGF-I signaling pathway in rat ovary Effects of methoxychlor on IGF-I signaling pathway in rat ovary. Biotech Histochem. Taylor & Francis; 2017;92(3):230–42.

Parvez S, Gerona RR, Proctor C, Friesen M, Ashby JL, Reiter JL, Lui Z, Winchester PD. Glyphosate exposure in pregnancy and shortened gestational length: A prospective Indiana birth cohort study. Environ Heal A Glob Access Sci Source. Environmental Health; 2018;17(1):1–12.

Pedersen T, Peters H. Proposal for a classification of oocytes and follicles in the mouse ovary. Reproduction. 2007;17(3):555–7.

Pepling ME, Spradling AC. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. Dev Biol. 2001;234(2):339–51.

Picut CA, Dixon D, Simons ML, Stump DG, Parker GA, Remick AK. Postnatal Ovary Development in the Rat:Morphologic Study and Correlation of Morphology to Neuroendocrine Parameters. Toxicol Pathol. 2015;43(3):343–53.

Poulsen MS, Rytting E, Mose T, Knudsen LE. Modeling placental transport: Correlation of in vitro BeWo cell permeability and ex vivo human placental perfusion. Toxicol Vitr. 2009;23(7):1380–6.

Ratia K, Light SH, Antanasijevic A, Anderson WF, Caffrey M, Lavie A. Discovery of Selective Inhibitors of the Clostridium difficile Dehydroquinate Dehydratase. Popoff MR, editor. PLoS One. Public Library of Science; 2014;9(2):e89356.

Ren X, Li R, Liu J, Huang K, Wu S, Li Y, Li C. Effects of glyphosate on the ovarian function of pregnant mice, the secretion of hormones and the sex ratio of their fetuses. Environ Pollut. 2018;243(Pt B):833–41.

Richard S, Moslemi S, Sipahutar H, Benachour N, Seralini GE. Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ Health Perspect. 2005;113(6):716–20.

Romano MA, Romano RM, Santos LD, Wisniewski P, Campos DA, de Souza PB, Viau P, Bernardi MM, Nunes MT, de Oliveira CA. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. Arch Toxicol. 2012;86(4):663–73.

Samsel A, Seneff S. Glyphosate, pathways to modern diseases II: Celiac sprue and gluten intolerance. Interdiscip Toxicol. 2013;6(4):159–84.

Samsel A, Seneff S, Samsel A, Seneff S. Glyphosate's Suppression of Cytochrome P450 Enzymes and Amino Acid Biosynthesis by the Gut Microbiome: Pathways to Modern Diseases. Entropy. Multidisciplinary Digital Publishing Institute; 2013;15(12):1416–63.

Scognamiglio V, Antonacci A, Patrolecco L, Ghuge SA, Lambreva MD, Rea G, Litescu SC. Analytical tools monitoring endocrine disrupting chemicals. TrAC Trends Anal Chem. Elsevier; 2016;80:555–67.

Seneff S, Swanson N, Li C. Aluminum and Glyphosate Can Synergistically Induce Pineal Gland Pathology: Connection to Gut Dysbiosis and Neurological Disease. Agric Sci. 2015;6:42–70.

Stefansdottir A, Fowler PA, Powles-Glover N, Anderson RA, Spears N. Use of ovary culture techniques in reproductive toxicology. Reprod Toxicol. Elsevier Inc.; 2014;49:117–35.

T.C. Tarım ve Orman Bakanlığı. Bitki Koruma Ürünleri Veri Tabanı. 2019.

USEPA. EPA 738-F-93-011. Registration Eligibility Decision (RED) for Glyphosate. Reregistration Eligibility Decis. Glyphosate. 1993.

USEPA. Revised Glyphosate Issue Paper: Evaluation of Carcinogenic Potential.EPA's Office of Pesticide Programs. 2017.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):178-184
DOI: 10.30607/kvj.478070

RESEARCH ARTICLE

Causes of Culling and Disease Incidences At First Production Year of Imported Brown Swiss and Simmental Cows From Austria

Zeynep KUCUK BAYKAN[1], Mustafa OZCAN[2*]

[1]Republic of Turkey, Ministry of Agriculture and Forestry, Saruhanlı District Agriculture Directorate, Manisa, 45800, Turkey
[2]Istanbul University-Cerrahpaşa, Veterinary Faculty, Department of Animal Breeding and Husbandry, Istanbul, 34320, Turkey

ABSTRACT

The purpose of this study is to determine the disease incidences and the causes of culling of Brown Swiss and Simmental cows before and after calving. The study material was consisted of 70 Brown Swiss and 282 Simmental pregnant heifers. 8.57% and 9.57% of Brown Swiss and Simmental cows had various diseases before calving, and the treatment success rates were 16.67% and 81.48%. After calving, the disease incidences of Brown Swiss cows was 75.39%, and the disease incidences of Simmental cows was 64.26%. While digestion and Genital diseases were more common before calving, Genital and udder problems were more common after calving. Simmental cows had a higher rate of treatment success before calving ($P<0.05$). Both breeds had similar rates of treatment success after calving. 7.14% of Brown Swiss cows were culled before calving, and 23.08% were culled after calving due to various reasons. For Simmental cows, the culling rate was 1.77% before calving and 14.08% after calving. Simmental cows were more disease-resistant, responded more positively to treatment and had a lower culling rate than Brown Swiss cows.

Keywords: Simmental, Brown Swiss, Disease, Culling, Adaptation

***

Avusturya'dan İthal Edilen İsviçre Esmeri ve Simmental İneklerin İlk Üretim Yılındaki Hastalık İnsidensleri ve Ayıklama Sebepleri

ÖZ

Çalışmanın amacı, İsviçre Esmeri ve Simmental ırkı gebe düvelerin buzağılama öncesi ve sonrası dönemde karşılaştıkları hastalıkların insidenslerini ve ineklerin sürü dışı edilme sebeplerini belirlemektir. Araştırmanın materyalini Avusturya'nın Innsbruck bölgesinden Türkiye'nin batısında bulunan Manisa iline getirilen 70 baş İsviçre Esmeri ve 282 baş Simmental ırklarından inekler oluşmuştur. İsviçre Esmeri ve Simmental inekler doğum öncesinde %8,57 ve %9,57 oranlarında değişik hastalıklar ile karşılaşmışlar, %16,67 ve %81,48 oranlarında tedavi başarısı elde edilmiştir. Buzağılama sonrası dönemde İsviçre Esmerlerinin hastalanma oranı %75,39 olurken, Simmental ineklerin oranı %64,26 olarak tespit edilmiştir. Sindirim ve genital sistem problemleri buzağılama öncesinde, genital sistem ve meme problemleri buzağılama sonrasında daha yoğun olarak ortaya çıkmıştır. Buzağılama öncesi dönemde Simmental ineklerin tedavi başarısı daha yüksek olurken ($P<0,05$), buzağılama sonrasında her iki ırktan ineklerin tedavi başarı oranları birbirine yakın bulunmuştur. İsviçre Esmeri ineklerin %7,14'ü buzağılama öncesinde, %23,08'i buzağılama sonrasında değişik sebeplere bağlı olarak sürü dışı edilmiştir. Simmentallerde ayıklama oranı buzağılama öncesinde %1,77 ve buzağılama sonrasında %14,08 olarak tespit edilmiştir. Genel olarak, Simmental ineklerin hastalıklara karşı daha dirençli olmuşlar, uygulanan tedaviye daha yüksek oranda pozitif yanıt vermişler ve daha az oranda ayıklamaya tabi tutulmuşlardır.

Anahtar Kelimeler: Sığır, Simmental, İsviçre Esmeri, Hastalık, Adaptasyon

To cite this article: Küçük Baykan Z. Özcan M. Causes of Culling and Disease Incidences At First Production Year of Imported Brown Swiss and Simmental Cows From Austria. Kocatepe Vet J. (2019) 12(2):178-184.

Submission: 02.11.2018 **Accepted**: 14.05.2019 **Published Online**: 18.05.2019
ORCID ID; ZKB: 0000-0002-6423-7985, MÖ: 0000-0002-6045-1394
*Corresponding author e-mail: muscan@istanbul.edu.tr

INTRODUCTION

In cattle enterprises, sustaining productivity in the following years depends on improving cattle's genetic capacity, and also optimizing the enterprise's environmental conditions, completing the cattle's adaptation to geographical, climatic and vegetative environment in a short period of time. It is reported that animals that are moved regionally or imported from abroad suffer from stress factors, and the negative conditions in terms of animal welfare and stress depress animal's immune system, increase disease incidence and prevalence and result in decreased productivity (Broom 2005).

The incidence of various health problems and treatment failures in dairy cattle cause extended periods of open days, delayed gestation, and extended periods between two calving. Those disorders in estrous cycle might lead to extended lactation periods in cows and decreases in lifetime milk yield and economic losses.

Various diseases affecting animals might also affect each other or trigger one another. A study found that there was a positive relation between cows' Genital insufficiencies and clinical mastitis cases, and since foot and leg problems prevent the visibility of heat symptoms, they postpone the insemination time and thus gestation (Maizon et al. 2004).

In dairy cattle enterprises, the disease incidences for per production year are considered as normal 2% for clinic mastitis per month and <3% for retentio secondinarium, 5-25% for metritis, <10% for cystic ovarian, <3% for abomasum displacement and <25% for lameness per year (Dinç 2016). One of the most important indicators of whether the herd management programs are successful in dairy cattle enterprises is the level of annual culling rates and the frequency of culling reasons. It is reported that the culling rate of enterprises per year is around 21-36% (Maher et al. 2008; Pinedo et al. 2010; Stojic et al. 2013), and while 12-18% is considered normal in terms of indicating heard health, this rate might reach 25% with the addition of voluntary culling (Dinç 2016).

The culling rates in herds change depending on the breed differences, disease incidences and frequency in herd, cows' age, lactation order, Genital and lactation performance and unexpected economic situations of enterprise. While Genital diseases are the most common causes of culling for cows in the herds, they are followed by mastitis, foot and hoof diseases and metabolism diseases respectively (Seegers et al. 1998, Fetrow et al. 2006, Pinedo et al. 2010, Stojic et al. 2013).

The study made by Pinedo et al. (2010) on 2054 Holstein herds found that the culling and mortality rates per year were 25.1% and 6.6% respectively. It reported that 20.6% of all of culling reasons were atypical deaths; this was followed by Genital problems (17.7%), injuries (14.3%) and low productivity and mastitis (12.1%). In this research, among all culled and dead cows; the culling rate of young cows at their first calving year was 20%. Stojic et al. (2013) found that, in herds with high yield, 32.5% of young cows that calved for the first time were culled for foot and hoof diseases, 19.9% were culled for Genital problems, and 11% were culled for metabolic diseases. They reported that the culling rate due to Genital problems were lower in herds with low milk yield than high productive herds.

The aim of this study is to determine the disease incidences and treatment success rates of pregnant Simmental and Brown Swiss cows, which were imported from Austria to a private breeding dairy cattle enterprise in Manisa, before and after calving, to identify the culling reasons at the first production year. According to diseases and treatment incidences and culling rates of Simmental and Brown Swiss cows, it is possible to make scientific comments about importation success and adaptation ability in the West Anatolia location and compare between Simmental and Brown Swiss breeds at their first production year.

MATERIAL and METHODS

Material Definition

The study was conducted in a private dairy cattle enterprise, which was located in Manisa and which has recently started livestock operations. Manisa is located in Western Anatolia Region between 27 08' and 29 05' eastern longitudes and 38 04' and 39 58' northern latitudes. It has Mediterranean continental climate. Precipitation generally occurs in winter, and summer is dry. The temperature is below zero for an average of 26 days per year (Municipality of Manisa 2017).

The first animal materials of the enterprise were contained 352 pregnant, disease free (such as tuberculosis, blue tongue, bovine spongiform encephalopathy, foot and mouth disease) heifers imported from Innsbruck/Austria. Also their body weights were between 400 kg and 550 kg. Of those cows, 70 were Brown Swiss and 282 were Simmental. The pregnant cows calved in the enterprise between June and December.

Material Care and Feeding

The cattle barns were constructed in a free range and semi-open system. When the cows were transported to the enterprise, they were first taken into quarantine, and then were placed in paddocks. The

cows were vaccinated with IBR, BVD, Clostridium, Pasteurella vaccines and feed to special feeding with forage (Net energy maintenance-NEm:1,37 Mcal/kg, NEg:0,8 Mcal/kg, CP: 12,5%DM, ADF: 31,4%DM, NDF: 48,9%DM) at this period.

The cows which were lactation period had three different rations, namely fresh ration, lactation and dry-period rations. After calving, cows were subjected to post-calving feeding for 3 weeks, and then lactation feeding. First period ration, nutrient information was NEL (Net energy lactation): 1,62 Mcal/kg, CP (crude protein): 17,2%DM (dry matter), ADF (acid detergent fiber): 20,22%DM, NDF (neutral detergent fiber): 34,68%DM, starch: 24,4%DM. The ration consisted of: 14% alfalfa, 48% corn silage, 3% oat-barley hay, 11% seasonal pulp, 24% factory lactation feed mix. In the lactation period, NEL: 1,61 Mcal/kg, CP: 16,89 % DM, ADF: 22,67 % DM, NDF: 37,33 % DM, starch: 23 % DM and 11% alfalfa, 50% corn silage, 4% oat-barley hay, 12% seasonal pulp, 23% factory lactation feed mix was given. Lactation feed was prepared according to the cows with high and low milk yields in the progressing milking periods. The dry-period feed consisted of: 19% alfalfa, 38% corn silage, 30% oat-barley hay, 4% seasonal pulp, 9% dry-period factory feed mix and NEL: 1,3 Mcal/kg, CP: 14,5 %DM, ADF: 31 %DM, NDF: 49 %DM, anion cation balance: 21,34, Ca/P: 1,9.

Disease Incidences

The diseases detected in 352 cows (70 Brown Swiss, 282 Simmental) were divided into two as before calving (after transport) and after calving. The period after transport covered the period between the day when the cows were brought to the enterprise and the first calving; the period after calving covered the period between the 1st calving and 2nd one. The incidence and treatment success of diseases detected in those two periods were expressed proportionately. The culling and mortality causes of cows at the first production year were assessed according to breeds and diseases.

All cows were observed in point of behavior differences, body conditions and disease symptoms every day. The diseases were diagnosed by the enterprise's veterinarian by using the cow's body temperature, auscultation, palpation, urine and feces examination, various observation data, blood samples and ultrasound data.

The disease definitions were as follows:

A. Digestive problems: Ketosis, abomasum displacement, diarrhea, tympany and constipation;

B. Respiratory problems: Pneumonia cases presenting with mucous or sera-mucous nasal flow and , abdominal panting;

C. Genital Diseases: Uterine prolapsed, metritis, ovarian cysts, dystocia, chronic infertility cases;

D. Foot problem: Lameness or being unable to stand on a foot condition in one foot or more, swelling in joints, bleeding, fractures or cracks in extremities;

E. Udder problems: Traumatic udder injuries, udder edema, udder blindness and acute or chronic mastitis cases;

F. Other disorders: Cachexia, ketosis, idiopathic/atypical cases that occur due to the mistakes (contraindication after wrong treatment protocols, unexpected secondary infections, short-term care and feeding mistakes, late observation of health problems etc.) of veterinarian, technician or caregiver or unknown reasons.

Statistical analysis

When calculating the disease incidences of cows, sub-groups were made such as before calving, after calving, disease incidence, treatment success and mortality cause; the conditions of cases were addressed; and the results were presented by using values expressed in percentages. "Pearson Chi–Square Test" and "Fisher's Exact Test" were used for comparisons between groups. SPSS (Statistical Package for the Social Sciences) program package was used for all statistical analysis (SPSS 1999).

RESULTS

The disease incidences of Brown Swiss and Simmental cows were addressed separately for the before calving (after transport) and after calving periods.

The diseases in the period before calving covered the diseases that the pregnant cows had since their arrival in the enterprise until their first calving or abort, and these are shown in Table 1. The detailed results of diseases are shown in Table 2. In the after transport/before calving period, 8.57% of Brown Swiss and 9.57% of Simmental cows were diagnosed various diseases. While there was no statistically significant difference between Brown Swiss and Simmental cows in terms of disease incidence, the positive response of sick cows to treatment was higher in Simmental cows (81.48%) and this was statistically significant (P<0,05). The overall treatment success rate was 16.67% for Brown Swiss.

In the period before calving, the cows had cases of diarrhea, constipation, pneumonia, lameness. In this period, the treatment of diseases such as cachexia, lameness, diarrhea, constipation and pneumonia had a success rate between 75% and 100%. It was seen that 12 cows, which were imported to the enterprise, were not pregnant or had early abort, and the cows were taken into treatment and re-insemination process. For the treatment of idiopathic infertility problems with unknown origins, treatment success rate was 71.43% for Simmental cows (P<0.05). However, the

treatments for Brown Swiss failed, and the cows were culled.

After calving, diseases of various origins were detected in 75.39% of Brown Swiss and 64.26% of Simmental cows. According to the disease incidence and treatment success rates of Brown Swiss and Simmental cows between their 1st and 2nd calving, the distribution of other diseases (except for other disorders) was statistically insignificant according to breed. While digestive and respiratory system diseases were more common in Simmental cows, Genital diseases, foot diseases and udder diseases were more common in Brown Swiss. However, the differences between breeds were statistically insignificant (P>0.05).

After calving, the most common cases were metritis, ovarian cysts, mastitis and cachexia. The rate of cachexia was 13.85% in Brown Swiss and 3.25% in Simmental, which was statistically significant (P<0.05). When treatment success rates were evaluated in general, uterine prolapse and uterus abscess cases had the lowest treatment success. Higher treatment success was obtained in treating metritis in Simmental cows (P<0.05).

Table 3 indicates the causes of cow mortality and culling before and after calving in the first production year. 10 cows died or were culled before calving, and 54 cows died or were culled after calving due to various reasons. Brown Swiss had more losses before and after calving. Before calving, Brown Swiss cows died or were culled only due to idiopathic infertility, while Simmental cows died or were culled due to diarrhea, constipation, pneumonia, lameness and idiopathic infertility etc. After calving, retentio secondinarium was the only condition that did not cause mortality/culling. For Brown Swiss, mortality/culling due to cachexia had a higher rate compared to Simmental (P<0.05). Cachexia is including anorexia in this research. It is looking as a consequence of the loss of appetite metabolic disorders in dairy farm. In postpartum period, some cows have shown anorexia and debility due to difficult birth. This status can cause the cows to weaken over time.

DISCUSSION and CONCLUSION

The imported Brown Swiss and Simmental cows had a disease ratio of 9.38% before calving, and the treatment success rate was 70%. Before calving, Simmental mostly had digestive problems, and Brown Swiss had genital diseases. Simmental cows had a higher treatment success rate in this period. This can be explained by the less group number of Brown Swiss cows in the enterprise and to be more resistant of Simmental cows to changing environmental conditions and transportation.

The most common diseases encountered by both breeds after 1st calving were: Genital diseases (33.33%), udder diseases (12.86%), digestive system problem (5.55%), foot diseases (5.26%), and respiratory system problem (4.09%) respectively. These results were consistent with the findings in literature, which reports that Genital and udder diseases were more common in dairy cattle enterprises (Seegers et al. 1998, Fetrow et al. 2006, Pinedo et al. 2010, Stojic et al. 2013). According to the incidence of specific diseases; the incidence of metritis (13.16%), mastitis (9.06%), ovarian cyst (8.18%), and retentio secondinarium (4.09%) were higher than other diseases.

The annual rates were considered normal for diseases in breeding dairy cattle enterprises are: <3% for retentio secondinarium, 5-25% for metritis, <10% ovarian cysts, 2% for clinic mastitis, <3% for abomasum displacement, <25% for lameness, and <35% for culling (Dinç 2016). Accordingly, while the incidences of metritis and ovarian cysts were reasonable in the enterprise, the incidences of mastitis and retentio secondinarium were above normal.

Table 1. Incidence and treatment success of system diseases in Brown Swiss and Simmental cows before and after calving (%)

DISEASES	BEFORE CALVING								AFTER CALVING							
	INCIDENCE				TREATMENT SUCCESS				INCIDENCE				TREATMENT SUCCESS			
	Brown Swiss (N:70)		Simmental (N:282)		Brown Swiss (N:70)		Simmental (N:282)		Brown Swiss (N:65)		Simmental (N:277)		Brown Swiss (N:65)		Simmental (N:277)	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Digestive system diseases	0	0	14	4.96	0	0	13	92.86	1	1.54	18	6.50	1	100.00	17	94.44
Respiratory system diseases	1	1.43	4	1.42	1	100.00	3	75.00	1	1.54	13	4.69	1	100.00	9	69.23
Genital system diseases	5	7.14	7	2.48	0	0.00[b]	5	71.43[a]	25	38.46	89	32.13	18	72.00	72	80.89
Foot diseases	0	0	1	0.35	0	0	1	100.00	4	6.15	14	5.02	4	100.00	9	64.29
Udder diseases	0	0	0	0	0	0	0	0	9	13.85	35	12.64	6	66.67	24	68.57
Other disorders	0	0	1	0.35	0	0	0	0	9	13.85[a]	9	3.25[b]	5	55.56	8	88.89
GENERAL	**6**	**8.57**	**27**	**9.57**	**1**	**16.67 [b]**	**22**	**81.48[a]**	**49**	**75.39**	**178**	**64.26**	**35**	**71.43**	**139**	**78.09**

a, b : Differences between the rates with different letters in the same lines are significant (P<0,05).

Table 2. Incidence and treatment success of specific diseases in Brown Swiss and Simmental cows before and after calving (%)

DISEASES	BEFORE CALVING								AFTER CALVING							
	INCIDENCE				TREATMENT SUCCESS				INCIDENCE				TREATMENT SUCCESS			
	Brown Swiss (N:70)		Simmental (N:282)		Brown Swiss (N:70)		Simmental (N:282)		Brown Swiss (N:65)		Simmental (N:277)		Brown Swiss (N:65)		Simmental (N:279)	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Ketosis	0	0	0	0	0	0	0	0	0	0	7	2.53	0	0	7	100.00
Abomasum Displacement	0	0	0	0	0	0	0	0	0	0	2	0.72	0	0	2	100.00
Diarrhea, Constipation	0	0	14	4.96	0	0	13	92.86	1	1.54	9	3.25	1	100.00	8	88.89
Pneumonia	1	1.43	4	1.42	1	100.00	3	75.00	1	1.54	13	4.69	1	100.00	9	69.23
Uterine prolapse	0	0	0	0	0	0	0	0	0	0	5	1.81	0	0	3	60.00
Metritis	0	0	0	0	0	0	0	0	10	15.38	35	12.63	7	70.00[b]	34	97.14[a]
Retentio secondinarium	0	0	0	0	0	0	0	0	4	6.15	13	4.69	4	100.00	13	100.00
Abscess adherence in uterus	0	0	0	0	0	0	0	0	1	1.54	13	4.69	0	0	8	61.54
Ovarian cysts	0	0	0	0	0	0	0	0	9	13.85	19	6.86	7	77.78	14	73.68
Idiopathic infertility	5	7.14	7	2.48	0	0.00[b]	5	71.43[a]	0	0	3	1.08	0	0	0	0
Dystocia	0	0	0	0	0	0	0	0	1	1.54	1	0.36	0	0	0	0
Mastitis	0	0	0	0	0	0	0	0	7	10.77	24	8.66	5	71.43	19	79.17
Traumatic injury of udder	0	0	0	0	0	0	0	0	1	1.54	5	1.81	1	100.00	5	100.00
Low milk yield	0	0	0	0	0	0	0	0	1	1.54	5	1.81	0	0	0	0
Udder Blindness	0	0	0	0	0	0	0	0	0	0	1	0.36	0	0	0	0
Lameness	0	0	1	0.35	0	0	1	100.00	4	6.15	14	5.05	4	100.00	9	64.29
Cachexia	0	0	1	0.35	0	0	1	100.00	9	13.85[a]	9	3.25[b]	5	55.56	8	88.89

a, b : Differences between the rates with different letters in the same lines are significant (P<0,05).

Table 3. Causes of culling in Brown Swiss and Simmental cows before and after calving (%)

DISEASES	BEFORE CALVING						AFTER CALVING					
	Brown Swiss (N:70)		Simmental (N:282)		Overall (N:352)		Brown Swiss (N:65)		Simmental (N:277)		Overall (N:342)	
	n	%	n	%	n	%	n	%	n	%	n	%
Diarrhea, constipation	0	0	1	20.10	1	10.00	0	0	1	2.56	1	1.85
Pneumonia	0	0	1	20.00	1	10.00	0	0	4	10.26	4	7.41
Uterine prolapse	0	0	0	0	0	0	0	0	2	5.13	2	3.70
Metritis	0	0	0	0	0	0	3	20.00	1	2.56	4	7.41
Retentio secondinarium	0	0	0	0	0	0	0	0	0	0	0	0
Abscess adherence in uterus	0	0	0	0	0	0	1	6.67	5	12.82	6	11.11
Ovarian cysts	0	0	0	0	0	0	2	13.33	5	12.82	7	12.96
Idiopathic infertility	5	100.00	2	40.00	7	70.00	0	0	3	7.69	3	5.56
Dystocia	0	0	0	0	0	0	1	6.67	1	2.56	2	3.70
Mastitis	0	0	0	0	0	0	2	13.33	5	12.82	7	12.96
Low milk yield	0	0	0	0	0	0	1	6.67	5	12.82	6	11.11
Udder blindness	0	0	0	0	0	0	0	0	1	2.56	1	1.85
Lameness	0	0	0	0	0	0	0	0	5	12.82	5	9.26
Cachexia	0	0	1	20.00	1	10.00	4	26.60[a]	1	2.56[b]	5	9.26
Idiopathic mortality	0	0	0	0	0	0	1	6.67	0	0	1	1.85
GENERAL	**5**	**7.14[a]**	**5**	**1.77[b]**	**10**	**2.84**	**15**	**23.08**	**39**	**14.08**	**54**	**15.79**

a, b : Differences between the rates with different letters in the same lines are significant (P<0,05)

Reasons such as the length of time required for cows' adaptation to machine milking after first calving, lack of experience of milking personal in the newly-established enterprise and failing to show enough sensitivity for milking hygiene might have increased mastitis rate.

Studies made in dairy farms with different breeds and different countries were reported that Retentio secondinarium incidence was 2,6-3%; Metritis incidence was 2,9-32,6%; Cystic Ovarian incidence was 9,6%; Mastitis incidence was 1,3-6,5%; Foot diseases incidence was 2-57,3% (Stanton et al. 2011, Sharifi et al. 2012, Stojic et al. 2013, Scköpke et al. 2013, Ansari-Lari et al. 2013, Hagiya et al. 2014, Koeck et al. 2014, Yin et al., 2014). The main reason for the difference between the reports and the cows after calving studied in this study might describe from the difference in researchers' disease definitions. Since the sub-clinic and clinic cases were assessed together in this study, it might cause having higher values than the sources calculating only clinic cases. In addition, the differences between studies in terms of breed, age, breeding location, breeding system (organic, conventional), season and disease appraisal period might be affected as the other reasons of differences in disease incidences (Seegers et al. 1998, Fetrow et al. 2006, Dinç A. 2016).

In this study, culling rate after calving was 15.79% (including voluntary culling and mortality), and the culling rate due to Genital problems among culled ones was 6.96%. Those values were lower than the overall culling rates reported for different cattle breeds (Sharifi et al. 2012, Ansari-Lari et al. 2013, Ferguson et al. 2013, Stojic et al. 2013). Moreover, the fact that the most common reason for culling in this farm was genital problems such as metritis, cystic ovary and uterus complications etc. was consistent with reports (Seegers et al. 1998, Fetrow et al. 2006, Pinedo et al. 2010, Stojic et al. 2013). Digestive system problems caused more culling rates in Brown Swiss than Simmental cattle.

Difficulties experienced in the preparation and implementation of feed and ration protocols in the cattle breeding enterprise, which has started production for the first time, and also the lack of experience of the animal care workers, who worked in the farm and had a high turnover rate, might have increased the culling rates. The higher culling rates of Brown Swiss cows before and after calving shows the difference in the adaptation capacities of cattle breeds.

When study results are assessed together, the imported all cows had disease incidences of 9.38% before calving, and the treatment success rate was 70%. Before calving, the most common diseases were digestive and Genital diseases. The success rate in treating diseases was higher for Simmental cows. The overall disease and culling incidences according to health parameters at the first production year of Simmental cows was equal or below the targeted values, which indicates that Simmental cows were higher adaptability than Brown Swiss cows in West Anatolia region of Turkey. These findings shows that Brown Swiss cows at the first production year are need to care under careful and attentive breeding and health program than Simmentals in this region.

ACKNOWLEDGEMENT

This study was arranged from a part of the first author's PhD thesis. This work was supported by Scientific Research Projects Coordination Unit of Istanbul University (Project Number: 23141). Küçük Baykan Z., Özcan M., "Causes of culling and disease incidences at first production year of pregnant Brown Swiss and Simmental cows", 5th International Vet-Istanbul Group Congress & 8th International Scientific Meeting Days of Veterinary Medicine-2018, Ohrid, MAKEDONYA, 23-27 Eylül 2018, pp.130-130. The research protocol of the current study was approved by the Ethic Committee of the Istanbul University (Approval number: 2012/31)

REFERENCES

Ansari-Lari M, Mohebbi-Fani M, Rowshan-Ghasrodashti A. Causes of culling in dairy cows and its relation to age at culling and interval from calving in Shiraz, southern Iran. Veterinary Research Forum. 2012; 3, 4: 233-237.

Broom DM. The effects of land transport on animal welfare. Rev. Sci. Tech. Off. Int. Epiz. 2005; 24, 2: 683-691.

Dinç A. Reproductive Performance Parameters of Dairy Cows. 4. Herd Health Symposium. 2016; 25-28 May 2016, Antalya, 111-128.

Ferguson JD, Skidmore A. Reproductive performance in a select sample of dairy herds. J. Dairy Sci. 2013; 96: 1269-1289.

Fetrow J, Nordlund KV, Norman HD. Culling: Nomenclature, definition and recommendations. J. Dairy Sci. 2006; 89: 1896-1905.

Hagiya K, Yamazaki T, Nagamine Y, Togashi K, Yamaguchi S, Gotoh Y, Kawahara T, Masuda Y, Suzuki M. Genetic correlations between production and disease traits during first lactation in Holstein cows. The Animal Consortium. 2014; 8, 2: 217–223.

Koeck A, Loker S, Miglior F, Kelton DF, Jamrozik J, Schenkel F. Genetic relationships of clinical mastitis, cystic ovaries, and lameness with milk yield and somatic cell score in first-lactation Canadian Holsteins. J. Dairy Sci. 2014; 97: 5806-5813.

Maher P, Good M, More SJ. Trends in cow numbers and culling rate in the Irish cattle population, 2003 to 2006. Irish Vet. J. 2008; 61, 7: 455-463.

Maizon DO, Oltenacu PA, Gröhn YT, Strawderman RL, Emanuelson U. Effects of diseases on reproductive performance in Swedish red and white dairy cattle. Preventive Vet. Med. 2004; 66: 113-126.

Municipality of Manisa (Date of access: 20.03.2017) (http://www.manisa.bel.tr/s23_manisa-cografyasi.aspx)

Pinedo PJ, De Vries A, Webb DW. Dynamics of culling risk with disposal codes reported by dairy herd improvement dairy herds. J. Dairy Sci. 2010; 93: 2250-2261.

Schöpke K, Weidling S, Pijl R, Swalve HH. Relationships between bovine hoof disorders, body condition traits, and test-day yields. J. Dairy Sci. 2013; 96: 679–689.

Seegers H, Beauddeau F, Fourichon C, Bareille N. Reasons for culling in French Holstein cows. Preventive Vet. Med. 1998; 36: 257-271.

Sharifi H, Kostoulas P, Bahonar A, Bokaie S, Vodjgani M, Hangdoost AA, Karamouzian M, Foroushani AR, Leontides L. Effect of health disorders on the hazard of culling on the first or second lactation in Iranian dairy herds. Preventive Vet. Med. 2012; 109: 144-147.

SPSS. Statistical Package For The Social Sciences, Release 10.0. 1999, SPSS Inc. IL, Chicago, USA.

Stanton AL, Kelton DF, Leblanc SJ, Wormuth Leslie KE. The effect of respiratory disease and a preventative antibiotic treatment on growth, survival, age at first calving, and milk production of dairy cows. J. Dairy Sci. 2011; 95: 4950–4960.

Stojic P, Beskorovajni R, Pantelic V, Novakovic Z, Kovacevic S, Stanojevic D. Causes for culling first calving cows on farms with different levels of production. Biotechnology In Animal Husbandry. 2013; 29, 2: 259-267.

Yin T, Bapst B, Von Borstel UU, Simianer H, König S. Genetic analyses of binary longitudinal health data in small low input dairy cattle herds using generalized linear mixed models. Livestock Sci. 2014; 162: 31-41.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):185-192

DOI: **10.30607/kvj.486698**

RESEARCH ARTICLE

An Assessment About The History of The Turkish Veterinary Medical Association Chamber of Veterinarian for Afyonkarahisar

Emine TÜRKMENOĞLU*

Yozgat Bozok University, Faculty of Veterinary Medicine, Department of Veterinary History and Deontology, Sorgun/Yozgat, Turkey

ABSTRACT

The first chambers of veterinarian became operative in Turkey after the enactment of Law number 6343 in 1954. Chambers of veterinarian are tasked with ensuring that the relevant professional legislation is duly applied. This study has been carried out to provide information about the historical development of the Turkish Veterinary Medical Association Chamber of Veterinarian for Afyonkarahisar Region (Afyonkarahisar CV). The study material is comprised of first hand documents obtained from the Afyonkarahisar CV Archive and the Turkish Veterinary Medical Association Archive and the relevant material has been assessed with the retrospective method used in historical research. Afyonkarahisar CV became operable in 1968. During its historical development process, the chamber has held meetings in search of solutions for professional problems, decision making and organized various training programs. It has been concluded that Afyonkarahisar CV is responsible for positive contributions to the professional development of veterinary medicine in Turkey.

Keywords: The Chamber of Veterinarian for Afyonkarahisar, The Turkish Veterinary Medical Association, History of Veterinary Medicine.

***

Türk Veteriner Hekimleri Birliği Afyonkarahisar Veteriner Hekimler Odasının Tarihi Hakkında Bir Değerlendirme

ÖZ

Türkiye'de ilk veteriner hekim odaları 6343 sayılı Kanunun yürürlüğe girmesinden sonra 1954'te faaliyete geçirildi . Veteriner hekim odaları meslekle ilgili mevzuatın gereği gibi uygulanmasını sağlamakla yükümlüdürler. Bu çalışma Türk Veteriner Hekimleri Birliği Afyonkarahisar Veteriner Hekimler Odası (Afyonkarahisar VHO)'nın tarihi gelişimi hakkında bilgi vermek amacı ile yapıldı. Çalışmanın materyalini Afyonkarahisar VHO Arşivi ve Türk Veteriner Hekimleri Birliği Arşivinden elde edilen ilk elden belgeler oluşturdu ve söz konusu materyal, tarih araştırmalarında kullanılan retrospektif yöntemle değerlendirildi. Afyonkarahisar VHO'nun 1968'de faaliyete geçtiği saptandı. Tarihi gelişim süreci içerisinde oda tarafından mesleki sorunlara çözüm yollarının arandığı toplantıların kararlaştırıldığı ve çeşitli eğitim programlarının düzenlendiği belirlendi. Afyonkarahisar VHO'nun Türkiye'de veteriner hekimliği mesleğinin gelişimine olumlu katkılarda bulunduğu sonucuna varıldı.

Anahtar Kelimeler: Afyonkarahisar Veteriner Hekimler Odası, Türk Veteriner Hekimleri Birliği, Veteriner Hekimliği Tarihi.

To cite this article: Türkmenoğlu E. An Assessment About The History of The Turkish Veterinary Medical Association Chamber of Veterinarian for Afyonkarahisar. Kocatepe Vet J. (2019) 12(2):185-192.

Submission: 22.11.2018 **Accepted**: 14.05.2019 **Published Online**: 22.05.2019

ORCID ID; ET: 0000 0002 3868 0908,

*Corresponding author e-mail: e_turkmenoglu@hotmail.com

GİRİŞ

Türk Veteriner Hekimleri Birliği (TVHB) 6343 sayılı Kanunla kurulmuş olan ve veteriner hekimliği mesleğinin Türkiye çıkarlarına en faydalı biçimde uygulanmasını sağlama, veteriner hekimlerin maddi ve manevi hak ve yararlarını koruma gibi görevleri bulunan kamu kurumu niteliğinde bir meslek kuruluşudur[1] . TVHB'yi oluşturan organlardan biri olan veteriner hekim odalarının ilk olanlarına 1954'te işlerlik kazandırıldı[2]. Veteriner hekim odaları, sınırları içinde en az 30 veteriner hekim bulunan illerde ya da bölgelerde kurulmaktadır. Odalar sahip oldukları genel kurul, yönetim kurulu, denetleme kurulu ve haysiyet divanı olmak üzere dört organ aracılığıyla meslekle ilgili mevzuatın gereği gibi uygulanmasını sağlamaya yönelik çalışmalarda bulunmaktadır[3] .

Günümüzde Türkiye'de faaliyet gösteren 56 veteriner hekim odasından sekizinin Ege Bölgesinde bulunduğu ve TVHB İzmir Veteriner Hekimler Odası (İzmir VHO)'ndan (1954) sonra TVHB Afyonkarahisar Veteriner Hekimler Odası (Afyonkarahisar VHO)nın bölgede kurulan ikinci oda olduğu belirlendi[4].

Türkiye'deki veteriner hekim odalarının tarihi konusunda TVHB Samsun-Sinop Bölgesi Veteriner Hekimler Odası (Samsun-Sinop VHO) (Sanal ve Melikoğlu Gölcü 2014) ve TVHB Kars Bölgesi Veteriner Hekimleri Odası (Kars VHO) (Kızıltepe 2017) hakkında birer adet bilimsel yayına rastlandı. Yapılan incelemelerde Afyonkarahisar VHO'nun 1968-1975 yıllarına ait herhangi bir evraka rastlanamadı. Bu araştırma Afyonkarahisar VHO'nun tarihsel gelişimi hakkında bilgi vermek amacı ile yapıldı.

MATERYAL VE METOT

Çalışmanın materyalini Resmi Gazete Arşivi, TVHB Arşivi ve Afyonkarahisar VHO Arşivinden sağlanan ilk elden belgeler oluşturdu. Ayrıca konuyla ilgili literatürden faydalanıldı. Orijinal belgelerin künyeleri dipnotlar halinde gösterildi. Mevcut materyal tarih araştırmalarında kullanılan retrospektif yöntem ile değerlendirildi.

BULGULAR

Afyonkarahisar VHO'nun 1968'de kurulduğu ve ilk oda yönetim kurulu başkanının Mehmet Eroğlu olduğu (1968-1969) belirlendi. Daha sonra sırası ile Yakup Hamza Çebi **(**1969-1970), Mehmet Eroğlu (1970-1971, 1971-1972), Abdullah Kardan (1972-1973), Süleyman Özkan (1973-1974) ve Sedat Öncel'in (1974-1975, 1975-1976) odaya başkanlık yaptıkları saptandı[5],[6]. Günümüze kadar seçilmiş 34 oda yönetim kurulu başkanı arasında hiç kadın veteriner hekimin bulunmadığı ve toplam yönetim kurulu üyeleri arasında bir kadın üyenin sadece bir dönem yer aldığı ve odanın diğer organlarında hiç kadın bulunmadığı belirlendi. Afyonkarahisar VHO organlarına seçilmiş veteriner hekimlerin isimleri Tablo 1-4'te sunuldu[7].

Resmi evraklarda odanın isminin 1976'dan itibaren "Afyon Bölgesi Veteriner Hekimler Odası" biçiminde yazıldığı[8] ve 1977'de odanın sorumluluk alanında Afyonkarahisar, Antalya, Burdur, Isparta ve Uşak illerinin bulunduğu tespit edildi[9]. Antalya'nın 1978, Burdur'un 1992[10], Isparta'nın 19. ile 20. dönem genel kurulları arasındaki süreçte[11], Uşak'ın ise 1998'de Afyonkarahisar VHO'dan ayrıldığı[12],[13] ve bundan sonra odanın isminin "Afyon Veteriner Hekimler Odası" biçiminde, 2006'dan itibaren ise "Afyonkarahisar Veteriner Hekimler Odası" biçiminde yazıldığı tespit edildi[14].

Antalya'da 30 Eylül 1976'da gerçekleşen genel kurul sonrasında Antalya Veteriner İşleri Baş Müdürü Sedat Öncel'den[15] boşalan oda başkanlığına Afyonkarahisar Veteriner İşleri Müdürü Necdet Demir'in gelmesinden sonra oda adresinin Afyonkarahisar Veteriner İşleri Müdürlüğü olduğu[16] ve 1979'da söz konusu müdürlüğün bir odasında il dışından gelen oda üyelerinin barınması için bir misafirhane kurulduğu saptandı[17],[18]. Veteriner Bölge Sağlık Kontrol Laboratuvar Müdürlüğünün yeni binasına taşınmasından sonra eski binasının uygun olan odalarından bazılarının Afyonkarahisar VHO'ya lokal ve misafirhane olarak ayrıldığı belirlendi[19]. Söz konusu misafirhanenin 1985'te kapatıldığı[20] ve 2005 yılında oda için Vakıflar Müdürlüğünde bir büro kiralanmasına karar verildiği[21] ve oda adresinin 3. Vakıf İş Hanı kat: 6, no: 42 olduğu saptandı[22]. Günümüzde ise Afyonkarahisar VHO'nun 2013'te satın alınan Çetinkaya mahallesi Uydukent İş Merkezindeki 22 no'lu dairede faaliyet gösterdiği belirlendi[23],[24].

[1] 09/03/ 1954 tarihli ve 6343 sayılı Veteriner Hekimliği Mesleğinin İcrasına, Türk Veteriner Hekimleri Birliği ile Odalarının Teşekkül Tarzına ve Göreceği İşlere Dair Kanun, 18/03/1954 tarihli ve 8661 sayılı Resmî Gazete.
[2] TVHB Arşiv Belgeleri (1954-2018).
[3] Türk Veteriner Hekimleri Birliği Hizmetlerinin Yürütülmesine İlişkin Uygulama Yönetmeliği, 13/09/2006 tarihli 26288 sayılı Resmi Gazete.
[4] Bkz: Dipnot 2.
[5] 23/01/1985 tarih ve 73/300 nolu gelen evrak. Afyonkarahisar VHO Arşivi.
[6] 31/01/1985 tarih ve 19 nolu giden evrak. Afyonkarahisar VHO Arşivi.
[7] 1976-2016 genel kurul seçim tutanakları. Afyonkarahisar VHO Arşivi.
[8] Afyonkarahisar VHO Arşivi.
[9] 1977-1978 dönemi yönetim kurulu çalışma raporu. Afyonkarahisar VHO Arşivi.
[10] Bkz: Dipnot 2.
[11] 1992-1994 dönemi yönetim kurulu çalışma raporu. Afyonkarahisar VHO Arşivi.
[12] 23/03/1998 tarih ve 20nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[13] 21/08/1998 tarih ve 30 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[14] 27/09/2006 tarihli genel kurul seçim tutanağı. Afyonkarahisar VHO Arşivi.
[15] 1976-1977 dönemi yönetim kurulu çalışma raporu. Afyonkarahisar VHO Arşivi.
[16] Bkz: dipnot 8
[17] 22/01/1979 tarih ve 8 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[18] 12/03/1979 tarih ve 11 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[19] 1980-1981 dönemi yönetim kurulu çalışma raporu. Afyonkarahisar VHO Arşivi.
[20] 13/05/1985 tarih ve 9 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[21] 15/02/2005 tarih ve 35 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[22] 03/11/2013 tarihli yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[23] 27/09/2013 tarihli yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[24] 30/09/2013 tarihli yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.

Tablo 1: Afyonkarahisar VHO Yönetim Kurulu (1976-2016)
Table 1: Afyonkarahisar VHO Executive Board (1976-2016)

Dönem	Başkan	Sekreter	Muhasip	Üye	Üye
1976-1977	Necdet Demir	O. Nuri Serdaroğlu	Semai Çelebi	Erol Çelebi Hasan Sarıbaş ↑	Mehmet Ayalp
1977-1978	Necdet Demir	O. Nuri Serdaroğlu	Hüseyin Kurtulmuş	Aydın Tuncer	Semai Çelebi
1978-1979	Necdet Demir	O. Nuri Serdaroğlu	Semai Çelebi	Mehmet Çakırgöz	Rıfat Açıkgöz
1979-1980	Necdet Demir	O. Nuri Serdaroğlu	Semai Çelebi	Mehmet Çakırgöz	Aydın Tuncer
1980-1981	Necdet Demir	O. Nuri Serdaroğlu	Semai Çelebi	Mehmet Çakırgöz	Ramazan Şimşek
1981-1982	Necdet Demir	Rıfat Açıkgöz	Ramazan Şimşek	Mehmet Çakırgöz	Hasan Aytekin Ali Ünsoy↑
1982-1984	Necdet Demir	Düzali Karataş	Ramazan Şimşek	Ümit Ülker	Hamza Akalın
1984-1986	Necdet Demir Bekir Aslan↑	Hüsamettin Öner	Bekir Aslan Ramazan Şimşek↑	Cengiz Tan Hüseyin Tan↑	Baki Uçman
1986-1988	Bekir Aslan Hüsamettin Öner↑	Hüsamettin Öner Semai Çelebi↑	Semai Çelebi Hüseyin Tan↑	Hüseyin Tan Düzali Karataş↑	O. Nuri Serdaroğlu
1988-1990	Semai Çelebi	Ali Ünsoy	Şükrü Şap	Ümit Ülker Halil Kalınbaçoğlu↑	O. Nuri Serdaroğlu
1990-1992	Semai Çelebi	O. Şükrü Aksu	Mehmet Çelik	Mehmet Çakırgöz	Ali Ünsoy
1992-1994	Ali Ünsoy	O. Şükrü Aksu	Bekir Aslan	Mehmet Çakırgöz	Semai Çelebi
1994-1996	Bekir Aslan	M. Azmi Gökteke	Halil Kalınbaçoğlu	Beyazıt Yılmaz Rıza Avşar↑	Semai Çelebi
1996-1998	O. Şükrü Aksu	Şiar Ayhan	M. Azmi Gökteke	Mehmet Çelik	Mahmut Salman
1998-2000	Y. Selim Kızıltepe	Hakan Gökberber Sebahattin Doğantaş↑	Sebahattin Doğantaş Hakan Gökberber↑	Kadir Ekin	Recep Aslan
2000-2002	H. Aziz Toker	Hakan Gökberber	Şükrü Şap	Hulusi Tütüncü	O. Şükrü Aksu
2002-2004	H. Aziz Toker	Hakan Gökberber	A. Kadir Şahin	Halil Kalınbaçoğlu	Levent Demirbaş
2004-2006	Fatih M. Birdane	Zekeriya Erdurmuş	Nuri Gümüş	Türkan İlhan	Celalettin Çankaya
2006-2008	Fatih M. Birdane	Zekeriya Erdurmuş	Nuri Gümüş	H. Ahmet Çelik	Hulusi Tütüncü
2008-2010	Fatih M. Birdane	Zekeriya Erdurmuş	Nuri Gümüş	H. Ahmet Çelik	Hulusi Tütüncü
2010-2012	Fatih M. Birdane	Ö. Buğrahan Solmaz	Nuri Gümüş	H. Ahmet Çelik	Hulusi Tütüncü
2012-2014	Fatih M. Birdane	Ö. Buğrahan Solmaz	Nuri Gümüş	H. Ahmet Çelik	Hulusi Tütüncü
2014-2016	Fatih M. Birdane	Hulusi Tütüncü	Nuri Gümüş	H. Ahmet Çelik	Ö. Buğrahan Solmaz
2016-2018	Fatih M. Birdane	Hulusi Tütüncü	Nuri Gümüş	H. Ahmet Çelik	Ö. Buğrahan Solmaz

↑ Görevinden ayrılmış olan bir üst satırdaki üyenin yerine atanmış üye

Tablo 2: Afyonkarahisar VHO Haysiyet Divanı (1976-2016)

Table 2: Afyonkarahisar VHO Discipline Board (1976-2016)

Dönem	Üyeler				
1976-1977	—	—	—	—	—
1977-1978	Süleyman Özkan	Ali Ünsoy	Yaşar Yanık	—	—
1978-1979	Süleyman Özkan	Ali Ünsoy	Yaşar Yanık	—	—
1979-1980	—	—	—	—	—
1980-1981	Basri Küçüközdemir	Ali Ünsoy	Yaşar Yanık	Ümit Ülker	Mevlüt Çelik
1981-1982	İsmet Kutlual	Ali Ünsoy	Yaşar Yanık	Baki Uçman	S. Zafer Onur
1982-1984	Semai Çelebi	Ali Ünsoy	O. Nuri Serdaroğlu	Mehmet Çakırgöz	S. Zafer Onur
1984-1986	Semai Çelebi	Cengiz Tan	Necdet Demir	Yaşar Uzel	İsmet Örmeci
1986-1988	Semai Çelebi	Baki Uçman	O. Nuri Serdaroğlu	Yaşar Uzel	Hüsamettin Öner
1988-1990	Semai Çelebi	Baki Uçman	Bekir Aslan	Namık Zengin	Ali Ünsoy
1990-1992	Mehmet Çelik	Ali Kutver	Yalçın Konak	Kadir Ekin	Alaattin Kaptanlar
1992-1994	Nevzat Ercan	Rıza Avşar	O. Nuri Serdaroğlu	Bekir Aslan	Şükrü Şap
1994-1996	Mehmet Çakırgöz	İhsan Karakadıoğlu	O. Nuri Serdaroğlu	Ali Ünsoy	Ergün Karagenç
1996-1998	Mehmet Çakırgöz	Semai Çelebi	Bekir Aslan	Rıza Avşar	Hulusi Tütüncü
1998-2000	Semai Çelebi	Hulusi Tütüncü	Halil Kalınbaçoğlu	Rıza Avşar	İhsan Karakadıoğlu
2000-2002	Mehmet Çakırgöz	Nevzat Erkan	Mehmet Çelik	Rıza Avşar	Kadir Ekin
2002-2004	Mehmet Çakırgöz	Mustafa Tekerli	Mehmet Çelik	Rıza Avşar	M. Fehmi Çağır
2004-2006	İsmail Türkmenoğlu	İsmail Kaynar	Mehmet Çelik	Hulusi Kaplan	Ahmet Karakoç
2006-2008	İsmail Türkmenoğlu	Hakan Gökberber	M. Ali Akkoyun	İsmail Atlı	Yıldırım Keskin
2008-2010	İsmail Türkmenoğlu	M. Fehmi Çağır	M. Ali Akkoyun	Yalçın Konak	Mustafa Tatar
2010-2012	İsmail Türkmenoğlu	Hakan Gökberber	M. Ali Akkoyun	Yalçın Konak	Ahmet Karakoç
2012-2014	İsmail Türkmenoğlu	Hakan Gökberber	M. Ali Akkoyun	Yalçın Konak	Ayhan Aytulun
2014-2016	İsmail Türkmenoğlu	Erkan Acar	M. Ali Akkoyun	Yalçın Konak	Ayhan Aytulun
2016-2018	İsmail Türkmenoğlu	Erkan Acar	M. Ali Akkoyun	Yalçın Konak	Ayhan Aytulun

— Üyenin ismine ulaşılamamıştır.

Tablo 3: Afyonkarahisar VHO Büyük Kongre Delegeleri (1976-2016)
Table 3: Afyonkarahisar VHO Grand Congress Delegates (1976-2016)

Dönem	Üyeler					
1976-1977	Necdet Demir	Hasan Sarıbaş				
1977-1978	Ethem Edincikli	Sami Tekin				
1978-1979	Basri Küçüközdemir	Ali Yancı				
1979-1980	—	—				
1980-1981	Ali Ünsoy	Necdet Demir				
1981-1982	Hamza Akalın	Necdet Demir				
1982-1984	Ali Ünsoy	Necdet Demir	Mehmet Çakırgöz	Baki Uçman		
1984-1986	Hamza Akalın	Necdet Demir	Baki Uçman			
1986-1988	Hüsamettin Öner	Yalçın Ünsal				
1988-1990	Hüsamettin Öner	Ali Ünsoy				
1990-1992	Mehmet Çakırgöz	Bekir Aslan				
1992-1994	Mehmet Çakırgöz	Bekir Aslan	Semai Çelebi	Yalçın Konak		
1994-1996	Y. Selim Kızıltepe	Kadir Ekin	İ. Sadi Çetingül	Yalçın Konak	Mahmut Salman	İbrahim Altıntaş
1996-1998	Sebahattin Eronat	Kadir Ekin	H. İbrahim Tekin	Şiar Ayhan	Nevzat Ercan	Erkan Acar
1998-2000	Mustafa Tekerli	Cemil Tosun	Halit İmik	H. Aziz Toker		
2000-2002	Mustafa Tekerli	Cemil Tosun	Halit İmik	Yalçın Konak	M. Atilla Çakmak	H. İbrahim Tekin
2002-2004	Seçkin Küçükerdem	Cemil Tosun	Şükrü Şap	H. Basri Yazıcı	M. Atilla Çakmak	H. İbrahim Tekin
2004-2006	Seçkin Küçükerdem	Cemil Tosun	Artay Yağcı	Aziz Bülbül	M. Atilla Çakmak	Ramazan Avcı
2006-2008	Vural Özdemir	Cemil Tosun	Artay Yağcı	Aziz Bülbül	H. Hüseyin Aşkın	Ramazan Avcı
2008-2010	Ahmet Karakoç	İsmail Atlı	H. İbrahim Tekin	Ceyan Tosun	H. Hüseyin Aşkın	Ramazan Avcı
2010-2012	Zekeriya Erdurmuş	İsmail Atlı	H. İbrahim Tekin	Mustafa Tatar	H. Hüseyin Aşkın	Ramazan Avcı
2012-2014	M. Nevzat Algan	İsmail Atlı	H. İbrahim Tekin	Mustafa Tatar	H. Hüseyin Aşkın	Ramazan Avcı
2014-2016	Mahmut Salman	İsmail Atlı	H. İbrahim Tekin	Mustafa Tatar	H. Hüseyin Aşkın	Ramazan Avcı
2016-2018	Mahmut Salman	İsmail Atlı	H. İbrahim Tekin	Mustafa Tatar	H. Hüseyin Aşkın	Ramazan Avcı

— Üyenin ismine ulaşılamamıştır.

Tablo 4: Afyonkarahisar VHO Denetleme Kurulu (1976-2018)
Table 4: Afyonkarahisar VHO Inspection Board (1976-2018)

Dönem	Üyeler	
1976-1977	—	—
1977-1978	Baki Uçman	Muhsin Uysal
1978-1979	Baki Uçman	Muhsin Uysal
1979-1980	—	—
1980-1981	Baki Uçman	Hamza Akalın
1981-1982	Baki Uçman	Hamza Akalın
1982-1984	Baki Uçman	Mehmet Çakırgöz
1984-1986	Baki Uçman	Mehmet Çakırgöz
1986-1988	Semai Çelebi	Hüseyin Tan
1988-1990	Bekir Aslan	Halil Kalınbaçoğlu
1990-1992	Hakan Boyar	Halil Kalınbaçoğlu
1992-1994	O. Şükrü Aksu	Erol Ünsoy
1994-1996	Sabahattin Doğantaş	Ali Ünsoy
1996-1998	Hakan Gökberber	Ahmet Karakoç
1998-2000	Hakan Karakoç	Azmi Gökteke
2000-2002	Ahmet Karakoç	Ali Kutver
2002-2004	Ahmet Karakoç	Ümit Dağdeviren
2004-2006	Turgut Çivri	A. Kadir Şahan
2006-2008	Zafer Avcı	A. Kadir Şahan
2008-2010	Zafer Avcı	A. Kadir Şahan
2010-2012	Zafer Avcı	A. Kadir Şahan
2012-2014	Zafer Avcı	A. Kadir Şahan
2014-2016	Numan Altıntaş	Mustafa Aslan
2016-2018	Numan Altıntaş	Mustafa Aslan

— Üyenin ismine ulaşılamamıştır.

05 Ağustos 1983'te yayınlanan 68 sayılı Kanun Hükmünde Kararname (KHK)[25] ile veteriner hekim odalarının genel kurullarının 31 Ekim 1983 tarihine kadar yapılması hükme bağlanırken Sıkı Yönetim Koordinasyon Kurulu kararı ile[26] bu maddenin işletilmesinin durdurulduğu ve 16 Eylül 1983'te yayınlanan 86 sayılı KHK[27] le veteriner hekim odalarının genel kurullarının 01 Aralık 1983'ten itibaren üç ay içerisinde yapılmasının hükme bağlandığı saptandı. Yukarıdaki nedenlerle Afyonkarahisar VHO'nun her yıl Eylül ayında gerçekleştirdiği olağan genel kurulun 1983'te yapılmadığı 22 Ocak 1984'te yapıldığı belirlendi[28]. Ayrıca mevzuatta yapılan değişiklikle[29] odaların olağan genel kurullarının 1984'ten itibaren iki yılda bir yapılmaya başlandığı tespit edildi. Kamuda çalışan veteriner hekimlerin odaya üye olma mecburiyetinin 68 sayılı KHK [30]ile kaldırılmasının hemen ardından Afyonkarahisar VHO'nun üye sayısının 63'den 49'a düştüğü tespit edildi[31]. Odaya kayıtlı üye sayısının 18 Temmuz 2018 tarihi itibarı ile 431 olduğu belirlendi[32]. Afyonkarahisar VHO'nun, ilaç satışı konusunda üyeleri arasındaki haksız rekabeti önlemek amacı ile Ocak 2003'te Afyonkarahisar Eczacılar Odası Başkanlığı ile birlikte içeriğini; her iki odanın da ilaç satışı konusunda ticari ahlak ve meslek etiği çerçevesinde haksız rekabeti önleyici tedbirleri alması, üyelerden protokolü uygulayacaklarına dair imza alınması; üyelerin aylık olarak belirlenecek fiyat listesine uyması ve listenin altında ücret alanlara yaptırım uygulanmasının oluşturduğu bir protokol

[25] 9/3/1954 Tarih ve 6343 Sayılı Veteriner Hekimliği Mesleğinin İcrasına, Veteriner Hekimleri Birliği ile Odalarının Teşekkül Tarzına ve Göreceği İşlere Dair Kanunun Bazı Maddelerinin Değiştirilmesi ve Bu Kanuna Bazı Maddeler Eklenmesi Hakkında 1/6/1983 Tarihli ve 68 Sayılı Kanun Hükmünde Kararname.05/08/1983 tarih ve18126 sayılı Resmî Gazete.

[26]TVHB 30. büyük kongre tutanağı (1984). TVHB Arşivi.

[27] 9/3/1954 Tarih ve 6343 Sayılı Veteriner Hekimliği Mesleğinin İcrasına, Veteriner Hekimleri Birliği ile Odalarının Teşekkül Tarzına ve Göreceği İşlere Dair Kanunun Bazı Maddelerinin Değiştirilmesi ve Bu Kanuna Bazı Maddeler Eklenmesi Hakkında 1/6/1983 Tarihli ve 68 Sayılı Kanun Hükmünde Kararnamenin Bazı Hükümleri ile 6343 Sayılı Kanun'un Bazı Hükümlerinin Değiştirilmesine Dair 6/9/1983 Tarihli 86 Sayılı Kanun Hükmünde Kararname. 16.09.1983 tarih ve 18167 sayılı Resmî Gazete.

[28] 1982-1984 dönemi yönetim kurulu çalışma raporu. Afyonkarahisar VHO Arşivi.

[29] 9/3/1954 Tarih ve 6343 Sayılı Veteriner Hekimliği Mesleğinin İcrasına, Veteriner Hekimleri Birliği ile Odalarının Teşekkül Tarzına ve Göreceği İşlere Dair Kanunun Bazı Maddelerinin Değiştirilmesi ve bu Kanuna Bazı Maddeler Eklenmesi Hakkında 1/6/1983 Tarihli ve 68 Sayılı Kanun Hükmünde Kararnamenin Bazı Hükümleri ile 6343 Sayılı Kanun'un Bazı Hükümlerinin Değiştirilmesine Dair 6/9/1983 Tarihli 86 Sayılı Kanun Hükmünde Kararnamenin Değiştirilerek Kabulü Hakkında Kanun (2993 Sayılı Kanun).07.04. 1984 tarih ve 18365 sayılı Resmî Gazete.

[30] Bkz: dipnot: 25

[31] Bkz: dipnot 28.

[32] 18/07/2018 tarihli oda üye kayıt listesi. Afyonkarahisar VHO Arşivi.

düzenledikleri tespit edildi[33]. Protokol çerçevesinde eczane ve veteriner hekim muayenehanelerini denetlemek için Afyonkarahisar VHO yönetim kurulundan da iki üyenin görevlendirildiği bir komisyonun oluşturulduğu[34] ancak Rekabet Kurulunun odaya yapmış olduğu tebligat ile söz konusu ilaç fiyat tespit listelerinin uygulamadan kaldırıldığı saptandı[35].

Afyonkarahisar VHO'nun Merkez Konseyden, diğer odalardan ve çeşitli yerlerden gelen yazıları üyelerine ileterek çeşitli konularda onların görüş ve düşüncelerini aldığı[36] ve odanın da gerekli zaman ve konularda Bakanlık ve Merkez Konseye görüş bildirdiği saptandı[37],[38]. Pek çok dönemde (1977-1992) odaya gelir sağlamak amacı ile bağış makbuzları bastırıldığı belirlendi[39]. 6343 sayılı Kanun'un 18. Maddesi, 2993 sayılı Kanun'un 19. Maddesi[40] ile yürürlükten kaldırılana kadar ikinci görev almak için odaya başvuran veteriner hekimlerin onay işlemlerinin yapıldığı tespit edildi[41],[42],[43]. Odada rutin olarak; üyelerin kayıt ve nakil işlemlerinin yapıldığı, asgari ücret tarifnamesinin düzenlendiği ve üyelerle mesleki sorunların görüşülüp tartışıldığı toplantıların kararlaştırıldığı, odanın, il meclis toplantılarında temsil edildiği, günün ve konunun gerektirdiği durumlarda dönemin ilgili siyasi ve mesleki şahıslarına tebrik ya da konu ile ilgilenilmesi hususunda telgraflar çekildiği, yazılar yazıldığı ve veteriner hekimliğinin kuruluş yıl dönümünde ve 2000 yılından itibaren Dünya Veteriner Hekimler gününde üyelerin aileleri ile birlikte katıldıkları kutlama programları düzenlendiği saptandı[44],[45].

Afyonkarahisar VHO tarihinden tespit edilen bazı önemli faaliyetler; müsteşarlık seviyesinde yeni bir kuruluşta toplanma talebinin devlet büyüklerine telgrafla pek çok kez iletilmesi (1979-1980 dönemi)[46], Merkez Konseye Veteriner Hekimler Yüksek Danışma Kurulunun kurulması önerisinin sunulması (1980-1981 dönemi)[47], üyelerle daha iyi iletişim kurabilmek için Burdur, Isparta ve Uşak illerinde birer temsilci görevlendirilmesi (1988-1990 dönemi)[48], devlet büyüklerine Hayvancılık Bakanlığının kurulması için telgraflar çekilmesi (1990-1992 dönemi)[49], Hayvancılık ve Su Ürünleri Müsteşarlığı kurulması talebinin devlet büyüklerine iletilmesi, dönemin Tarım ve Köy İşleri Bakanının ziyaret edilerek (16/03/1996) mesleki sorunların dile getirilmesi (1994-1996 dönemi)[50], çeşitli tarihlerde suni tohumlama kursları (4-8/10/2004[51], 24-28/07/2006[52],) akredite veteriner hekim kursları (11-13/03/2006, 18-20/03/2006)[53] ve hizmet içi eğitim kursları düzenlenmesi (18/11/2011[54], 19-21/12/2011[55],14-15/06/2012[56], 23-24/12/2012[57]) olarak tespit edildi.

Afyonkarahisar VHO'da değerlendirilen toplam beş disiplin vakasına rastlandı[58],[59],[60],[61] ve odanın1976'dan günümüze kadar herhangi bir yayın organının bulunmadığı belirlendi[62].

TARTIŞMA VE SONUÇ

TVHB'nin tarihinin incelendiği bir araştırmada (Melikoğlu ve Kızıltepe 2008) TVHB arşivinin çok yetersiz olduğunun; belirlendiği benzer şekilde bu çalışmada da hem TVHB Arşivi hem de Afyonkarahisar VHO Arşivinde oda ile ilgili 1968-1975 yıllarına ait bir evraka rastlanılmadığı, bu durumun ise söz konusu arşivler açısından bir eksiklik olduğu dolayısı ile daha düzenli ve tam bir arşivin tutulması gerektiği ileri sürülebilir.

Samsun-Sinop VHO'da seçilmiş (1969-2012) toplam 32 (Sanal ve Melikoğlu Gölcü 2014) ve Kars VHO'da seçilmiş (1989-2017) toplam 15 oda yönetim kurulu başkanı (Kızıltepe 2017) arasında hiç kadın veteriner hekimin bulunmadığı benzer şekilde Afyonkarahisar VHO' da da seçilmiş (1968-2016) 34 başkan arasında hiç kadın bulunmadığı[63], (Tablo 1-4) bu durumun ise kadınların çeşitli nedenlerle oda başkanlığına seçilmediğini gösterdiği ileri sürülebilir. Diğer veteriner hekim odalarında kadınların oda başkanı olma durumları yapılacak çalışmalarla ortaya çıkarılabilir.

Çeşitli tarihlerde Afyonkarahisar VHO tarafından devlet bünyesinde veteriner hekimliğe daha özelleşmiş bir birimin kurulması talebinin pek çok kez devlet büyüklerine iletilmesinin, mesleği geliştirme çabası açısından önemli olduğu söylenebilir[64].

Özen ve arkadaşları yapmış oldukları bir araştırmada (Özen ve ark. 2010) veteriner hekimlerin ilaç satış yetkisinin en olumsuz yönünün haksız rekabet olduğu tespit etmişlerdir Rekabet Kurulunun tebliği ile

[33] 21/01/2003 tarih ve 7 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[34] 27/01/2003 tarih ve 8 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[35] 03/11/2003 tarih ve 17 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[36] 1977-1981 yönetim kurulu çalışma raporları. Afyonkarahisar VHO Arşivi.
[37] 08/03/1991 tarih ve 22 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[38] 07/03/2007 tarih ve 756 sayılı gelen evrak. TVHB Arşivi.
[39] 1977-1992 yönetim kurulu çalışma raporları. Afyonkarahisar VHO Arşivi.
[40] Bkz: dipnot 29
[41] 05/10/1978 tarih ve 3 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[42] 20/11/1980 tarih ve 5 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[43] 04/10/1982 tarih ve 3 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[44] 1976-2017 yönetim kurulu karar defterleri. Afyonkarahisar VHO Arşivi.
[45] 1976-2000 yönetim kurulu çalışma raporları, 24/09/1977, 05/10/1078, 27/09/1980, 19/09/1981, 18/09/1982; 22/01/1984, 23/09/1990, 20/09/1992, 25/09/1994, 29/09/1996 ve 01/10/2000 tarihli genel kurul belgeleri. Afyonkarahisar VHO Arşivi.
[46] 1979-1980 dönemi yönetim kurulu çalışma raporu, 27/09/1980 tarihli genel kurul belgeleri. Afyonkarahisar VHO Arşivi.
[47] 1980-1981 dönemi yönetim kurulu çalışma raporu, 19/09/1981 tarihli genel kurul belgeleri. Afyonkarahisar VHO Arşivi.
[48] 1988-1990 dönemi yönetim kurulu çalışma raporu, 23/09/1990 tarihli genel kurul belgeleri. Afyonkarahisar VHO Arşivi.
[49] 1990-1992 dönemi yönetim kurulu çalışma raporu, 20/09/1992 tarihli genel kurul belgeleri. Afyonkarahisar VHO Arşivi.
[50] 1994-1996 dönemi yönetim kurulu çalışma raporu, 29/09/1996 tarihli genel kurul belgeleri. Afyonkarahisar VHO Arşivi.
[51] 01/10/2004 tarih ve 26 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[52] 01/08/2006 tarih ve 70 noluyönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[53] 13/02/2006 tarihli 396 sayılı gelen evrak. TVHB Arşivi.
[54] 26/12/2011 tarih ve 90184 nolu gelen evrak. TVHB Arşivi.
[55] 02/01/2012 tarih ve 0700/1338 nolu giden evrak. TVHB Arşivi.
[56] 18/06/2012 tarih ve 2012-130 nolu gelen evrak. TVHB Arşivi.
[57] 25/12/2012 tarih ve 2012-305 sayılı gelen evrak. TVHB Arşivi.
[58] 08/05/1997 tarih ve 9 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[59] 22/07/2014 tarih ve 14 nolu yönetim kurulu kararı. Afyonkarahisar VHO Arşivi.
[60] 10/04/2017 tarih ve 303/177 sayılı giden evrak. TVHB Arşivi.
[61] 20/06/2017 tarih ve 303/295 sayılı giden evrak.TVHB Arşivi.
[62] Bkz: dipnot 8.
[63] Bkz: dipnot 7.
[64] Bkz: dipnot 46,49,50.

sonradan uygulamadan kalkmış da olsa odanın 2003'te Afyonkarahisar Eczacılar Odası ile birlikte ilaç satışı konusunda haksız rekabeti önlemek için düzenledikleri protokol[65] Afyonkarahisar VHO'nun söz konusu sorunun çözümüne yönelik gayret göstermesi açısından olumlu olarak değerlendirilebilir. Samsun-Sinop VHO'da (Sanal ve Melikoğlu Gölcü 2014) ve Kars VHO'da (Kızıltepe 2017) veteriner hekimlere yönelik eğitim programlarının düzenlendiği benzer biçimde bu araştırmada Afyonkarahisar VHO'da da bu programların düzenlendiği ve bu eğitimlerin ise üyelerin mesleki bilgi ve becerilerinin gelişimi açısından olumlu faaliyetler olduğu ifade edilebilir.

Kars VHO'nun yayın organı olan derginin tarihi boyunca (1989-2017) sadece bir sayı yayınlandığı (Kızıltepe 2017), Afyonkarahisar VHO'nun ise tarihi boyunca (1968-2017) hiç yayın faaliyetinin bulunmadığı[66] bu durumun ise oda için bir eksiklik olduğu ileri sürülebilir.

Kızıltepe'nin tez çalışmasında (Kızıltepe 2010) Marmara Bölgesi ve Ege Bölgesinde çalışan serbest veteriner hekimlerle ilgili reklam yapma ve klinik açmadan çalışma ile ilgili deontolojik-etik ihlallere Türkiye'nin diğer bölgelerinden daha sık rastlandığı Tong ve arkadaşlarının araştırmasında (Tong ve ark. 2014) İzmir VHO Haysiyet Divanının 1992-2014 yılları arasında 74 karar aldığı . Afyonkarahisar VHO'da ise 1976-2018 yılları arasında beş disiplin vakasına rastlandığı bu durumun ise İzmir VHO ile kıyaslandığında görece düşük bir rakam olduğu ileri sürülebilir..

Sonuç olarak Afyonkarahisar VHO'nun tarihi gelişim sürecinde, saptanan eksiklikleri bir tarafa sorumluluk bölgesi özelinde mesleğin çıkarlarına ve mesleki sorunların çözümüne yönelik girişimleri ve yapmış olduğu eğitim çalışmaları ile Türkiye'de veteriner hekimliği mesleğinin gelişimine ve dolayısı ile toplum sağlığına katkıda bulunduğu ifade edilebilir.

KAYNAKLAR

Kızıltepe, A. (2010). Türkiye'de Klinik Veteriner Hekimliği Uygulamalarında Karşılaşılan Deontolojik Etik Sorunlar Üzerine Bir Araştırma, Doktora Tezi, AÜ. Sağlık Bilimleri Enstitüsü, Ankara.

Kızıltepe, A. (2017). Türkiye'de Veteriner Hekim Odalarının Çalışmalarına Yönelik Bir İnceleme: Kars Bölgesi Veteriner Hekimler Odası Örneği. Kafkas Univ Vet Fak Derg, 23: 757-765.

Melikoglu, B., Kızıltepe, A. (2008).Türk Veteriner Hekimleri Birliği: Tarihten Notlar. Türk Veteriner Hekimleri Birliği Dergisi, 1-2: 94-101.

Özen, A., Yüksel, E., Doğan, Ö. (2010) Veteriner İlaçları Satış Yetkisinin Veteriner Hekimliği Açısından Değerlendirilmesi: I. Klinisyenlerin Ilaç Satış Yetkisi Konusundaki Tutumları; Kafkas Univ Vet Fak Derg,16, 805-812.

Sanal, S,., Melikoglu Gölcü, B. (2014). Dünden bugüne Samsun-Sinop Bölgesi Veteriner Hekimler Odası. IV. Ulusal Veteriner Hekimliği Tarihi ve Mesleki Etik Sempozyumu Bildiriler Kitabı, Otak Form Ofset, Samsun.sf. 439-446.

Tong, E., Özdemir, H.G., Aslım, G. (2014). İzmir Veteriner Hekimler Odası Haysiyet Divanı kararlarının "gizliliğe saygı ilkesi" çerçevesinde değerlendirilmesi. Vet Hekim Der Derg,85: 35-41.

[65] Bkz: dipnot 32-34.
[66] Bkz: dipnot 8

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):193-199
DOI: 10.30607/kvj.538979

RESEARCH ARTICLE

Effect of *Pomegranate Molasses* on Egg Quality Traits During Different Storage Time in Laying Hens

İ.Sadi ÇETİNGÜL, Aamir IQBAL*, İsmail BAYRAM, Eyüp Eren GÜLTEPE, Cangir UYARLAR, Ümit ÖZÇINAR

Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03030 Afyonkarahisar, Turkey.

ABSTRACT

The present study trial was conducted to investigate the effects of pomegranate molasses on egg quality at different storage periods. A total of 240 Babcock white laying hens (58 weeks old) were divided into 5 groups (n=48) with 8 subgroups having 6 hens in each. Pomegranate molasses was added in the drinking water to experimental groups with 0%, 0.1%, 0.25 %, 0.5%, and 1%, respectively during 4 weeks. At the end of study (30 days), total of 320 eggs were collected randomly and eighty eggs (80) were analyzed at 0 day of storage while other eggs were stored at 4°C temperature for 10, 20 and 30 days. The result reveals that the egg weight, egg shell thickness and albumin index remained non-significant (P>0.05) during the whole trial Moreover , Haught unit showed positive response in the group B and D at 10 day storage as compared with control group however, it was remained non-significant (P<0.05) at 0, 20 and 30 days of storage. It is concluded that pomgranate mollases has shown positive effect on some egg quality traits during several storage periods 0, 10, 20, 30 days.

Keywords: Laying hens, pomgranate mollases, Egg storage, Egg quality triats

****

Yumurta Tavuklarında Nar Ekşisinin Yumurtaların Farklı Depolama Sürelerinde Yumurta Kalitesi Üzerine Etkisi

ÖZ

Bu araştırma, yumurta tavuklarında içme suyuna katılan nar ekşisinin, 0, 10, 20 ve 30 günlük sürelerle depolanan yumurtaların bazı kalite özelliklerine etkisini belirlemek amacıyla gerçekleştirilmiştir. Toplam 240 adet 58 haftalık yaşta Babcock beyaz yumurtacı tavuk her birinde 48 adet olmak üzere 5 gruba ayrılmıştır. Deneme grupları ayrıca her birinde 6 adet tavuk bulunan 8 alt gruba ayrılmıştır. Çalışmada deneme grupları içme sularına (A, B, C, D ve E) 4 hafta boyunca sırasıyla %0, %0.1 ,%0.25, %0.5 ve %1 oranlarında nar ekşisi ilave edilmiştir. Araştırmanın 30. gününde, toplam 320 yumurta gruplardan rastgele toplanmıştır. Toplanan yumurtaların 80 adedi aynı gün analiz edilirken (0. Gün), diğer yumurtalar ise 4 ° C sıcaklıkta 10, 20 ve 30 gün boyunca depolanmıştır. Bu yumurtalardan 80 yumurta 10 gün sonra analiz edilirken, kalan yumurtaların 80 adedi 20. Gün, 80 adedi ise 30 günlük depolamadan sonra analiz edilmiştir. Araştırmada içme sularına nar ekşisi katılan tavukların farklı sürelerle depolanan yumurtaların ağırlıklarının gruplar arası karşılaştırmasında anlamlı farklılıklar görülmemiştir (P> 0.05). İlave olarak, Haugh birimi değeri B ve D gruplarında 10 günlük depolamada kontrol grubuna kıyasla yüksek bulunmuştur. Sonuç olarak, nar ekşisinin 0, 10, 20, 30 günlük sürelerle depolama periyodunda bazı yumurta kalitesi özellikleri üzerinde olumlu etki gösterdiği sonucuna varılmıştır.

Anahtar Kelimeler: Yumurta tavuğu, Nar ekşisi, Yumurta depolama, Kalite

To cite this article: Çetingül İ.S. Iqbal A. Bayram İ. Gültepe E.E. Uyarlar C. Özçınar Ü. Effect of Pomegranate Molasses on Egg Quality Traits During Different Storage Time in Laying Hens. Kocatepe Vet J. (2019) 12(2):193-199.
Submission: 12.03.2019 **Accepted**: 21.05.2019 **Published Online**: 02.06.2019
ORCID ID; İSÇ: 0000-0002-7608-6176, AI: 0000-0003-4473-2329, İB: 0000-0002-9993-7092, EEG: 0000-0002-2404-1232, CU: 0000-0002-7803-2454, ÜÖ: 0000-0002-1143-1215
*Corresponding author e-mail: aamir_vet@yahoo.com

INTRODUCTION

Poultry is the most important sector in livestock of any country. It is the passion of farmer to supply humankind with pure food including the meat and eggs. Poultry is the foremost widely used and cheaper source of protein. Although science contributed a lot to develop this industry, still there are some limitations in quality. Layer farmers need the better egg production in terms of egg shelf life during storage and its internal quality parameters like yolk height and width, albumin height and width, Egg shell thickness, yolk color and egg weight. The internal quality of eggs is more porn to replenish after they are laid by the layers. There are some factors like age of flock (Williams 1992), genetic factors that play a role in egg quality after storage. Not only these, but there are also some critical and value able parameters like storage time and temperature(Samli et al. 2005) that are noticed for egg quality. Egg quality change is directly related to the carbon dioxide loss through the shell pores in freshly laid eggs during storage (Scott et al. 1971). Egg quality parameters can be controlled and evaluated during different storage times by the dietary supplementation of different plant based products. The internal quality of eggs has more chances to replenish after they are laid by the layers. There is a need to focus on the use of herbs in diet to increase the quality of eggs during and after storage. Many researchers have played role in this field. Many researchers have got positive effects of herbal extracts and oils on performance parameters including egg production and quality (Rahimi et al. 2011, Khan et al. 2012).

Turkey is famous for cultivation and it comes at 4th position all over the world (Özkal And Dinç 1993). Aril is an edible part of pomegranate and it is composed of 52% (w/w) of the total fruit mass and 78% juice and 22% seeds. The seeds contain polyunsaturated fatty acids (PUFA), polyphones, minerals, sugars, vitamins and polysaccharides (Miguel et al. 2004). Pomegranates are studied as a phytochemical source and used as antifungal and antiviral. It decreases the impacts of paralysis, rectocele, leucorrhea, menorrhagia, colic, and dysentery (Wu et al. 2016).

Pomegranate juice contains polyphenol antioxidants (ellagic acid and punicalagin) and the antioxidant content of pomegranate juice is among the highest of any foods (Guo et al. 2004). Pomegranate juice contains cyanidin-3-O-glucoside, cyanidin-3,5-di-O-glucoside, delphinidin-3-O-glucoside, delphinidin-3,5-di-O-glucoside, pelargonidin-3-O-glucoside, and pelargonidin-3,5-di-Oglucoside (Lansky et al. 1993).
Traditional methods are adapted to make pomegranate molasses. The process of its concentration is done by boiling pomegranate without adding any sugar or other additives. The procedure of processing involves cleaning, crushing, extraction, filtration, clarification and evaporation in open vessel or under vacuum. There are different type's production methods (Ezra et al. 2010, Gunal et al. 2006). The final product is highly nutritive because this is a concentrate and especially the presence of high mineral contents makes it more nutritious. The strong antioxidant activity of it is also important for egg quality and human health. It is anti-carcinogenic and due to its interaction with the process of angiogenesis, circulation, invasion, and tumor cell proliferation, its use is enhanced. Many study trials approved and conducted for the use of pomegranate juice as anti-inflammatory, antioxidant, anti-atherogenic anti-carcinogenic and antimicrobial effects. Its potential against harmful effects of ultraviolet rays and cardiovascular diseases diabetics and cancer has attracted a number of researchers to investigate more (Yılmaz and Usta 2010). Linseed oil with pomegranate juice was used in broilers for 6 weeks and examination of the adipose tissue, fatty acid profiles, blood parameters, liver enzymes, and serum profiles showed an increase in white blood cells for 0.5% and 1.0% dose group and decrease in the cholesterol level for the group being offered 1.5 % pomegranate with linseed oil (Manterys et al. 2016).

According to (Kalida and Jayanty 2010) 500mg/kg of whole pomegranate extract was given to rats in India and anti-depressant effect was noticed in the trial. It has been published that no negative effect was seen on yield parameters after the addition of 5% and 10% levels in drinking water to chicken for one month. (Rahman et al. 2017). Pomegranate seed pulp may be used as potential feed supplement up to 5% for the laying hens and no adverse effect on egg quality and production was reported (Saki et al. 2014c).Pistacia terebinthus seeds at 20 and 40 g kg−1 supplementation levels could be used to extend the shelf life of eggs without any adverse effect on egg quality(Gultepe 2018).

Pomegranate seed oil is rich in the CLA (conjugated linolic acid) isomer known as punicic acid and it have been shown that the concentration of punicic acid in pomegranate seed oil ranges from 45 to 70%. Many researchers have shown that CLA is readily incorporated into egg yolk (Windisch et al. 2008). Commercial table eggs are therefore, used as a vehicle to enhance human nutrition. Many studies have shown that the exogenous use of CLA containing feeds affects the sensory properties and shelf life of the eggs. It improves the texture of egg yolks. Manterys et al. (2016), reported that there was significant increase in the hardness of CLA enriched eggs. The textures of yolks from eggs containing CLA were rubbery and elastic and these yolks were

relatively difficult to brake using an Instron (Ahn et al. 1999).

Keeping in view the above points, the present study was designed to investigate the effect of pomegranate molasses on egg quality traits during storage in laying hens.

MATERIALS and METHODS

The current study was performed at the Experimental Animal Research farm of Afyon Kocatepe University, Turkey after the approval of the Local Ethics Committee of the faculty of veterinary medicine under approval No: AKÜHADYEK-146-16, on 03-01-2016.

Experimental design and management

The present study trial was conducted to investigate the effects of pomegranate molasses on egg quality after storage for different periods. A total of 240 Babcock white laying hens (58 weeks old) were divided into 5 groups (n=48) with 8 subgroups having 6 hens in each. Pomegranate molasses was added in the drinking water to experimental groups with 0 %, 0.1%, 0.25 %, 0.5%, and 1%, respectively during 4 weeks. The 16 hours light and 8 hours dark period was given.In this study all treatment groups were fed the basal diet, which was prepared according to the (National Research Council 1994) recommendation to meet the bird's requirement. (Table.1.0) the diet offered was isocaloric and isonitrogenous.

POM molasses ingredients analyses were performed as a comparative examination regarding the qualitative and quantitative evaluation of phenolic compounds by HPLC in molasses obtained from pomegranate. This method has a good repeatability, since several analyses carried out on the same sample produce a repeatability coefficient of percent variation (CV%) of 7%, which is similar to the values reported by other Authors (Angerosa et al. 1996) (Table.2.0).

Data Collection and Analyses

Pomegranate molasses was poured in fresh drinking water on daily basisA total of 320 eggs were collected at 30th day randomly. 80 eggs were analyzed at the 0 day and remaining were stored at 4°C temperature for 10, 20, 30 days. 80 eggs were analyzed at 10 day and 20 eggs were analyzed after 20 days storage and 30 eggs were analyzed after the 30 day storage. The egg analysis was done at the central laboratory of the concerned university department.

Statistics

The model assumptions of normality and homogeneity of variance were examined by Shapiro-Wilk and Levene tests, respectively. The statistical analysis was performed with MedCalc software (MedCalc Software bvba, Ostend, Belgium, version 17.5). One-way ANOVA was used for group comparison followed by Tukey-Kramer for post-hoc. All data were expressed as mean ± SEM. The significance level was considered as $p < 0.05$.

RESULT

The result of the recent study showed that there was no significant effect ($p > 0.05$) on egg weight during the whole study protocol (Table.3.0)

However, the yolk color showed positive response ($P<0.05$) in groups D and E at 30 day of storage within the groups as compared with control group. In contrast, the yolk color ($P<0.05$) decreased at 0 and 10th day of storage period between the treatment groups as compapred with control group (Table. 4.0)

Haught unit showed positive response in the group B and D at 10 day storage as compared with control group however, it was remained non-significant ($P<0.05$) at 0, 20 and 30 days of storage (Table. 5.0)

Egg shell thickness remained non-significant ($p>0.05$) during the whole trial of egg analysis. (Table. 6)

Egg Albumin index was decreased significantly within the group B that was offered 0.25mg/L of pomegranate molasses juice however it was remained non-significant in all groups as compared with control group (Table. 7.0) A significant impact ($P<0.05$) was seen within all groups at 0, 10, 20, 30 day storage analysis on yolk index however, no positive effect ($P<0.05$) were observed between the groups at different storage time (Table. 8.0)

Table 1. Composition of basal diet

Ingredients	%, as fed basis
Corn	54.90
Vegetable oil	0.34
Sunflower meal (%32 CP)[1]	16.93
Full fat soya	10.00
Soybean meal (%44 CP)[1]	7.39
Limestone	7.87
Dicalsium phosphate	1.73
Common salt	0.40
Vitamin-mineral premix[2]	0.25
L-Lysine HCl	0.10
DL-methionine	0.10
Calculated values	
CP,%	17.00
ME,kcal/kg	2750
Ca	3,71
Av.P	0.38
Na,%	0,20
Met+Sis	0.71
Lysine,%	0.83
Treonin,%	0.61
Triptophane,%	0.20
Linoleic acid,%	2.36

1.Providedper kg of diet:Vitamin A:12.000.000 IU, Vitamin D3:3.000.000IU, Vitamin E:35.000, Vitamin K3:3.500,Vitamin B1:2.750IU, Vitamin B2:5.500IU, Nicotinamid: 30.000IU,Ca-D-Panthotenate:10.000IU,Vitamin B6: 4.000IU, Vitamin B12-15IU, Folic acid:1.000IU, D-Biotin: 50IU,Cholin clorid:150.000IU, Manganese: 80.000mg, Iron: 60.000 mg, Zinc:60.000 mg, Copper:5.000 mg, Iodine:2.000 mg, Cobalt: 500 mg, Selenium: 150 mg, Antioxidant:15.000 mg

Table 2. Ingredients of Pomegranate Molasses

Caffeic (ppm)	4240.998
P-coumaric (ppm)	0.799
Cinnamic (ppm)	5.338
2,5 dihydrokci (ppm)	819.052
Epicatechin (ppm)	1356.114
Ellagic (ppm)	883.340
Acetic acid (ppm)	273.60
Propoinic acid (ppm)	624.72
Butyric acid (ppm)	5417.97

Composition of Molasses (Anonymous, 2001)	**Amount**
Water Soluble Dry Matter, %, minimum	68.0
Titration acidity (as citric acid), %, minimum	7.5
PH	3.0
HMF, mg/100g, maximum	50

Table 3. Effect of Pomegranate molasses on egg weight at 0, 10, 20 and 30 days of storage

Group	0th day		10th day		20th day		30th day		p
	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	
A	67.991	1.4814	66.390	1.4364	69.089	1.8174	66.670	1.4408	0.578
B	65.704	1.9121	65.723	1.6124	65.733	1.5049	67.216	1.5188	0.887
C	66.684	1.8620	67.505	2.0790	65.704	1.9736	64.489	1.8607	0.692
D	68.809	1.4948	62.675	1.5095	66.643	1.5735	66.899	1.7489	0.380
E	68.904	1.0693	65.636	1.5709	65.063	1.3452	65.638	1.4265	0.225
P	0.474		0.346		0.497		0.705		

Table 4. Effect of Pomegranate molasses on yolk color at 0, 10, 20 and 30 days of storage

Group	0th day		10th day		20th day		30th day		p
	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	
A	12.750^{ab}	0.2189	13.344^{b}	0.2027	12.656	0.1921	13.187	0.1700	0.318
B	12.125^{a}	0.3461	12.969^{ab}	0.1736	12.875	0.1963	12.875	0.3146	0.084
C	12.406^{ab}	0.2709	13.000^{ab}	0.2092	12.781	0.2457	12.906	0.3169	0.406
D	13.219^{Bb}	0.1706	12.281^{Aa}	0.1644	13.031^{AB}	0.2014	13.469^{B}	0.1852	***0.001***
E	12.656^{Aab}	0.1563	12.562^{Aa}	0.1819	13.187^{B}	0.1760	13.594^{B}	0.1838	***0.001***
P	***0.028***		***0.002***		0.394		0.129		

Table 5. Effect of Pomegranate molasses on Haugh Unit at 0, 10, 20 and 30 days of storage

Group	0th day		10th day		20th day		30th day		p
	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	
A	86.613	2.2182	83.175^{ab}	2.4428	84.909	1.7575	87.516	1.3208	0.296
B	92.624^{B}	1.4553	85.269^{Aab}	1.7388	85.383^{A}	1.2128	84.769^{A}	1.6554	***0.002***
C	88.938	1.5555	84.630^{ab}	1.6822	84.315	1.7763	85.633	1.4148	0.129
D	86.968^{AB}	1.8400	88.951^{Bb}	1.6551	82.134^{A}	2.2285	86.814^{AB}	1.4201	***0.029***
E	86.636	1.9472	79.998^{a}	2.3444	85.725	1.0874	88.529	2.2252	0.130
P	0.113		***0.036***		0.496		0.563		

Table 6. Effect of Pomegranate molasses on eggshell thickness at 0, 10, 20 and 30 days of storage

Group	0th day		10th day		20th day		30th day		P
	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	
A	0.363	0.01011	0.379	0.006612	0.371	0.007663	0.369	0.005039	0.465
B	0.361	0.006612	0.380	0.006831	0.381	0.008004	0.368	0.009627	0.221
C	0.371	0.008778	0.358	0.007201	0.367	0.007344	0.359	0.01076	0.631
D	0.379	0.009463	0.380	0.009083	0.391	0.01074	0.364	0.01060	0.278
E	0.388	0.005261	0.382	0.008427	0.388	0.009408	0.374	0.01144	0.598
P	0.116		0.170		0.282		0.831		

Table 7. Effect of Pomegranate molasses on albumin index at 0, 10, 20 and 30 days of storage

Group	0th day		10th day		20th day		30th day		P
	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	
A	9.013	0.4927	8.404	0.4271	8.416	0.4119	9.234	0.3624	0.332
B	10.539^{B}	0.5069	8.566^{A}	0.6147	8.727^{A}	0.3952	8.528^{A}	0.4100	***0.009***
C	9.687	0.5003	8.747	0.4742	8.443	0.3615	9.394	0.4038	0.092
D	8.863	0.4698	9.615	0.4802	8.763	0.5486	9.386	0.5020	0.407
E	8.876	0.5714	7.629	0.5320	8.628	0.2665	9.405	0.5190	0.062
P	0.087		0.064		0.967		0.574		

Table 8. Effect of Pomegranate molasses on yolk index at 0, 10, 20 and 30 days of storage

Group	0th day		10th day		20th day		30th day		p
	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	
A	41.549^{Aab}	0.6374	41.532^{Aa}	0.5949	42.864^{AB}	0.7364	45.228^{B}	0.8922	***0.001***
B	43.483^{Ab}	0.6791	41.679^{Aa}	0.5469	42.018^{A}	0.7639	46.745^{B}	0.7562	***0.001***
C	41.621^{Aab}	0.4060	41.519^{Aa}	0.5342	41.827^{A}	0.6695	44.507^{B}	0.8972	***0.007***
D	40.422^{Aa}	0.5347	39.975^{Ab}	0.6186	42.591^{AB}	0.8178	44.463^{B}	0.8698	***0.001***
E	41.826^{Aab}	0.7131	39.603^{Ab}	0.6515	43.854^{AB}	1.0322	45.455^{B}	0.7185	***0.001***
P	***0.015***		***0.031***		0.489		0.294		

DISCUSSION

According to (Dei et al. 2008) average daily gain (ADG) was increased by treatment of pomegranate molasses and no effect is seen on water intake (Shabtay et al. 2008). In contrast to this (Saki et al. 2014a) had observed no positive impact on FCR, feed intake , egg weight and egg mass in layers hens. Previous researchers observed that using thyme and oregano in the ration of laying hens positively increase the egg mass and egg weight with the supplementation of thyme and oregano (Abdel-Wareth et al. 2013). However, other studies did not find any positive results on egg weight with the supplementation of herbs in the diet of Laying Japanese Quails (Christaki et al. 2011). (Rahman et al. 2017) study indicated that supplementation of Mentha Piperita oil and its juice in the laying hen's diet had no significant effect on egg quality traits during storage for 15 and 30 days at 4°C. Similarly, in another study, no significant loss of egg weight was observed at 5°C and 21°C temperature till 10 days in normal fed diet layers hens (Jin et al. 2011). Similarly, the recent study showed that there was no significant effect ($p>0.05$) on egg weight during the whole study period.

Some researchers observed that with supplementation of 5% carrots in the diet of laying hen increased egg yolk color however, Haugh unit, eggshell thickness, and egg shape index remained unaffected (Ishikawa et al. 1999). For egg yolk, supplementation of 70g/day purple carrots increased egg yolk index. Moreover, purple carrots showed positive affect on egg yolk (Hammershøj et al. 2010). However, in our study the egg yolk color was significant between the groups at zero day storage and after ten day egg storage analysis, yolk color was increased significantly and egg yolk color was significantly increased at twenty and thirty day egg storage analysis within the 1mg/L and 2mg/L pomegranate molasses groups as compared to the control group.

Dietary supplementation of pomegranate molasses had no positive affect on the egg quality parameters such as the egg shell thickness, Haugh unit, eggshell weight, eggshell breaking strength. (Saki et al. 2014a) Some of the researcheres like (Navid et al. 2014) reported non-significant impact on egg quality trait of

plant extracts. Similarly in our research trial the Haugh unit was decreased significantly between the groups at 10 day storage analysis as compared to zero day analysis and it was also decreased during 30 day storage analysis within the groups of 0.25mg/L and 1.0mg/L. Moreover, egg shell thickness also remained non-significant during the whole trial of egg analysis. Feeding laying hen with pomegranate pulp had no positive result on yolk and albumen indexes and also Haugh unit was remained non-affected by dietary treatments, which could confirm the low effect of nutrition on Haugh unit (Saki et al. 2014c) In our study, egg albumin index was decreased significantly within the 0.25mg/L of pomegranate molasses juice group. However, it was remained non-significant in all other supplemented groups over control**.** Similarly, a good effect was observed on the yolk index within the groups and between the groups and the yolk index was increased between the groups at zero day and 10 day storage analysis and yolk index was increased in group 0.25mg/L and also increased within all groups at thirty day of analysis.

CONCLUSION

In conclusion, Pomegranate molasses had no effect on egg weight, egg shell breaking strength and eggshell thickness while positive affect was observed effect on yolk color, albumin index and Haugh unit. Overall, pomegranate molasses supplementation was found beneficial to preserve the egg texture and quality. It was suggested to do more research on egg external and internal parameters after storage for prolonged duration at relatively higher temperature.

ACKNOWLEDGEMENT

The current study was performed at the Experimental Animal Research farm of Afyon Kocatepe University, Turkey after the approval of the Local Ethics Committee of the faculty of veterinary medicine under approval No: AKÜHADYEK-146-16, on 03-01-2016.

REFERENCES

Abdel-WA, Ismail ZSH, Sudekum K. Effects of thyme and oregano on performance and egg quality characteristics of laying hens. Worlds. Poult. Sci. J. 2013;69(1): 1–7.

Ahn DU, Sell JL., Jo C. Chamruspollert, M., & Jeffrey, M. Effect of dietary conjugated linoleic acid on the quality characteristics of chicken eggs during refrigerated storage. *Poultry Science*, 1999;*78*(6), 922-928.

Angerosa F, d'Alessandro N, Corana F, Mellerio G. Characterization of phenolic and secoiridoid aglycons present in virgin olive oil by gas chromatography-chemical ionization mass spectrometry. *Journal of Chromatography A*, 1996; *736*(1-2), 195-203.

Christaki EV, Bonos EM, Florou-Paneri PC. Comparative evaluation of dietary oregano, anise and olive leaves in laying Japanese quails. *Brazilian Journal of Poultry Science*, 2011;*13*(2), 97-101.

Dei HK, Rose SP, Mackenzie AM, Amarowicz R. Growth performance of broiler chickens fed diets containing shea nut (Vitellaria paradoxa, Gaertn.) meal fermented with Aspergillus niger. Poult. Sci. 2008;87(9): 1773–8.

Ezra D, Gat T, Skovorodnikova Y, Vardi Y, Kosto I. First report of Alternaria black spot of pomegranate caused by Alternaria alternata in Israel. Australas. Plant Dis. Notes. 2010;5(1): 1–2.

Gunal M, Yayli G, Kaya O, Karahan N, Sulak O. The effects of antibiotic growth promoter, probiotic or organic acid supplementation on performance, intestinal microflora and tissue of broilers. Int. J. Poult. Sci. 2006;5(2): 149–55.

Guo FC, Kwakkel RP, Williams BA. Effects of mushroom and herb polysaccharides on cellular and humoral immune responses of Eimeria tenella-infected chickens. Poult. Sci. 2004;83(7): 1124–32.

Gultepe EE. Bayram I. IQBAL A. Revista Brasileira de Zootecnia Effects of Pistacia terebinthus seed meal and different storage times on egg quality of laying hens. 2018.

Hammershøj M, Kidmose U, Steenfeldt S. Deposition of carotenoids in egg yolk by short-term supplement of coloured carrot (Daucus carota) varieties as forage material for egg-laying hens. J. Sci. Food Agric. 2010;90(7): 1163–71.

İshıkawa S, Murakamı, H, Yamazakı, M, Takemasa, M. Effect of Carrot Leaf Supplementation on Egg Yolk β-Carotene Content and Egg Quality. *Japanese poultry science*, 1999;*36*(4), 275-283.

Jin YH, Lee KT, Lee WI, Han YK. Effects of storage temperature and time on the quality of eggs from laying hens at peak production. *Asian-Australasian Journal of Animal Sciences*, 2011;*24*(2), 279-284.

Khan RU, Nikousefat Z, Tufarelli V, Naz S. Garlic (Allium sativum) supplementation in poultry diets: Effect on production and physiology. Worlds. Poult. Sci. J. 2012;68(3): 417–24.

Lansky P, Schılcher, H. P, J.D., Loew D. Plants that lower cholesterol. First World Congr. Med. Aromat. Plants Hum. welfare, Maastricht, Netherlands, Acta- Hortic. 1993. p. 131–136.

Manterys A, Franczyk ZM, Czyzynska CI. Haematological parameters, serum lipid profile, liver function and fatty acid profile of broiler chickens fed on diets supplemented with pomegranate seed oil and linseed oil. Br. Poult. Sci. 2016;57(6): 771–9.

Miguel, G, Fontes C, Antunes ., Neves A, Martins D. Anthocyanin concentration of "Assaria" pomegranate fruits during different cold storage conditions. *BioMed Research International*, *2004*(5), 338-342.

National Research Council. Nutrient Requirements of Poultry: Ninth Revised Edition, 1994. Natl. Acad. Press. 1994.

Navid J, Mozaffar M, Kazem K. Effect of dietary medicinal herbs on performance, egg quality and immunity response of laying hens. Adv. Environ. Biol. 2014;7(13): 4382–9.

Özkal N, Dinç S: Nar Meyvesinin Kimyasal Bileşimi Ve Biyolojik Aktiviteleri. *Ankara Eczacılık Fakültesi Dergisi*, 1993, 22: 1-2.

Rahimi S, Teymouri Z, Karimi TMA, Omidbaigi R, Rokni H. Effect of the three herbal extracts on growth performance, immune system, blood factors and intestinal selected bacterial population in broiler chickens. J. Agric. Sci. Technol. 2011;13(4): 527–39.

Rahman A, Eren GE, Uyarlar C, Cetıngul S, Iqbal A , Bayram I. Effect of Mentha Piperita (Peppermint) Extract and its Juice on Egg Quality Traits during Different Storage Time in Laying Hens. Kocatepe Vet. J. Kocatepe Vet J Kocatepe Vet J. 2017;10(101): 14–20.

Kalita D, Jayanty SS. Comparison of polyphenol content and antioxidant capacity of colored potato tubers, pomegranate and blueberries. *Journal of Food Processing & Technology*, 2014 *5*(8), 1.

Saki AA, Aliarabi H, Hosseini SA, Salari J, Hashemi M. Effect of a phytogenic feed additive on performance, ovarian morphology, serum lipid parameters and egg sensory quality in laying hen. Vet. Res. forum an Int. Q. J. 2014a;5(4): 287–93.

Saki AA, RM, Zamani P, Yousefi A. The Effects of Different Levels of Pomegranate Seed Pulp with Multi Enzyme on Performance , Egg Quality and Serum Antioxidant in Laying Hens. 2014b;4(May): 803–8.

Saki AA, Rabet M, Zamani P, Yousefi A. The Effects of Different Levels of Pomegranate Seed Pulp with Multi Enzyme on Performance, Egg Quality and Serum Antioxidant in Laying Hens. Iran. J. Appl. Anim. Sci. 2014c;4(4): 803–8.

Samli HE, Agma A, Senkoylu N. Effects of Storage Time and Temperature on Egg Quality in Old Laying Hens. Poult. Sci. 2005;14(1): 548–53.

Scott ML, Hull SJ, Mullenhoff PA. The calcium requirements of laying hens and effects of dietary oyster shell upon egg shell quality. Poult. Sci. [Internet]. 1971;50(4): 1055–63. Available from: http://ps.oxfordjournals.org/content/50/4/1055.abstract

Shabtay A, Eitam H, Tadmor Y, Orlov A, Meir A, Kerem Z. Nutritive and antioxidative potential of fresh and stored pomegranate industrial byproduct as a novel beef cattle feed. J. Agric. Food Chem. 2008;56(21): 10063–70.

Yılmaz B, Usta Ç. Nar'ın (Punica granatum) terapötik etkileri. Türkiye Aile Hekimliği Dergisi 2010; 14: 146-53.

Williams KC. Some factors affecting albumen quality with particular reference to Haugh unit score. Worlds. Poult.Sci.J.1992;48(01):5–16.Availablefrom: http://www.journals.cambridge.org/abstract_S0043933992000023

Windisch W, Schedle K, Plitzner C, Kroismayr A. Use of phytogenic products as feed additives for swine and poultry. J. Anim. Sci. 2008;86(14): E140–8.

Wu J, Jahncke ML, Eifert JD, O'Keefe SF, Welbaum GE. Pomegranate peel (Punica granatum L) extract and Chinese gall (Galla chinensis) extract inhibit Vibrio parahaemolyticus and Listeria monocytogenes on cooked shrimp and raw tuna. Food Control. Elsevier Ltd; 2016;59: 695–9. Available from: http://dx.doi.org/10.1016/j.foodcont.2015.06.050

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):200-204
DOI: 10.30607/kvj.554074

RESEARCH ARTICLE

Investigation of Anti-*Toxoplasma gondii* Antibodies in Slaughtered Sheep in Abattoir of Nigde Using ELISA Test

Serkan GÜLER[1], Mustafa KARATEPE[2]*

[1]Niğde Ömer Halisdemir University, Graduate School of Natural and Applied Sciences, Biology Department, 51240, Niğde, Turkey
[2]Niğde Ömer Halisdemir University, Arts and Sciences Faculty, Biotechnology Department, 51240, Niğde, Turkey

ABSTRACT

The objective of this study is to determine seroprevalence of anti-*Toxoplasma gondii* antibodies with ELISA test in the sheep slaughtered in Nigde slaughterhouse. Blood samples were taken from one year and older 175 sheep between January and April. The serum samples obtained from the blood samples were examined with ELISA test, and anti-*T. gondii* antibodies were detected in 11 out of the 175 samples (6.28%). Serological examination of the sheep shows that the highest seropositivity rate of 12.5% (5/40) was detected in March, while the seropositivity rate in January was 5.9% (2/34), and in February it was 6.7% (4/60). However, none of the sheep inspected in April were seropositive for *T. gondii*. Comparison of the seropositivity rates of the sheep with regards to months did not reveal statistical significance (P>0.05). In conclusion, the presence of *T. gondii* in the sheep slaughtered in Nigde province have been determined.

Keywords: *Toxoplasma gondii*, Sheep, Nigde, Slaughterhouse, ELISA

***

Niğde Mezbahasında Kesilen Koyunlarda Anti-*Toxoplasma gondii* Antikorlarının ELISA Testi ile Araştırılması

ÖZ

Bu çalışmada, Niğde mezbahasında kesilen koyunlarda anti-*Toxoplasma gondii* antikorlarının ELISA testi ile belirlenmesi amaçlanmıştır. Bu amaçla Ocak ve Nisan ayları arasında bir yaş ve üzerindeki toplam 175 koyundan kan alınmıştır. Serumları çıkarılan kan örnekleri ELISA testi ile incelenmiş ve toplam 175 koyunun 11 (%6.28)'inde anti-*T. gondii* antikorları saptanmıştır. Koyunların serolojik incelemesinde en yüksek seropozitiflik oranı %12.5 (5/40) ile Mart ayında belirlenirken, Ocak ayında %5.9 (2/34), Şubat ayında %6.7 (4/60) oranında seropozitiflik belirlenmiştir. Buna karşılık Nisan ayında muayene edilen koyunların hiçbirinde *T. gondii* seropozitifliği tespit edilememiştir. Aylar açısından koyunlardaki seropozitiflik oranları karşılaştırıldığında istatistiksel olarak bir farklılık bulunamamıştır (P>0.05). Sonuç olarak, Niğde yöresinde mezbahada kesilen koyunlarda *T. gondii*'nin varlığı ortaya konulmuştur.

Anahtar Kelimeler: *Toxoplasma gondii,* Koyun, Niğde, Mezbaha, ELISA

To cite this article: Güler S. Karatepe M. Investigation of Anti-Toxoplasma gondii Antibodies in Slaughtered Sheep in Abattoir of Nigde Using ELISA Test. Kocatepe Vet J. (2019) 12(2):200-204.

Submission: 15.04.2019 **Accepted**: 23.05.2019 **Published Online**: 02.06.2019
ORCID ID; SG: 0000-0002-9241-5494, MK: 0000-0002-2944-6837
*Corresponding author e-mail: mkaratepe@ohu.edu.tr

GİRİŞ

Toxoplasmosisin etkeni olan *Toxoplasma gondii* ilk kez Nicolle ve Monceaux tarafından 1908 yılında Kuzey Afrika'da *Ctenodactylus gundii* adı verilen ve parazitin tür ismini aldığı bir kemiricide tanımlanmıştır. Cins ismini ise organizmanın yay şekline benzemesi nedeniyle Yunan dilinde aynı anlamdaki toxon kelimesinden almıştır (Dubey 1998, Dubey ve Beattie 1988).

Etken zorunlu hücre içi paraziti olup eritrositler hariç tüm hücrelerde gelişim gösterebilir. Parazitin konak türüne ve enfeksiyon dönemine göre değişen takizoit, bradizoit ve ookistten oluşan üç farklı yaşam formu bulunmaktadır (Dubey 1998, Dubey ve Beattie 1988). Parazitin yalnızca kedigillerde bulunan ookist formu 10x12µm boyutlarında, oval şekilli olup kalın ve dayanıklı duvara sahiptir. Kedi dışkısı ile dış ortama çıktığında henüz enfeksiyöz olmayan ookistler, uygun ısı ve nem varlığında sporlanarak enfeksiyöz hale gelir. Sporulasyon süresi ortamdaki ısı ve oksijenine bağlı olarak değişmektedir. Olgun ookistteki sporozoitler, enfekte hayvandaki takizoitler ve kistlerdeki bradizoitler, hem kediler için hem de diğer konak ve insanlar için enfeksiyon kaynağıdır (Dubey 1998, Dubey ve Beattie 1988).

Zonooz karakterli toxoplasmosis koyun ve diğer hayvanlarda subklinik bir seyir göstermektedir. Türkiye'de koyunlarda *Toxoplasma gondii*'nin varlığını belirlemek için çok sayıda seroprevalans çalışması yapılmış olup bu çalışmalarda %2.8-98.9 arasında anti-*T. gondii* antikorları saptanmıştır (Aktaş ve ark. 2000a, Çiçek ve ark. 2011). Niğde yöresinde toxoplasmosis ile ilgili daha önce yapılan çeşitli çalışmalarda; koyunlarda SFDT ile %50.90 oranında seroprevalans saptanmış bunun yanında at, sığır, keçi, hindi, güvercin, bıldırcın ve rodent gibi ara konaklarda ve son konak kedilerde enfeksiyonun varlığı ortaya konulmuştur (Karatepe ve ark. 2003, 2004a,b, 2008, 2010, 2011, Babür ve ark. 2008, Kılıç ve ark. 2017).
Bu çalışmada, Niğde mezbahasında kesilen koyunlarda anti-*Toxoplasma gondii* antikorlarının ELISA testi kullanılarak belirlenmesi amaçlanmıştır.

MATERYAL ve METOT

Bu çalışmanın gerçekleştirilmesi için, Niğde ili mezbahasında Ocak-Nisan 2011 tarihleri arasında kesilen bir yaşın üzerindeki 175 koyundan kan alınmıştır. Kanlardan usulüne uygun olarak serumları çıkarılmış ve serolojik analizler yapılıncaya kadar laboratuvarda -20^{o}C'lik derin dondurucuda saklanmıştır. Ayrıca, mevcut çalışma için HADMEK'in 05.06.2009 tarih ve "Araştırma kapsamı dışında teşhis ve tedavi amaçlı hayvanlara yapılan; klinik uygulamalar, ölü hayvanla veya ölmüş hayvan dokusu ile yapılan çalışmalar, mezbaha materyalleri, atık fetuslar, süt sağma, dışkı veya altlık örneği toplama, kan alma, swap ile örnek alma vb. müdahalelerde Hayvan Deneyleri Yerel Etik Kurulundan izin alınmasına gerek olmadığına" dair 12 sayılı kararına istinaden etik kurul onayı alınmamıştır.
Çalışmada, *T. gondii* antikorlarının araştırılması amacıyla CHEKIT-Toxotest ELISA Test Kiti (IDEXX, Switzerland AG) kullanılmıştır. ELISA testi, CHEKIT-Toxotest ELISA Test Kiti ile üretici firmanın prosedüründe belirtildiği şekilde yapılmıştır. ELISA testi sonucunda mikrotiter plateler 450nm'de ELISA mikroplate okuyucusunda (MR-96A) okutulmuş ve saptanan değerler üretici firmanın kit prosedüründe belirttiği formül ile hesaplanmıştır.

Formül;

$$\% \text{ Değer} = \frac{\text{O.D. örnek} - \text{O.D. negatif}}{\text{O.D. pozitif} - \text{O.D. negatif}} \times 100$$

O.D. örnek : Örneklerin Optikal Yoğunluğu
O.D. pozitif : Pozitif Kontrollerin Optikal Yoğunluğu
O.D. negatif : Negatif Kontrollerin Optikal Yoğunluğu

% Değer;
≥%100 ise pozitif,
≥%30 - <%100 ise zayıf pozitif,
≥%20 - <%30 ise şüpheli,
<%20 ise negatif olarak değerlendirilmiştir.

Araştırmada koyunların aylara göre seropozitiflik oranlarının istatistiksel olarak değerlendirilmesinde Ki-kare testi kullanılmıştır.

BULGULAR

Niğde mezbahasında kesilen bir yaş ve üzerindeki toplam 175 koyunun 11'inde ELISA testi ile *Toxoplasma gondii*'ye karşı antikorlar tespit edilmiş ve *T. gondii*'nin seroprevalansı Niğde yöresi koyunlarında %6.28 oranında saptanmıştır.

Tablo 1'e göre aylar açısından; Ocak ayında 34 koyunun 2 (%5.88)'sinde, Şubat ayında 60 koyunun 4 (%6.66)'ünde, Mart ayında 40 koyunun 5 (%12.5)'inde *T. gondii* yönünden seropozitiflik tespit edilmiştir. Buna karşılık Nisan ayında incelenen 41 koyunun hiçbirinde seropozitiflik saptanmamıştır. Seropozitif hayvanlar arasında aylar açısından seropozitifliğin istatistiksel yönden önemsiz olduğu belirlenmiştir ($P>0.05$).

Tablo 1. Koyunlarda *Toxoplasma gondii* seropozitifliğinin aylara göre dağılımı
Table 1. Distribution of *Toxoplasma gondii* seropositivity in sheep according to months

Aylar	Muayene Edilen Hayvan Sayısı	Pozitif Hayvan Sayısı	Seropozitiflik (%)
Ocak	34	2	5.88
Şubat	60	4	6.66
Mart	40	5	12.5
Nisan	41	-	-
Toplam	**175**	**11**	**6.28**

TARTIŞMA ve SONUÇ

Toxoplasmosis gibi tüm dünyada yaygın olarak görülen zoonoz protozoer enfeksiyonlar halk sağlığı açısından önemli bir problem oluşturmakla beraber hayvancılık ekonomisinde de önemli kayıplara sebep olmaktadır. Toxoplasmosis, genellikle latent enfeksiyonlara neden olduğundan gözden kaçmakta ve ciddi ekonomik kayıplar oluşturmaktadır. *Toxoplasma gondii,* enfekte koyunlarda et, süt ve yapağı gibi verim kayıplarının yansıra meydana getirdiği atıklarla da önemli protozoon parazitlerdendir. (Dubey ve Beattie 1988).

Türkiye'de koyunlarda ilk çalışma 1967 yılında Ekmen tarafından yapılmış ve SFDT ile %43.1 ve CFT ile %20 seropozitiflik oranı bulunmuştur (Ekmen 1967). Daha sonra ülkenin birçok bölgesinde koyunlarda toxoplasmosisin yaygınlığı üzerine SFDT, ELISA, IFAT, IHA ve LAT gibi farklı serolojik testler kullanılarak yapılmış araştırmalarla Orta Anadolu, Adana, Elazığ, Ankara, Çankırı, KKTC, Kayseri, Konya, Malatya, Kars, Kırıkkale, Amasya, Yozgat, Hatay, Şanlıurfa, Mersin, Aydın, Van, Afyon, Yalova, Samsun ve Nevşehir'de sırası ile %36, %22-25.5, %22.5-46.8, %14.6-72, %88.7, %52.1, %33.7, %10.1-13, %33.2, %51.4,-95.7, %63.9, %66.6, %45.4, %53.3, %42.7-55.6, %48, %72, %46, %54.6-98.9, %65-66.6, %50, %10 oranları tespit edilmiştir (Arda ve ark. 1987, Öz ve ark. 1995, Dumanlı ve ark. 1991, Zeybek ve ark. 1995, Babür ve ark. 1996, 1997, İnci ve ark. 1999, Nalbantoğlu ve ark. 1999, Aktaş ve ark. 2000a, b, Sevinç ve ark. 2000, Aslantaş ve Babür 2000, Yıldız ve ark. 2000, Karatepe ve ark. 2001, Babür ve ark. 2001, Kamburgil ve ark. 2001, Aslan ve Babür 2002, Öztürk ve ark. 2002, Paşa ve ark. 2004, Tütüncü ve ark. 2001, Çiçek ve ark. 2004, 2011, Öncel ve ark. 2005, Sevgili ve ark. 2005, Mor ve Arslan 2007, Acici ve ark. 2008, Aköz ve ark. 2009).
Niğde yöresinde mezbahada kesilen koyunlar üzerinde yapılan bu çalışmada ELISA ile %6.28 seropozitiflik oranı saptanmıştır. Konya'da, Sevinç ve ark. (2000) ile Aköz ve ark. (2009)'nın yaptıkları çalışmalarda elde edilen %10.1 ve %13'lük oranlar bu çalışmada elde edilen sonuca benzerlik göstermektedir. Bu durum çalışmaların yapıldığı illerin aynı coğrafi bölgede bulunmasından kaynaklanabilir. Yine Çakmak ve Karatepe (2017)'nin Nevşehir'de ELISA ile yaptıkları çalışmada tespit edilen %10'luk sonuç, bu çalışmada saptanan sonuca oldukça yakındır. Bunun sebebi aynı test tekniğinin kullanılması ve komşu şehir olan Niğde ile Nevşehir illerinin sahip olduğu coğrafi benzerlik olabilir. Türkiye'de yapılan bazı çalışmalarda çok yüksek seropozitiflik oranları elde edilmiştir. Bu farklılıkta, bölgeler arasında iklim ve konum farklılıkları ile birlikte kullanılan metodun da etkili olduğu düşünülmektedir. Bunun yanında, araştırmanın yürütüldüğü Niğde yöresinde Karatepe ve ark. (2004) tarafından SFDT ile koyunlar üzerinde yapılan bir çalışmada %50.90 oranında seropozitiflik saptanmıştır. Aynı yörede yapılan bu iki çalışma arasındaki farklılık, analizde kullanılan serolojik testlerin farklı olmasından ve çalışmaların farklı yıl ve farklı koyunlarda yapılmış olmasından kaynaklanabilir. Niğde yöresinde koyunlarda gerçekleştirilen bu çalışmayı dünyada koyunlar üzerinde yapılan araştırmalar ile karşılaştığımızda benzer sonuçları görmek mümkündür. O'Donoghue ve ark. (1987)'nın Kuzey Avustralya'da IHAT ile 1159 koyun üzerinde yaptıkları araştırmada %7.4 oranında seropozitiflik saptanmıştır. Pakistan'da Zaki (1995) 40 koyun üzerinde LAT ile yaptığı araştırmada %2.5 oranında ve Sharma ve ark. (2008) Bangladeş'te aynı yöntemi (ELISA) kullanarak yaptıkları serolojik çalışmada 186 koyunda %0.27 oranında seropozitiflik elde etmişlerdir. Aynı testin kullanılması ve farklı sonuçların elde edilmesine coğrafik koşulların farklılığı sebep olabilir.

Ülkemizde ve dünyada çeşitli oranlarda yaygınlık gösteren toxoplasmosisin korunma ve kontrolünde öncelikli olarak parazitin zorunlu olan konağı olan kedilerle mücadele etmek gerekmektedir. Çiftlik hayvanları ile yemleri, kedi ve kedi dışkılarından uzak tutulmalıdır. Hastalık zoonoz olduğundan özellikle hamilelerin kediler ile temastan kaçınması gerekir. Çiğ et ve çiğ et bulunan yiyecekleri tüketme alışkanlığının enfeksiyonun yayılmasında etkili olduğu unutulmamalıdır.

Sonuç olarak, toplam 175 koyundan ELISA ile elde ettiğimiz %6.28 seropozitiflik oranına bakarak toxoplasmosisin Niğde mezbahasında kesilen

koyunlarda yaygın olmasa da varlığının tespit edildiğini söyleyebiliriz. Toxoplasmosisin insan ve hayvan sağlığı açısından etkisinin tam olarak ortaya çıkarılabilmesi ve gerekli kontrol ve korunma önlemlerinin alınabilmesi için Niğde yöresinde daha geniş çapta serolojik ve moleküler çalışmalar yapılmasının gerekli olduğu düşünülmektedir.

TEŞEKKÜR

Bu çalışma Niğde Ömer Halisdemir Üniversitesi Bilimsel Araştırma Projeleri Koordinasyon Birimince desteklenmiş (Proje No : SSB-2010/01), Serkan GÜLER'in Yüksek Lisans tezinden özetlenmiş ve 19. Ulusal Parazitoloji Kongresi'nde (05-09 Ekim 2015, Erzurum) sunulmuştur.

KAYNAKLAR

Acici M, Babur C, Kilic S, Hokelek M, Kurt M. Prevalance of antibodies to *Toxoplasma gondii* infection in humans and domestic animals in Samsun province, Turkey. Trop Anim Health Prod. 2008; 40: 311-315.

Aköz M, Aydın İ, Kamburgil K, Handemir, E. Konya'nın Karapınar ilçesindeki abort yapan ve yapmayan koyunlarda *Toxoplasma gondii*'nin seroprevallansının İndirekt Fluoresan Antikor (IFA) tekniği ile belirlenmesi. Vet Bil Derg. 2009; 25: 37-43.

Aktaş M, Dumanlı N, Babür C, Karaer, Z, Öngör H. Elazığ yöresinde gebe ve yavru atmış koyunlarda Sabin-Feldman(SF) testi ile *Toxoplasma gondii* yönünden seropozitiflik oranının belirlenmesi. Türk J Vet Anim Sci. 2000a; 24: 239-241.

Aktaş M, Babür C, Düzgün A. Malatya yöresinde koyunlarda *Toxoplasma gondii*'nin seroprevalansı. FÜ Sağlık Bil Derg. 2000b; 14: 65-67.

Arda M, Bisping W, Aydın N, İstanbulluoğlu E, Akay Ö, İzgür M, Diker S, Karaer Z. Orta Anadolu bölgesi koyunlarında abortus olgularının etiyolojisi ve serolojisi üzerinde bir çalışma. Ankara Üniv Vet Fak Derg, 1987; 34: 195-206.

Aslan G, Babür C. Şanlıurfa'da koyun ve sığırlar ile mezbaha çalışanlarında *Toxoplasma gondii* seroprevalansı. Türk Mikrobiyal Cem Derg. 2002; 32: 102-105.

Aslantaş Ö, Babür C. Kars yöresinde sığır ve koyunlarda Bruselloz ve Toxoplazmoz üzerine seroepidemiyolojik araştırmalar. Etlik Vet Mikrobiyol Derg. 2000; 11: 47-55.

Babür C, Karaer, Z, Çakmak A, Yaralı C, Zeybek H. Ankara yöresinde Sabin-Feldman(SF), İndirekt Floresan Antikor(IFA), Latex Aglutinasyon (LA) testleri ile koyun toxoplasmosisinin prevalansı. FÜ Sağlık Bil Derg. 1996; 10: 273-277.

Babür C, İnci A, Karaer Z. Çankırı yöresinde koyun ve keçilerde *Toxoplasma gondii* seropozitifliğinin Sabin-Feldman boya testi ile saptanması. Türkiye Parazitol Derg. 1997; 21: 409-412.

Babür C, Esen B, Bıyıkoğlu G. Yozgat'ta koyunlarda *Toxoplasma gondii*'nin seroprevalansı. Türk J Vet Anim Sci. 2001; 25: 283-285.

Babür C, Karatepe B, Kılıç S, Karatepe M, Ergün G. A note on seroprevalence of *Toxoplasma gondii* in turkeys. Indian Vet J. 2008; 85: 994.

Çakmak ÖD, Karatepe B. Seroprevalence of *Toxoplasma gondii* in Sheep from Nevşehir Province in Turkey. Türkiye Parazitol Derg, 2017; 41: 148-51.

Çiçek H, Babür C, Karaer Z. Afyon yöresinde Sabin-Feldman (SF) boya testi ile koyunlarda *Toxoplasma gondii* seroprevalansı. AÜ Vet Fak Derg. 2004; 51: 229-231.

Çiçek H, Babür C, Eser M. Afyonkarahisar İlinde Pırlak Irkı Koyunlarda *Toxoplasma gondii*'nin Seroprevalansı. Türkiye Parazitol Derg, 2011; 35: 137-9.

Dubey JP. Advances in the life cycle of *Toxoplasma gondii*. International Journal for Parasitology. 1998; 28: 1019-1024.

Dubey JP, Beattie CP. Toxoplasmosis of Animals and Man. CRC Press, Boca Raton, Florida, 1988.

Dumanlı N, Güler S, Köroğlu E, Orak S. Elazığ yöresinde koyunlarda *Toxoplasma gondii*'nin yayılışı. Doğa Tr Vet Anim Sci. 1991; 16: 10-18.

Ekmen H. Toxoplasmozis'te enfeksiyon kaynakları, Koyun ve sığırlarda *Toxoplasma* antikorları. Mikrobiyol Bült. 1967; 1(4): 243-248.

İnci A, Aydın N, Babür C, Çam Y, Akdoğan C, Kuzan Ş. Kayseri yöresinde sığır ve koyunlarda toxoplazmozis ve brusellozis üzerine seroepidemiyolojik araştırmalar. Pendik Vet Mikrobiyol Derg. 1999; 30: 41-46.

Mor N, Arslan MÖ. Kars yöresindeki koyunlarda *Toxoplasma gondii*'nin seroprevalansı. Kafkas Üniv Vet Fak Derg. 2007; 13: 165-170.

Nalbantoğlu S, Babür C, Çakmak A, Karaer Z, Korudağ E. Kuzey Kıbrıs Türk Cumhuriyeti'nde Sabin-Feldman Dye testi ile koyun ve keçilerde *Toxoplasma gondii*'nin seroprevalansı. Türk Hij Den Biyol Derg. 1999; 56: 83-86.

Kamburgil K, Durgut R, Handemi, E. Hatay yöresinde atık problemi olan koyun sürülerinde toxoplasmozisin seroprevalansı. Veterinarium. 2001; 12: 1-4.

Karatepe M, Babür C, Karatepe B. Gümüşhacıköy (Amasya) Yöresi Koyunlarında *Toxoplasma gondii*'nin Sabin-Feldman Boya Testi ile Seroprevalansı. Türkiye Parazitol Derg. 2001; 25: 110-112.

Karatepe B, Babür C, Karatepe M, Çakmak A, Nalbantoğlu S. Niğde Yöresinde Sığırlarda *Toxoplasma gondii*'nin Sabin-Feldman Dye Testi ile Seroprevalansı. Etlik Vet Mikrob Derg. 2003; 14: 18-21.

Karatepe B, Babür C, Karatepe M, Çakmak A, Nalbantoğlu S. Seroprevalance of toxoplasmosis in sheep and goats in the Niğde province of Turkey. Indian Vet J. 2004a; 81: 974-976.

Karatepe M, Babür C, Karatepe B, Kılıç S, Çakır M. Prevalence of *Toxoplasma gondii* Antibodies in Anatolian Ground Squirrels, *Spermophilus xanthophrymnus (Rodentia : Sciuridae)* from Nigde, Turkey. Revue Med Vet. 2004b; 155: 530-532.

Karatepe B, Babür C, Karatepe M, Kılıç S, Dündar B. Prevalence of *Toxoplasma gondii* antibodies and intestinal parasites in stray cats from Nigde, Turkey. Ital J Anim Sci. 2008; 7: 113-118.

Karatepe B, Babür C, Karatepe M, Kılıç S. Seroprevalence of toxoplasmosis in horses in Niğde Province of Turkey. Trop Anim Health Prod. 2010; 42: 385-389.

Karatepe M, Kılıç S, Karatepe B, Babür C. Prevalence of *Toxoplasma gondii* Antibodies in Domestic (*Columba livia domestica*) and Wild (*Columba livia livia*) Pigeons in Niğde region, Turkey. Türkiye Parazitol Derg. 2011; 35: 23-26.

Kılıç S, Karatepe M, Babür C, Karatepe B. Niğde Yöresinde Bıldırcınlarda (*Coturnix coturnix japonica*) *Toxoplasma gondii*'nin Seroprevalansı. Kocatepe Vet J. 2017; 10: 129-133.

O'Donoghue JP, Riley MJ, Clarke JF. Serological survey for *Toxoplasma* infections in sheep. Aust Vet. J. 1987; 64: 40-45.

Öncel T, Vural G, Babür C, Kılıç S. Detection of Toxoplasmosis gondii seropozitivity in sheep in Yalova by Sabin-Feldman dye test and latex agglutination test. Türkiye Parazitol Derg. 2005; 29(1): 10-12.

Öz İ, Özyer M, Çorak M. Adana yöresi sığır, koyun ve keçilerinde ELISA ve IHA testleri ile toxoplasmosisin yaygınlığının araştırılması. Etlik Vet Mikrob Derg. 1995; 8: 80-86.

Öztürk C, Babür C, Aslan G. Mersin yöresinde koyunlarda ve mezbaha çalışanlarında Sabin-Feldman boya testi ile anti-*Toxoplasma gondii* antikorlarının araştırılması. Genel Tıp Derg. 2002; 12: 21-24.

Paşa S, Babür C, Kılıç S, Gazyağcı S, Bayramlı G. Seroprevalance of toxoplasmozis in sheep in Aydın region in Turkey. Indian Vet J. 2004; 81: 376-377.

Sevgili M, Babür C, Nalbantoğlu S, Karaş G, Vatansever Z. Determination of seropositivity for *Toxoplasma gondii* in sheep in Şanlıurfa province. Turk J Vet Anim Sci. 2005; 29: 107-111.

Sevinç F, Kamburgil K Dik B, Güçlü F, Aytekin H. Konya yöresinde atık yapan ve yapmayan koyunlarda indirekt fleurosan antikor (IFA) testi ile toxoplazmozis araştırması. FÜ Sağlık Bil Derg. 2000; 24: 180-185.

Sharma S, Sandhu KS, Bal MS, Kumar H, Verma S, Dubey JP. Serological survey of antibodies to *Toxoplasma gondii* in sheep, cattle and buffaloes in Punjab, India. J Parasitol. 2008; 94: 1174-1175.

Tütüncü M, Akkan HA, Babür C, Ayaz E, Karaca M. The seroprevalence of *Toxoplasma gondii* in sheep detected by Sabin Feldman Dye Test in the region of Van, Turkey. YYÜ Vet Fak Derg. 2001; 12(1-2): 33-35.

Yıldız K, Babür C, Kılıç S, Aydenizöz M, Dalkılıç İ. Kırıkkale Mezbahası'nda kesilen koyun ve sığırlar ile mezbaha çalışanlarında anti-*Toxoplasma* antikorlarının araştırılması. Türkiye Parazitol Derg. 24: 2000; 180-185.

Zaki M. Seroprevalance of *Toxoplasma gondii* in domestic animals in Pakistan. J Pak Med Assoc. 1995; 45: 4-5.

Zeybek H, Yaralı C, Nishikawa F, Nishikawa F, Dündar B. Ankara yöresi koyunlarında *Toxoplasma gondii*'nin prevalansının saptanması. Etlik Vet Mikrob Derg. 1995; 8: 80-86.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):205-212
DOI: 10.30607/kvj.493594

RESEARCH ARTICLE

Traumatic Diaphragmatic Hernia in Cats: A Retrospective Study of 15 Cases (2016-2017)

Rahime YAYGINGÜL*, Zeynep BOZKAN, Zeynep BİLGEN ŞEN, Büşra KİBAR KURT, Ali BELGE

Adnan Menderes University, Veterinary Faculty, Department of Surgery, 09016-Isikli, Aydin –Turkey

ABSTRACT

In this study, evaluation of clinical and operative results of cats with diaphragmatic hernia presented with respiratory failure was aimed. The cats were in different age, breed and sex. All of the cats were treated surgically with 73.3% survival rate. The highest incidence of herniation was in the liver and small intestine, but stomach and spleen also was encountered. All deaths occurred during surgery, the cases completed surgical procedure were healed. The cats which died had multiple organ herniation as well as laceration of the lungs and fluid accumulation in the thoracic cavity were noticed. It was seen that intensive care cabin applications and Ventolin - Pulmicort support through nebulizer provided marked contribution on survival rate.

Keywords: Cat, diaphragm, hernia, intensive care

***

Kedilerde Travmatik Diyafram Fıtkı: 15 Olgu (2016-2017)

ÖZ

Bu çalışmada, kliniğimize solunum güçlüğü şikâyeti ile getirilen kedilerde karşılaşılan diyafram fıtıklarının klinik ve operatif sonuçlarının değerlendirilmesi amaçlanmıştır. Kediler farklı yaş, ırk ve cinsiyette idiler. Tüm kediler % 73.3 sağ kalım oranıyla cerrahi olarak tedavi edildi. En fazla fıtıklaşan organ karaciğer ve ince bağırsak olmakla birlikte mide ve dalakla da karşılaşıldı. Tüm ölümler operasyon sırasında meydana geldi, operasyonu tamamlanan olguların tamamı yaşadı. Ölen kedilerde çoklu organ fıtıklaşması yanı sıra gerek fıtıklaşan organ gerekse akciğerlerde laserasyon, göğüs boşluğunda sıvı toplanması dikkati çekti. Yoğun bakım kabin uygulamaları ve nebulizator aracılığı ile ventolin- pulmicort desteği sağ kalım üzerine olumlu katkı sağladığı görüldü.

Anahtar Kelimeler: Kedi, diyafram, fıtık, yoğun bakım

To cite this article: Yaygıngül R. Bozkan Z. Bilgen Şen Z. Kibar Kurt B. Belge A. Traumatic Diaphragmatic Hernia in Cats: A Retrospective Study of 15 Cases (2016-2017). Kocatepe Vet J. (2019) 12(2):205-212.

Submission: 07.12.2018 **Accepted**: 22.05.2019 **Published Online**: 08.06.2019
ORCID ID; RY: 0000-0001-7402-9031, ZB: 0000-0003-4233-6496, ZBŞ: 0000-0002-4020-9779, BKK: 0000-0002-1490-8832, AB: 0000-0003-3346-6926
*Corresponding author e-mail: ryaygingul@hotmail.com

INTRODUCTION

Diaphragm is a musculocutaneous structure that separates abdominal and thoracic cavity, supports ventilation, and plays a role in lymphatic fluid flow. Embryologically it develops from the septum transversum in the ventral side, two pleuroperitoneal leaves and the mesentery of the small intestines in the dorsal side (Orton 2017, Randall 2018). Diaphragmatic hernia is a defect or hole in the diaphragmatic muscle which allows the one or more organs located in the abdominal cavity to move into the thoracic cavity. It may be occurred congenital or acquired and, is encountered in cats and dogs as a common problem (Fossum 2002, Gibson et al. 2005). Congenital diaphragmatic hernia is caused from the failure of the development of transversal septum. Since clinical findings are not very clear, the herniation usually determined incidentally after thorax radiography. Acquired hernias generally occur after the blunt traumas (traffic accidents, fall from high places etc.). Traumatic diaphragm hernias in cats and dogs are more common than congenital hernias and treated with surgically (Fossum 2002, Schmiedt et al. 2003, Minihan et al. 2004, Randall 2018). Initial approach to the patient with respiratory distress and, management of pre/post-operative period including oxygen therapy, fluid therapy, control of body temperature, using of the inhaler therapeutics have great role on prognosis (Mazzaferro et al. 2013, Nelson 2015). In this study, to share preoperative medical management, surgical treatment and results of the diaphragmatic hernia cats represented our clinic was aimed.

MATERIAL and METHOD

The study was conducted on 15 cats of different ages, breed and sexes. Place of hernia, herniated organs, complications and survival rates of cats were recorded to the trauma form. All cats were brought to our clinic with respiratory complaints. Clinical, radiological, hematological and biochemical analyzes were performed on all animals. Ventro-dorsal and latero-lateral, radiological position (Figure 1) (positive contrast celiography in 1 case, Iohexol Omnipaqua, Amersham, USA) was taken and it has been interpreted. Diaphragmatic hernia was diagnosed by clinical and radiographic findings (Figure 1A-B).

Preoperatively, the patients were to the intensive care cabin and oxygen support was provided for 24 hours. Serum and antibiotic (cefazolin sodium of 20 mg / kg, Iespor®, IE Ulagay, Istanbul) were administered intravenously for prophylaxis. Induction of the general anesthesia induction was performed with intravenous propofol of 4 mg / kg (Diprivan®, Astra Zeneca). Then, anesthesia was maintained with 1-3% MAC isoflurane (Isoflurane, Adeka, Samsun) by intubation with 3-3.5 cuffed endotracheal tubes. The patients were placed in a dorsal recumbency on the operating table tilted 40 degrees in caudal direction. During the operation, Ringer's lactate solution of 10 ml/kg/ h (500 ml, Polypharma) was administered intravenously. Operation area was prepared aseptically and covered sterile drapes. The diaphragm was approached by ventral median line laparotomy extending from the xiphoid cartilage to the umbilicus (Figure 2). The organs displaced to the thoracic cavity (Figure 3) were re-poisoned to the abdomen. Diaphragmatic defect (Figure 4A) was repaired with simple continuous or interrupted suture techniques with non-absorbable material (Figure 4B)(Prolene, Ethicon, Johnson &Johnson, Brussels, Belgium, USP 2/0-3/0). The negative pressure of the thoracic cavity was created before the last stitches. Abdominal cavity was lavaged with saline (Isotonic Sodium Chloride, 500 ml, Koçak Farma), the operation area was closed routinely. Polypropylene mesh (Polypropylene mesh)(Prolene®, Ethicon) implantation was needed in only one case to close of the abdominal cavity. No chest tube was placed in any of the patients. All cases were observed in the intensive care unit for 3 days. Meanwhile, oxygen support is provided and methylprednisolone of 1 mg/kg (Prednol®, Mustafa Nevzat, Turkey) were administered intramuscularly. Also salbutamol sulfate (Ventolin Nebules® 2.5mg/ml, GlaxoSmithKline) – budesonide (Pulmicort® Nebulizer sus. 0.5mg/ml Astra Zeneca) was given with mask via nebulizer twice a day (Figure 6). Additionally, cefazolin sodium administration continued for postoperative one week.

RESULTS

The cats were between 5 months and 15 years old, 2 male and 13 female, mean 2-5.5 kg body weight (Table 1). Etiologies of the diaphragmatic hernias were motor vehicle accidents in 5 cases, falling from high places in 4 cases and unknown traumas (as a result of run away from home) in 6 cases. Clinical presentation durations of the cases were between 1 to 5 days after trauma Tachypnea (15 cases), dyspnea and orthopnea (5 cases), inappetence (5 cases), open mouth respiration (3 cases), abdominal respiration (3 cases) exercise intolerance (2 cases) and vomiting (1 case) were determined in clinical and physical examination. Direct radiographies provided sufficient information in 14 cases, but positive contrast celiography was performed in one cases. Radiographic examination revealed that loss of diaphragmatic shadow and heart silhouette and displaced abdominal organs in the thorax. Celiography revealed that the contrast medium was distributed in thorax. The results of hematological and biochemical analysis were within normal limits. The diaphragmatic defects were on dorsal region in one case, ventral region in other cases (right in 6 cases, central in 4 cases, left in 3 case, right and left in 1 case). The herniated organs were single organ in 5

cases (2 small intestines, 3 liver), multiple organs in 10 cases (spleen, liver, stomach and intestine in 3 cases; small intestine and liver in 4 cases; stomach, spleen and small intestine in 2 cases; spleen and small intestine in 1 case). Four patients were died during the operation. The cases died had marked laceration of the lungs and large amount fluid accumulation into the thoracic cavity.

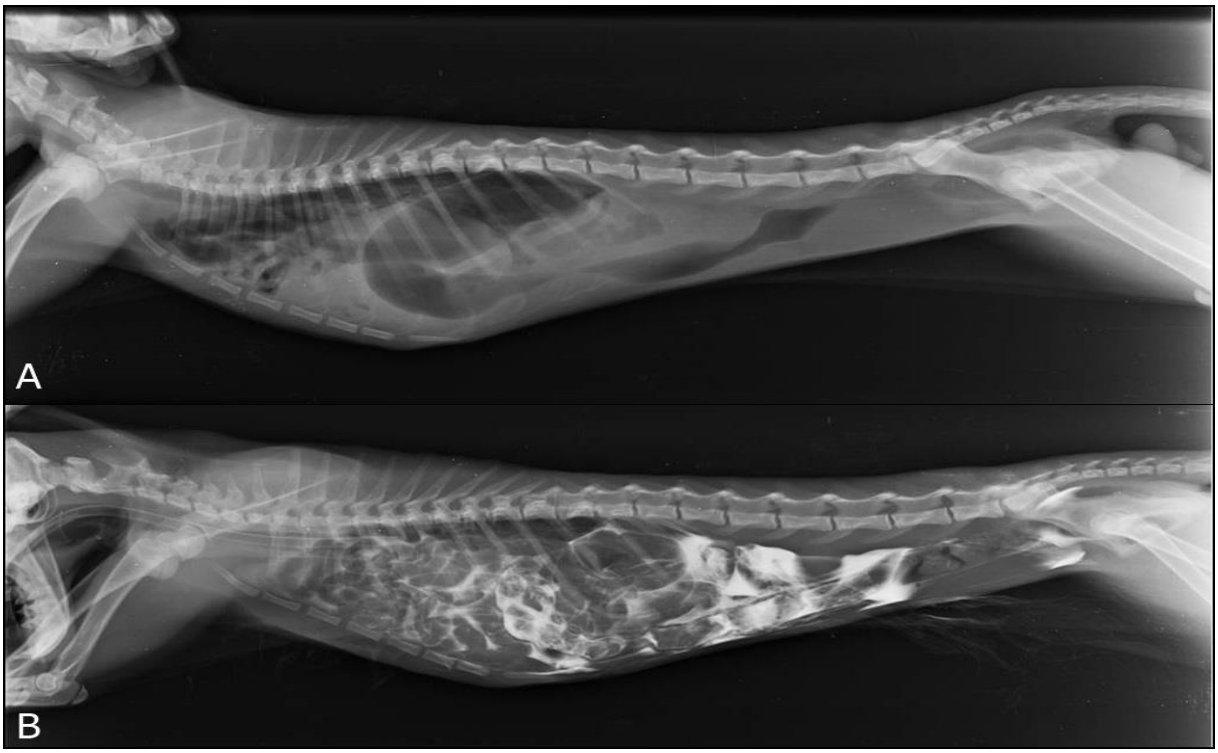


Figure 1. Direct radyography (A) and peritoneography (B).

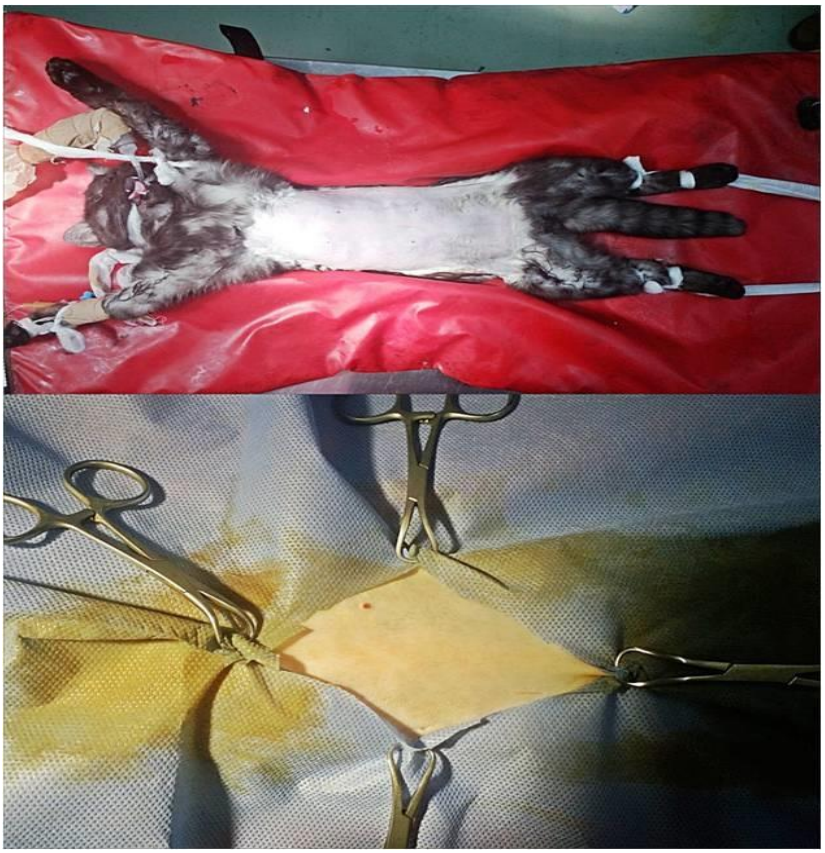


Figure 2. Limitation of the operation side with surgical drapes.

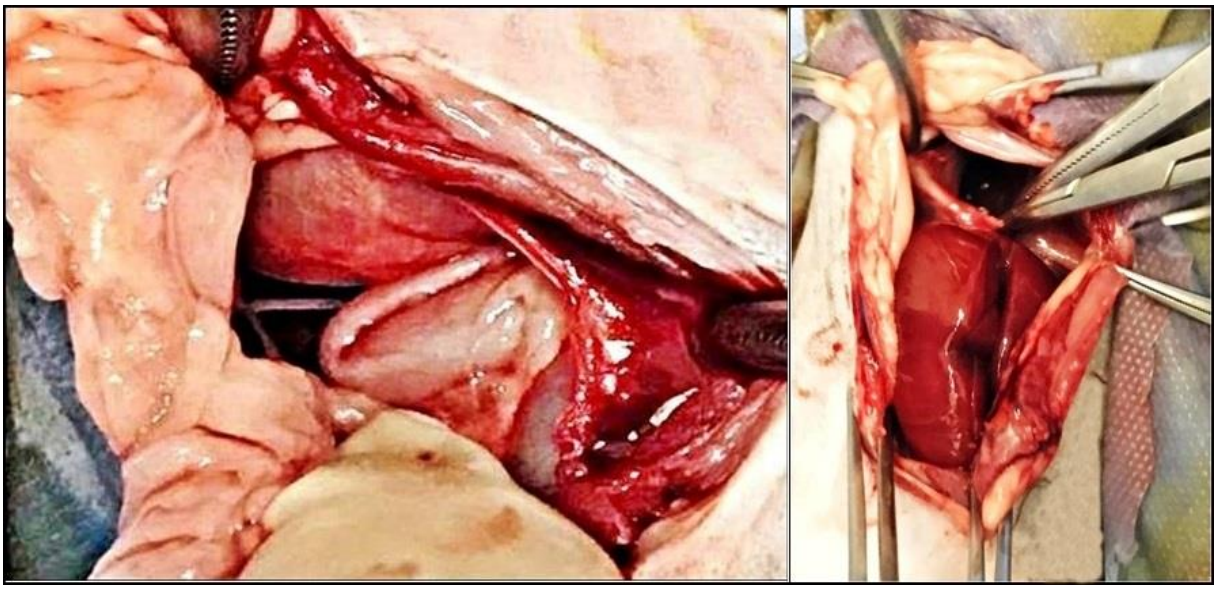


Figure 3. Intestine in thoraks.

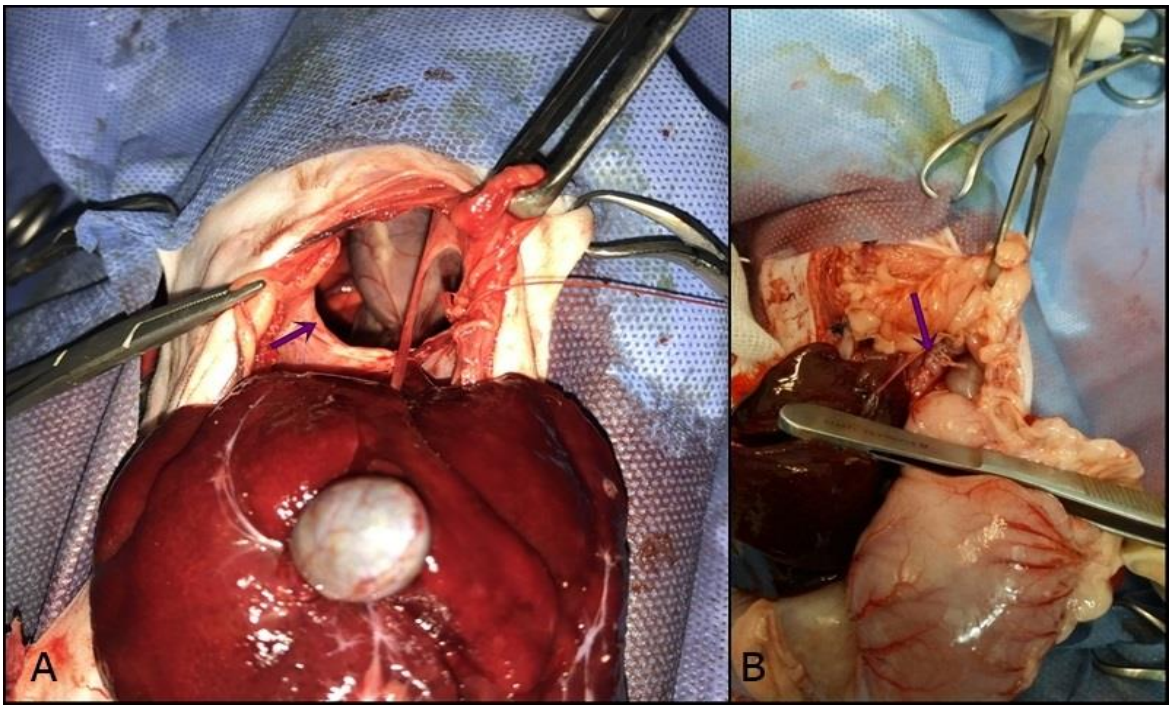


Figure 4: Diaphragmatic herni (A). Repair of the diaphragmatic hernia (B).

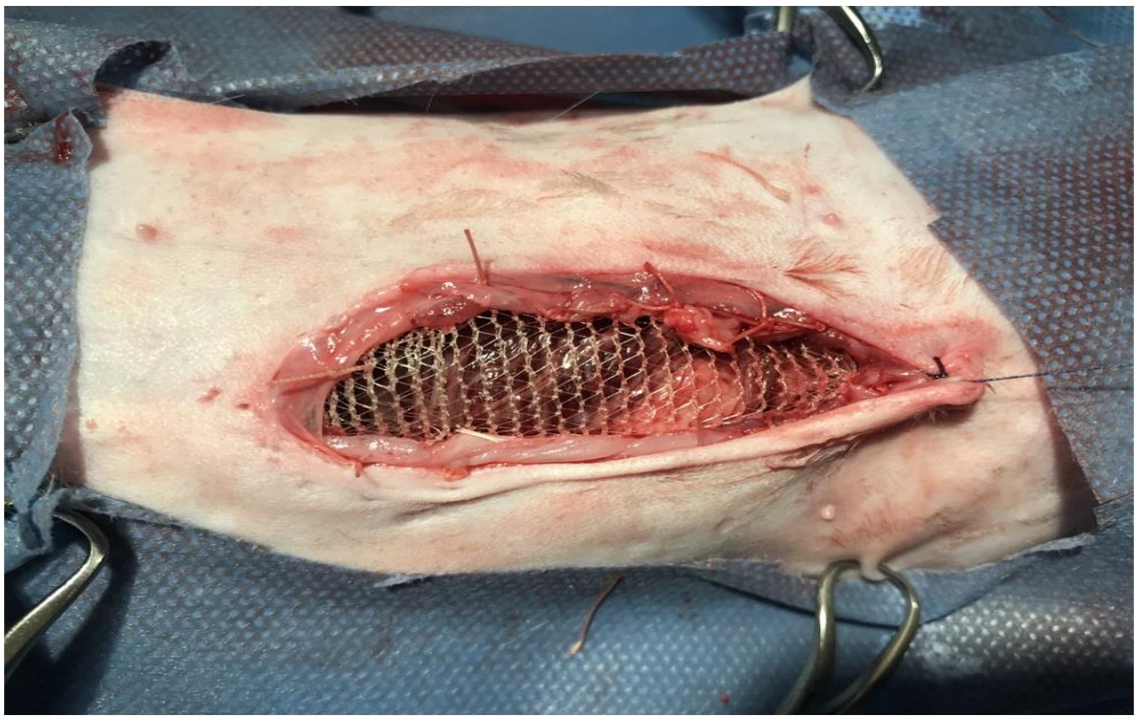


Figure 5: Polypropylene mesh.

Figure 6: Postoperative nebulisation.

Table 1. Cats information

	Age	Sex	Location of Hernia	Hernia organs	Cause
1	8 months old	Male	Dorsal-Central	Spleen, Small intestines	
2	12 years old	Female	Ventral -Left	Liver	
3	2 years old	Female	Ventral-Right	Small intestines	
4*	3 months old	Female	Ventral-Central	Spleen, Liver, Stomach, Small intestines	
5*	2 years old	Female	Ventral -Left	Spleen, Liver, Stomach, Small intestines	
6	5 months old	Female	Ventral- Right	Liver, Small intestines	
7*	1 years old	Female	Ventral-Central	Liver, Small intestines	
8	8 months old	Female	Ventral-Central	Liver, Small intestines	Trauma
9	7 months old	Female	Ventral-Right	Liver	
10	5 months old	Female	Ventral-Right	Small intestines	
11	1 years old	Female	Ventral-Right	Spleen, Liver, Stomach, Small intestines	
12	1 years old	Female	Ventral-Right-Left	Spleen, Stomach, Small intestines	
13	4 months old	Female	Ventral- Left	Spleen, Liver, Stomach, Small intestines	
14	15 years old	Female	Ventral- Right	Liver	
15*	4 months old	Male	Ventral- Central	Liver, Small intestines	

* Four patients were died during the operation

DISCUSSION

Diaphragmatic hernia is a defect or hole in the diaphragmatic muscle which allows the one or more organs located in the abdominal cavity to move into the thoracic cavity. It has very high incidence in cats after trauma. Survival rate after surgery of diaphragmatic hernia is reported as 54-90% (Schmiedt et al. 2003, Minimal et al. 2004, Gibson et al. 2005, Besalti et al. 2011, Legallet et al. 2017). Following the procedure which used in this study 73.3% survival rate was accomplished.

Many studies (Minimal et al. 2004, Hyun 2004, Besalti et al. 2011) have stated that diaphragmatic hernia cases occur more in male cats than females. However, 13 of the cats with diaphragmatic hernia were female and only 2 cases were male in our study.

Clinical manifestations of diaphragmatic herniation in animals include respiratory problems, increased respiratory rate, collapse, regurgitation, vomiting and muffled heart sounds. These findings may not be found in chronic cases (Fossum 2002, Schmiedt et al. 2003, Minimal et al. 2004, Gibson et al. 2005, Ozer et al. 2007, Besalti et al. 2011). Radiological findings include loss of the diaphragmatic shadow and heart silhouette, pleural effusion, gas findings of the gastrointestinal organs and shadow of the other abdominal organs in the thoracic cavity. Some patients may have no radiographic findings (Voges et al. 1997, Hyun 2004). Such cases can be diagnosed by contrast radiography of the gastrointestinal tract, peritoneography, thorax or abdominal ultrasonography and computed tomography (Gibson et al. 2005, Kibar et al. 2006). Transition of radiographic contrast agents injected into the peritoneal cavity to the thoracic cavity is evaluated by performing peritoneography (Sullivan and Lee 1989, Kibar et al. 2006, Tillson 2014). In this study, all cases were presented with complain of respiratory problems. Clinical and radiological examinations revealed diaphragmatic hernia. Except one case which diagnosed by celiography, because direct radiography and clinical examination findings were sufficient for

diagnosis in all cases, to perform other examination methods such as peritoneography, ultrasonography and computed tomography was not needed.

No specific laboratory findings are usually found in hernia cases. Some studies state that the cases with hernias of the liver may have high levels of aminotransferase and serum alkaline phosphatase (Fossum 2007, Ozer et al. 2007). In our study no abnormal laboratory finding were determined in any case, even in the cats with liver herniation.

The diaphragmatic rupture should be closed with a simple surgical suture. For this purpose, the absorbable or non-absorbable suture material should be used, and the needle tip has to be round. Autogenous and synthetic grafts may be needed in some cases where the defect cannot be repaired completely. By providing inspiration, the lungs are brought in fully expanded form to provide negative pressure in the thoracic cavity before final suture (Fossum 2002). In our study, non-absorbable suture material was used to repair the defect. Negative pressure before last suture was provided. No grafting material was needed to close the diaphragmatic defects. Only in one case, a synthetic mesh was used for the closure of the abdomen, because of enlargement of herniated organs.

Depending on the size and location of the diaphragmatic defects and the mobility of the abdominal organs, the herniated organ varies, but usually is liver. In addition, the small intestine, stomach, spleen, omentum, pancreas, colon, uterus can also herniate. According to some authors, the diaphragmatic rupture is mostly seen on the right side, and the liver, the small intestine, the pancreas is usually herniated from this defect. The stomach, spleen and small intestines is largely herniated from the left side defects (Schmiedt et al. 2003, Hyun 2004, Gibson et al. 2005, Bainess 2016). In our study, the diaphragmatic defects were on dorsal region in one case, ventral region in other cases. The ventral defects were localized on right side in 6 cases, central in 4 cases, left side in 3 case, right and left in 1 case. Among the herniated organs, the most common was liver and small intestine, but stomach and spleen were also encountered.

Surgery of the diaphragmatic hernia has a greater anesthesia risk than other abdominal operations. Dorsal recumbency on the operation table causes increased pressure in the lungs and displacement of the heart resulting exacerbated cardiovascular and respiratory disorders. Longer operation and longer anesthesia duration and higher soft tissue damage and need of oxygenation rises mortality rate in cats and dogs (Legallet et al. 2017). In addition, localization of hernia, amount of herniated organs, elapsed time after trauma to the operation also affect perioperative mortality (Besalti et al. 2011, Legallet et al. 2017). Therefore, the tine between anesthesia induction and herniorrhaphy should be as short as possible and oxygenation should be provided before and after anesthesia (Besalti et al. 2011, Yool 2014). In our study anesthesia duration before the surgical intervention was kept as short as possible. Oxygen support was supported before and after the operation and the cats.

Oxygen therapy is a well-known indispensable procedure for respiratory distress patient. But also to keep the animal calm and prevent getting stress has high important choosing the way of providing oxygen (Sharp et al. 2013). Therefore, the most recommended method is the oxygen cage (Mazzaferro et al. 2013, Mackay 2001). All cats included our study got oxygen support by using intensive care cabin a day before and 3 days after surgery. Also usage of the intensive care cabin provided keeping animal warm and preventing hypothermia.

The fluid therapy is another important step to improve tissue perfusion and oxygen delivery associated with bradycardia, hypotension and hypovolemia in the cats with diaphragmatic hernia (Nelson 2015). During the operation, Ringer's lactate solution of 10 ml/kg/h was administered intravenously to the all cats.

Aerosol therapy is known a functional method of drug delivery to direct therapy at the site of the problem (Miller et al. 2003). Aerosol agents include physiologic saline, some antibiotics, glucocorticoids and bronchodilators (Kirschvink et al. 2006). Usage of the aerosol glucocorticoids limits systemic complications and provides immediate relief of bronchoconstriction. Prednisone or prednisolone treatment in the cats works well as an anti-inflammatory agent but concurrent use of oral bronchodilators generally is usually recommended for bronchoconstriction (Rozanski et al. 2007). In our study ventolin and pulmicort were administered twice a day with mask by aid of nebulizer concurrently systemic methylprednisolone therapy. Thus, all cats who survived surgery (73.3%) lived no complication and healed completely in a short time.

As a result, it was concluded that the management procedures include fluid therapy, oxygen and, ventolin and pulmicort therapy concurrent with systemic methylprednisolone, and doing all of these in intensive care cabin to keep the animal away from stress contributes the survival rates of the cats.

REFERENCES

Baines SJ. Diaphragmatic herniorrhaphy. General Abdominal Surgery. Section 6, Chapter 52. p.375-376, 2016

Besalti O, Pekcan Z, Calıskan M, Aykut ZG. A retrospective study on traumatic diaphragmatic hernias in cats. Ankara Univ Vet Fak Derg. 2011; 58: 175-179.

Fossum TW. Traumatic Diaphragmatic Hernia. In: TW Fossum (Ed), Small Animal Surgery, 2nd ed. Mosby: Missouri p.795-798, 2002.

Fossum TW. Surgery of the lower respiratory system: pleural cavity and diaphragm. In: Fossum TW (Ed.): Small Animal Surgery. 3rd edn. Mosby Elsevier, St. Louis p.896-929, 2007.

Gibson TWG, Brisson BA, Sears W. Perioperative survival rates after surgery for diaphragmatic hernia in dogs and cats: 92 cases (1990-2002). J Am Vet Med Assoc. 2005; 227: 105-109.

Hyun C. Radiographic diagnosis of diaphragmatic hernia: Review of 60 cases in dogs and cats. J Vet Sci. 2004; 5: 157-162.

Kibar M, Bumin A, Kaya M, Alkan Z. Use of peritoneograhy (positive contrast cheliography) and ultrasonography in the diagnosis ofdiaphragmatic hernia: review of 35 cats. Rev Med Vet. 2006; 157(6): 331-335.

Kirschvink N, Leemans J, Delvaux F, et al. Inhaled fluticasone reduces bronchial responsivenessand airway inflammation in cats with mild chronic bronchitis. J Feline Med Surg. 2006; 8: 45–54.

Legallet C, Mankin KT, Selmic LE. Prognostic indicators for perioperative survival after diaphramatic herniorrhaphy in cats and dogs: 96 cases (2001-2013). BMC Vet Res. 2017; 1316: 1-7.

Mazzaferro E, Powell LL. Fluid therapy for the emergent small animal patient: crystalloids, colloids, and albumin products. Vet clin North Am. Small Anim Pract. 2013; 43(4): 721-734.

Mackay A. Practical approach to the dyspnoeic cat. In practice. 2001; 23(4): 198-207.

Miller CJM, McKiernan BC, Hauser C, Fettman MJ. Gentamicin aerosolization for the **treatment of infectious tracheobronchitis. J Vet Intern Med. 2003; 17: 386.**

Minihan AC, Berg J, Evans KL. Chronic diaphragmatic hernia in 34 dogs and 16 cats. J Am Anim Hosp Assoc.2004; 40: 51-63.

Nelson D. Patient care report for the stabilisation of a cat with a traumatic diaphragmatic rupture. The Veterinary Nurse.2015; 6(8): 498-503.

Orton EC. Diaphragm. Small Animal Thoracic Surgery John Wiley Sons p.139-146.

Ozer K, Güzel Ö, Devecioğlu Y, Aksoy Ö. Diaphragmatic hernia in cats: 44 cases. Med Weter. 2007; 63: 1564-1567.

Randall EK. Canine and Feline Diaphragm. In: Textbook of Veterinary Diagnostic Radiology. Ed: Thrall DE. 7th Ed. 33-648, 2018.

Rozanski EA, Bach JF, Shaw SP. Advances in respiratory therapy. Vet Clin North Am: Small Anim Pract. 2007; 37(5): 963-974

Schmiedt C, Tobias K, Stevenson M. Traumatic diaphragmatic hernia in cats: 34 cases (1991- 2001). JAVMA. 2003; 222 (9): 1237-1240.

Sullivan M, Lee R. Radiological features of 80 cases of diaphragmatic rupture. J Small Anim Pract.1989; 30: 561-566.

Sharp CR, Rozanski EA. Physical examination of the respiratory system. Top Companion Anim Med. 2013; 28(3): 79-85.

Tillson DM. Diaphragmatic hernia. Small Animal Surgery Secrets. Chapter 43. 2nd Ed. Ed: Joseph Harari. Hanley & Belfus Inc, Spokane, Washington. 141-145, 2014.

Voges AK, Bertrand S, Hill RC, Neuwirth L, Schaer M. (1997) True diaphragmatic hernia in a cat. Vet Radiol Ultrasound. 1997; 2: 116-119.

Yool DA. Diaphragm. Feline Soft Tissue and General Surgery. Ed: Langley-Hobbs SJ, Demetriou J, Ladlow J. Saunders, pp.521-530, 2014.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):213-220
DOI: 10.30607/kvj.512713

CASE REPORT

Reconstruction of Bilateral Upper Eyelid Coloboma in a Domestic Shorthaired Cat Using Roberts and Bistner Technique

Aynur DEMİR*, Gülşen SEVİM KARAGÖZOĞLU

Istanbul-Cerrahpaşa University, Faculty of Veterinary Medicine, Department of Surgery, Istanbul, Turkey

ABSTRACT

A 1-year-old, male domestic short haired cat was presented to our Faculty of Veterinary Medicine, University of Istanbul-Cerrahpaşa due to complaints of blepharospasm and epiphora in both eyes present since birth. In the ocular examination, absence of a part of palpebra in both eyes and persistent pupillary membrane (PPM) in the left eye were observed. There was trichiasis irritating the corneas in both eyes. Bilateral palpebral defects and secondary trichiasis were repaired using a Roberts and Bistner technique. This technique provided a good cosmetic appearance and functional outcome to the patient. Roberts and Bistner technique is recommended in treating congenital, bilateral eyelid coloboma.

Keywords: Cat, eyelid margin, persistent pupillary membrane, trichiasis, upper eyelid coloboma.

***

Bir Kedide Bilateral Üst Göz Kapağı Kollobomunun Roberts ve Bistner Tekniği ile Rekonstrüksiyonu

ÖZ

Bir yaşında erkek sarman kedi, İstanbul-Cerrahpaşa Üniversitesi, Veteriner Fakültesi'ne, iki gözünde doğduğundan beri devam eden blefarospazm ve epifora şikayeti ile getirildi. Oftalmolojik muayenede her ikisinde mevcut olan üst göz kapağı agenezisi ve sol gözünde persistent pupillar membran olgusuna rastlandı. Bunlara ek olarak korneaları irrite eden trişiyazis de tespit edildi. Olguda teşhis edilen bilateral göz kapağı agenezisi ve buna bağlı oluşan sekonder trichiasis, Roberts ve Bistner tekniği kullanılarak düzeltildi. Bu teknik hastaya iyi bir kozmetik görünüm ve işlevsel sonuç sağladı. Konjenital, bilateral göz kapağı kolobomunun tedavisinde Roberts ve Bistner tekniği önerilmektedir.

Anahtar Kelimeler: Göz kapağı kenarı, kedi, persistent pupillar membran, trisiyazis, üst göz kapağı kolobomu.

To cite this article: Demir A. Sevim Karagözoğlu G. Reconstruction of Bilateral Upper Eyelid Coloboma in a Domestic Shorthaired Cat Using Roberts and Bistner Technique. Kocatepe Vet J. (2019) 12(2):213-220.

Submission: 14.01.2019 **Accepted**: 12.03.2019 **Published Online**: 10.04.2019
ORCID ID; AD: 0000-0002-7856-2789, GSK: 0000-0002-3945-4101
*Corresponding author e-mail: Aynurdemir_1903@hotmail.com

INTRODUCTION

Eyelid agenesis, or commonly known as coloboma, is a congenital developmental anomaly characterized by lacking all or part of the eyelid (Ocelli and Neaderland 2011, Esson 2014). This problem has been reported in cats, dogs, horses, sheeps, Snow leopards, Texas cougar and humans (Cheng at al. 2006, Etemadi at al. 2013). It is a rare disease in small animals but is more common in cats than in dogs (Ocelli and Neaderland 2011, Etemadi at al. 2013, Reed at al. 2018).

This lesion, which affects the lateral portion of upper eyelid in cats, is mostly bilateral (Cheng at al.2006, Ocelli and Neaderland 2011). The aetiology has not been known exactly, but several causes containing a recessive genetic disorder and teratogenic effects have been reported (Whittaker at al.2010, Etemadi at al. 2013,Esson 2014). The clinical symptoms of the disease vary depending on the width and localization of the defect. The contact of the skin hairs to the cornea due to the absence of palpebral margin, causes ocular irritation and excessive tear production(Reed at al. 2018). Due to insufficient closure of the palpebral fissure, secondary exposure keratitis and keratoconjunctivitis sicca may occur(Whittaker at al.2010, Ocelli and Neaderland 2011, Etemadi at al. 2013). Medical treatment may be used in small eyelid lesions that do not cause severe ocular lesions, but this treatment may be inadequate because this lesion is usually in the form of large defects (Cheng at al.2006). Many surgical techniques have been reported, ranging from simple closure of smaller lesions to complex reconstruction procedures that was used for repairing large lesions of the eyelids (Cheng at al.2006,Whittaker at al.2010, Reed at al. 2018).
In this report, the treatment of bilateral, congenital eyelid coloboma in a cat using Roberts and Bistner surgical technique was evaluated.

CASE REPORT

A 1-year old male, domestic shorthair cat, was presented to Faculty of Veterinary Medicine, University of İstanbul-Cerrahpaşa with a history of ocular discomfort, blepharospasm and epiphora since birth. Ophthalmic examination revealed bilateral absence of part of upper palpebra and margins, skin hairs irritating the corneas, causing epiphora and blepharospasm (Figure 1-2). The palpebral fissures were not completely closed during the blink. Since the ocular surface was partially exposed, secondary keratitis and corneal vascularization developed in both eyes. Also there was a PPM that adhered to the cornea in the left eye (Figure 3). No other ocular and systemic anomaly was found in the examination. Because of the corneal surface lesions and serious ocular discomfort in the patient, it was decided to correct the defects with surgical reconstruction method in both eyes. General anaesthesia was maintained with xylazine (1mg/kg, IV, Basilazine, Bavet, Turkey) and ketamine (5mg/kg IV, Ketalar®, Pfizer, Turkey) followed by 2% isoflurone (Forane®, Abbott, Turkey) in oxygen. Analgesia was provided by meloxicam (0.1-0.2mg/kg, SC, Melox, Nobel, Turkey) 2 hours before surgery. Cephalosporin (25 mg/kg IV, Iesef 500mg, Ibrahim Ethem Ulugay, Turkey) was given 30 min prior to surgery. Defects were repaired with rotational pedicle eyelid flaps that were formed from the lateral region of the lower eyelid.

SURGERY

Skin that containing trichiasis was dissected from the adjacent palpebral conjunctiva and then excised with tenotomy scissors. Thus, first eyelid recipient beds were formed (Figure 4). Rotational pedicle flaps that were formed from the lateral region of lower eyelids were used to fill the defect cavities. That flaps were created with 3 step incisions. The first incision of the flap was dorsal incision at a distance of 1-2 mm from the edge of the lower eyelid. The ventral incision was large enough to close the defect of the upper eyelid and was parallel to the dorsal incision. The incisions of the flap were made slightly more than the length of the defect. The incisions parallel to each other were combined with a vertical incision that was made from the region close to the medial canthus of the eyelid (Figure 5). The width of the flap incision was made wide enough to cover the defect to prevent secondary lower eyelid ectropion (Figure 6). The lower eyelid flap that containing skin, muscle and tarsus, was rotated to cover the upper eyelid defect and ıt was sutured to the recipient bed atraumatically with absorbable 8/0 PGA, simple sutures (Figure 7-8). The dissociated conjunctiva in the temporal region was fixed to the skin of the flap by simple continuous sutures to support the feeding of the flap and to prevent secondary necrosis.

Postoperatively, an Elizabethan collar was worn to prevent self trauma. Topical 0.3% ofloxacin (Exocin, Abdi Ibrahım, Turkey) 4 times a day and systemic amoxicillin and clavulanic acid (Synulox 50mg, Pfizer, Turkey) 2 times a day 12.5 mg/kg PO antibiotics against secondary infection;for inflammation topical % 0.15 dıclofenac sodıum (Inflased®, Bilim, Turkey) 2 times a day and systemic meloxicam (Metacam®, Boehringer Ingelheim, Australia) 0.05mg/kg PO were used.

24 hours after the operation, minimal swelling occurred in the eyelids. Warm compress was

applied 2-3 times daily for 1 week to accelerate the blood circulation of the eyelids. Although the eyelids and flaps were slightly oedematous, no necrosis occurred at the flaps tip or trunk (Figure 9). The swelling of the cat's eyelid disappeared completely, the ocular discomfort has decreased and sutures were taken within 2 weeks. There were no complications such as flap loss, infection or ectropion. There was no problem with flap feeding and corneal protection in the patient was fully provided (Figure 10). The cat was followed for 4 months after the operation, but there was no recurrence similar to the first in the affected eyes (Figure 11).

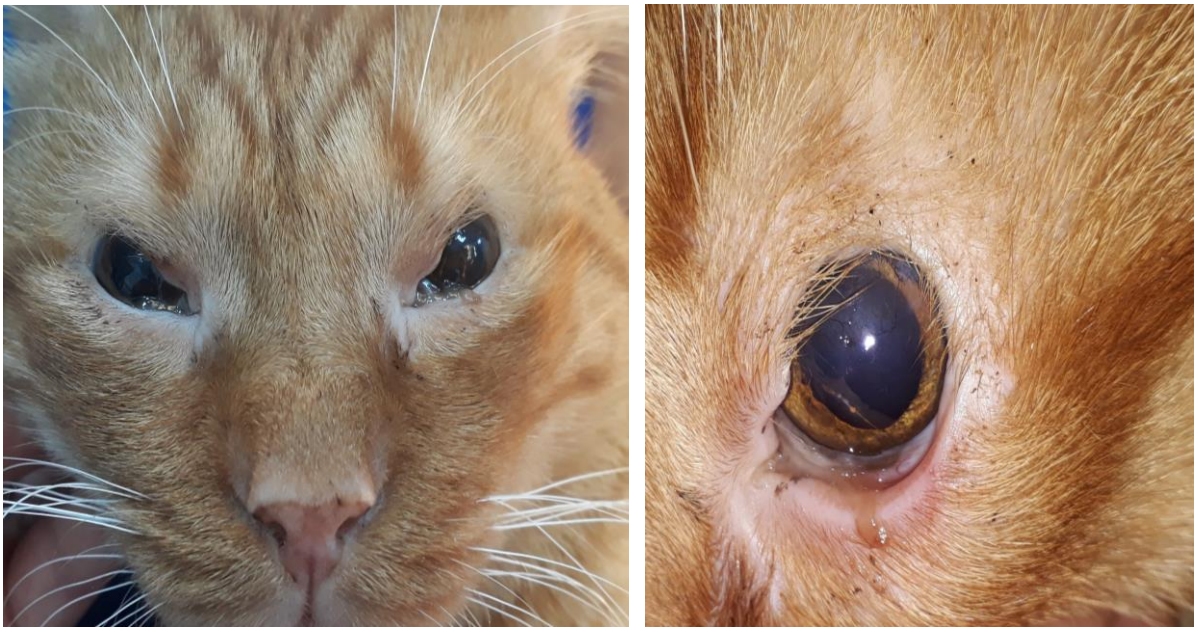


Figure 1-2. Bilateral absence of temporal upper eyelid in a cat and presence of severe trichiasis that irritated the cornea.

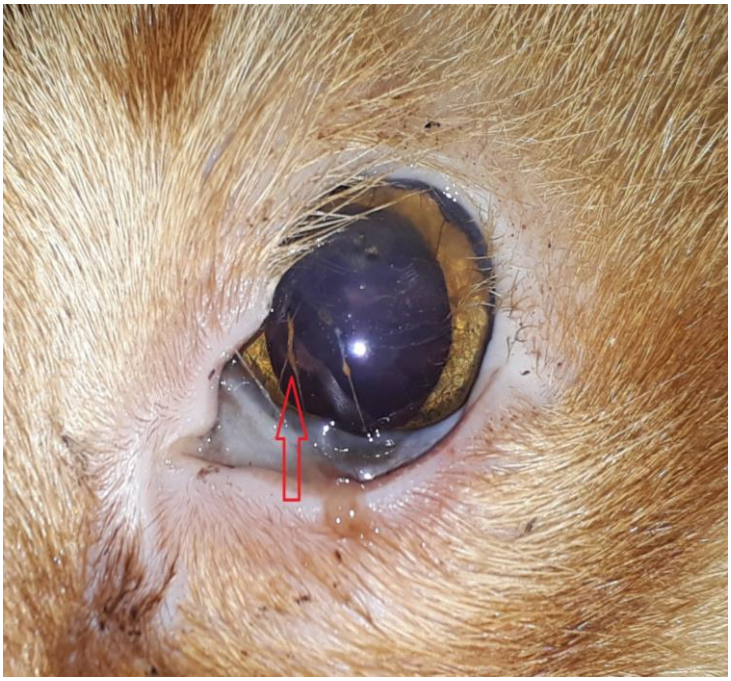


Figure 3. The cat also had PPM that adherent to cornea in the left eye.

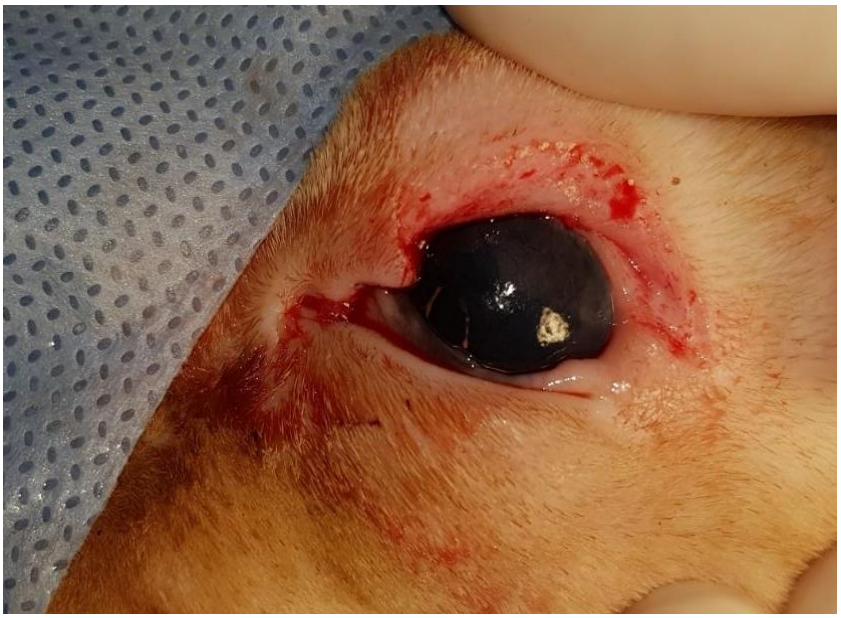


Figure 4. First, the upper eyelid recipient bed was formed by excising the colobomatous area and hairs that irritated the cornea.

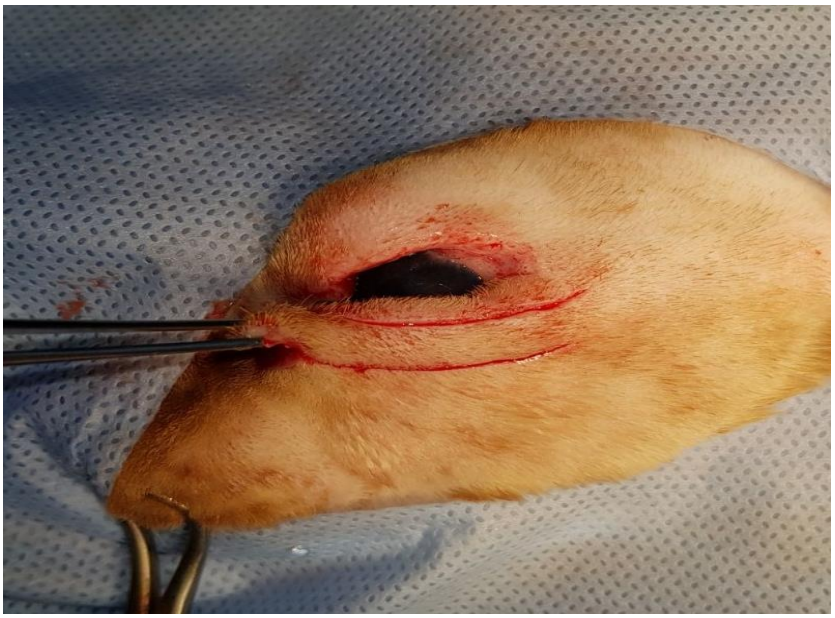


Figure 5. Surgical incisions of flap.

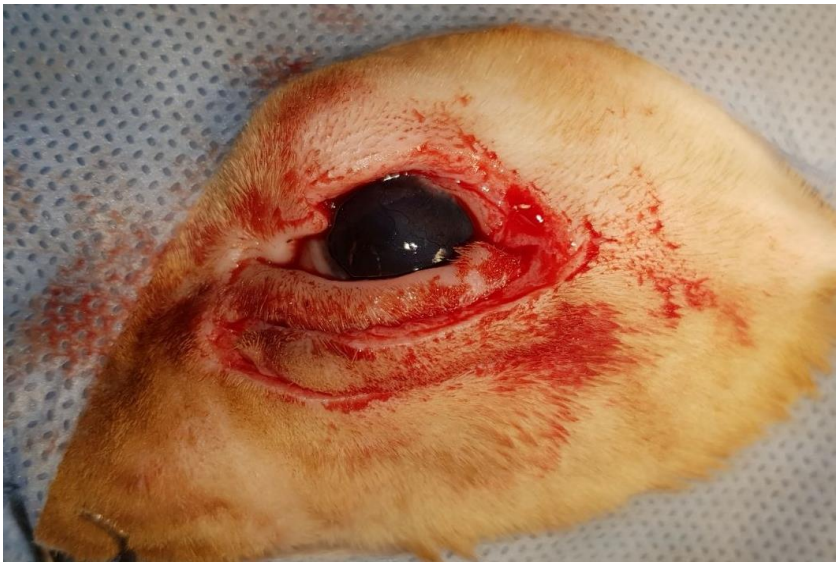


Figure 6. The flap is wide enough to close the defect.

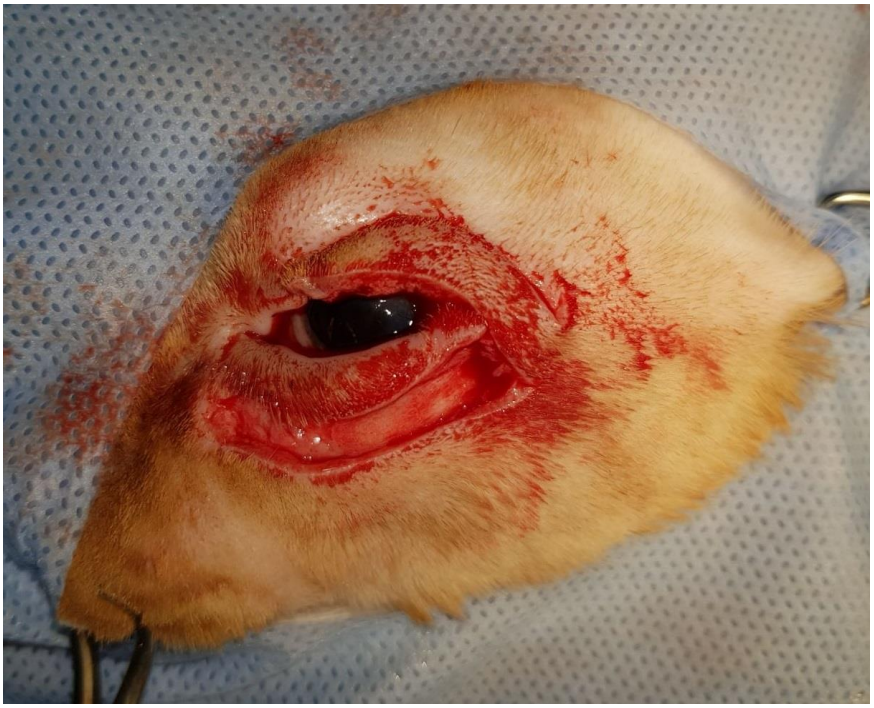


Figure 7. The lower eyelid flap is moved to the upper eyelid recipient bed.

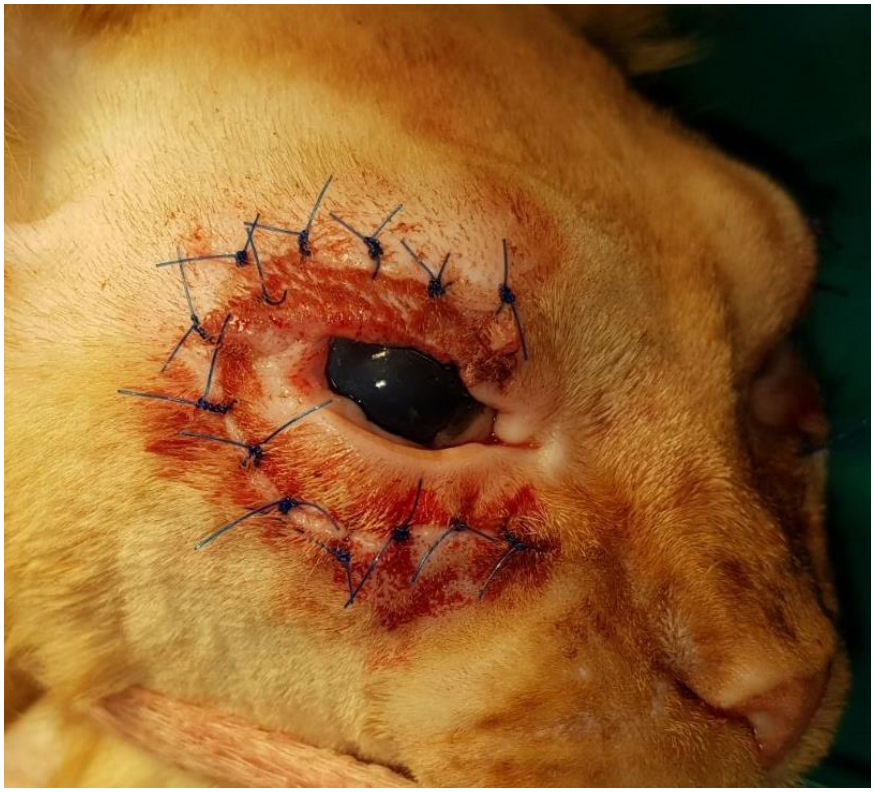


Figure 8. The lower eyelid flap is sutured to the upper eyelid recipient bed with simple sutures using 3/0 nylon.

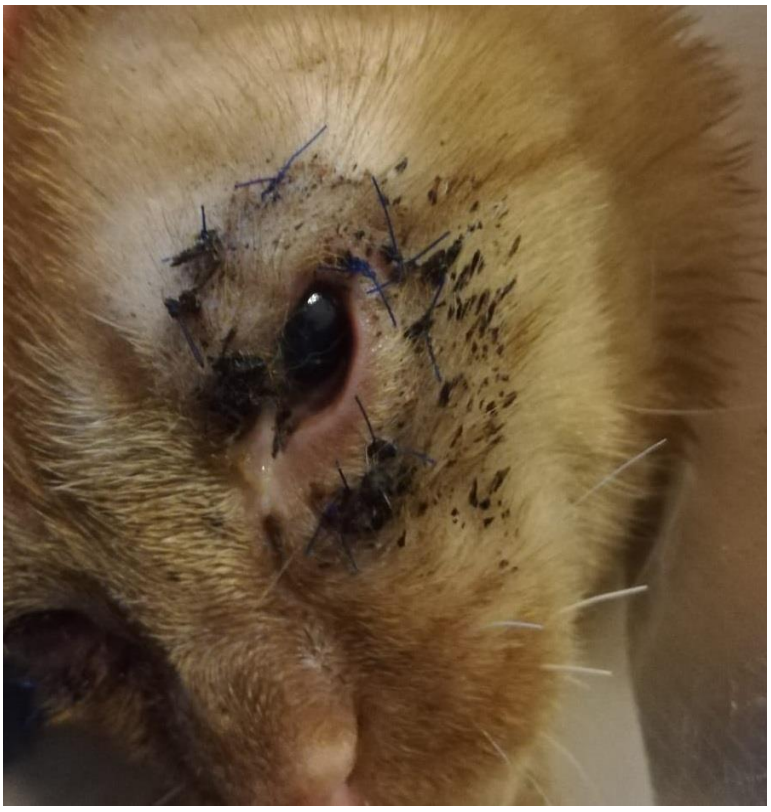


Figure 9. The appearance of the eyelid 1 week after surgery. No necrosis was seen, but milds swelling on the donor flap

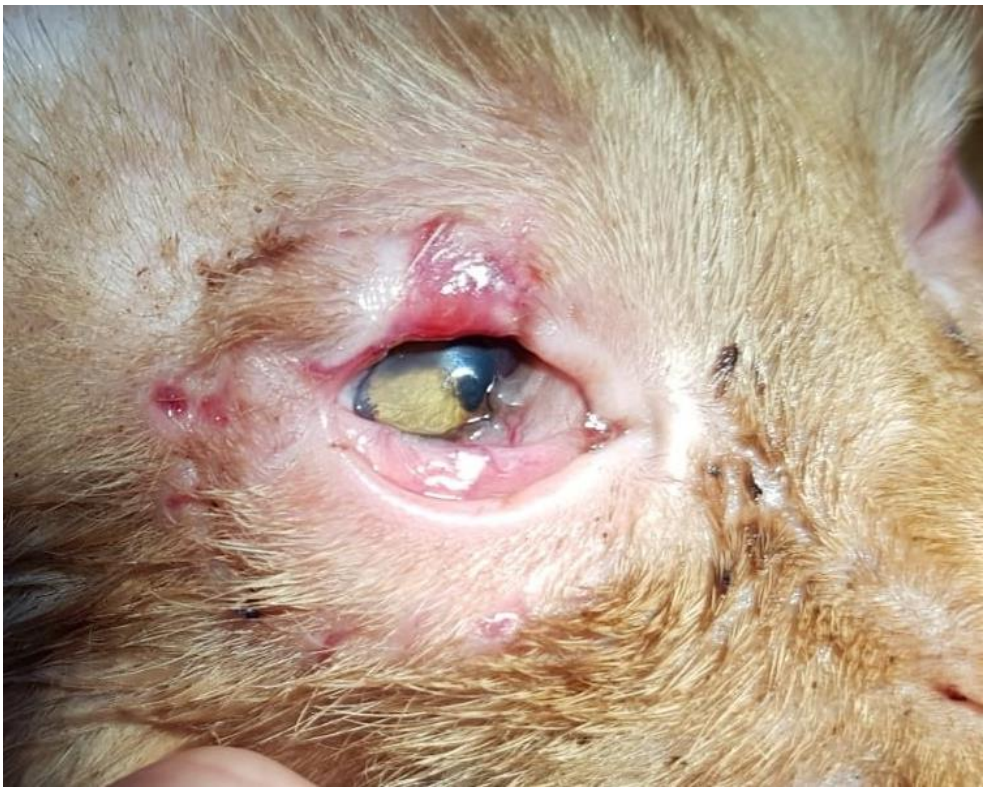


Figure 10. The appearance of the cat 2 weeks after surgery. Eyelid swelling disappeared, ocular discomfort was reduced but still had conjunctival hyperaemia.

Figure 11. 3rd control of the cat 4 months after surgery. The patient had a perfect cosmetic appearance and a functional upper eyelid.

DISCUSSION

Congenital eye anomalies are less common than other organ anomalies. The eyelid coloboma is one of the important anomalies of the eye characterized by a partial or complete absence of the palpebra. The most common site of colobomas is the upper eyelid, especially the lateral part in cats(Reed at al. 2018). In this case, coloboma of the lateral 2/3 of the upper eyelid was diagnosed in both eyes.

It has been reported that occurs more frequently in Domestic Shorthair, Persian, Birman cats, although there is no breed predisposition (Cheng at al.2006, Etemadi et al.2013, Gelatt 2014). Eyelid coloboma may be a single lesion or associated with other ocular disorders such as microphthalmia, dermoids, PPM, lacrimal gland aplasia, retinal dysplasia, cataracts and anophthalmos (Cheng at al.2006, Etemadi et al.2013). In the present case, PPM was present in addition to the eyelid lesion in the left eye.

If treatment is not started in the early period except for cosmetic disorders, complications occur due to prolonged exposure of the ocular surface to external factors. It is very difficult to get a good result with medical treatment in ocular lesions of eyelid defects,so surgical treatment is often indicated. The method of surgical repair is determined by the size of the defect. If the size of defect is smaller than 1/3 of the width of the eyelid, primary closure is the ideal treatment after wedge-shaped excision. Larger defects require reconstructive techniques that are more complex as the size and content of the defect increases (Whittaker at al.2010, Reed at al. 2018).

Reconstruction is indicated for all defects that may cause secondary complications when not repaired. These complications include exposure keratitis, keratoconjunktivitis sicca, epiphora and lagophthalmus (Gelatt 2014). The main purpose of the reconstruction is to provide eyelid function, protect the eyeball and obtain a cosmetic appearance (Esson 2001, Gelatt 2014). The reconstruction of eyelid components including skin, muscle, tars and conjunctiva is important for the function of the upper eyelid (Etemadi et al. 2013, Trbolova 2014). In cases where primary suture is insufficient, various eyelid reconstruction techniques; Cutler-Beard, Mustarde, Bucket handle, sliding skin such as semi-circular and z-plasty skin flap have been described (Esson 2001, Reed 2018). The bilateral palpebral defect of this case was repaired using Robert and Bistner technique. This technique is used for repairing of full-thickness defects of the upper eyelid. In this technique, a rotational flap that was rotated from the lower eyelid, is a preferred surgical treatment intervention. The advantages of this flap include reconstructing the eyelid defect with similar tissue (Cheng at al.2006, Trbolova 2014, Reed at al.2018). Since there is no marginal area like normal eyelid edge in the rotational flap, hairs on the palpebral skin may usually turn towards the cornea and cause discomfort in the eye after surgery (Cheng at al.2006). 4 weeks after the operation, no such

complication was encountered in our case.In addition, this procedure may cause complications such as ectropion of the lower eyelid after the operation (Reed at al. 2018). Ectropion was common in this disease compared to other complications, which did not develop in this patient.

In conclusion, a large number of upper eyelid reconstruction options are available. When evaluating them and choosing the appropriate one, it is necessary to select the method that gives the best result and causes the least damage.

The rotation flap of the lower eyelid can be said to be a technique that provides satisfaction results in both cosmetic and functional aspects in the treatment of rare eyelid coloboma.

REFERENCES

Cheng, S. H., Yeh, L. S., Lin, C. T. Case report: Eyelid coloboma in a domestic short haired cat. 2006; 32(4): 265-270

Esson, D. A. modification of the Mustardé technique for the surgical repair of a large feline eyelid coloboma. Veterinary ophthalmology. 2001; (2): 159-160

Etemadi, F., Fard, R. M. N., Tamimi, N., Rezaei, M., Vahedi, S. M., Golshahi, H., Aldavood, S. J. Coincidence of upper eyelid coloboma with posterior synechia in a cat: A case report.Journal of Veterinary Science & Technology. 2013; 4(4): 1-2.

Gelatt, K. N. Congenital and developmental eyelid disorders. In: KN, Gelatt, editor. Essentials of veterinary ophthalmology.3th ed. Wiley-Blackwell, 2014; USA p.

Occelli, L., Neaderland, M. Reconstruction of an iatrogenic eyelid defect in a Bichon Frisé. Pratique Médicale et Chirurgicale de l'Animal de Compagnie, 2011; 46(3-4): 99-105.

Reed, Z., Doering, C. J., Barrett, P. M. Use of 2-octyl cyanoacrylate for wound closure in a modified Roberts-Bistner procedure for eyelid agenesis in five cats (nine eyes). Journal of the American Veterinary Medical Association. 2018; 252(2): 215-221.

Trbolova, A. Eyelid agenesis in a cat, clinical case. Pol J Vet Ophthalmology, 2014; 4: 1-6

Whittaker, C. J. G., Wilkie, D. A., Simpson, D. J., Deykin, A., Smith, J. S., Robinson, C. L. Lip commissure to eyelid transposition for repair of feline eyelid agenesis. Veterinary ophthalmology, 2010; 13(3): 173-178

Wolfer, J. C. Correction of eyelid coloboma in four cats using subdermal collagen and a modified Stades technique.Veterinary ophthalmology, 2002; 5(4): 269-272.

Kocatepe Veterinary Journal

Kocatepe Vet J (2019) 12(2):221-225
DOI: 10.30607/kvj.442900

CASE REPORT

True Hermaphroditism in a Dog: A Case Report

Ebubekir YAZICI[1], Mehmet Fatih BOZKURT[2], Oktay YILMAZ[1*]

[1]Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03300 Afyonkarahisar, Turkey
[2]Department of Pathology, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03300 Afyonkarahisar, Turkey

ABSTRACT

True hermaphroditism in a dog is described in this case report. An eight month old cross-breed dog with enlarged structure protruding from the vulva was brought for an ovariohysterectomie operation. Physical examinations exposed the presence of an os clitoris and urethral orifice at the base of the os clitoris. Exploratory laparotomy was performed as a diagnostic and therapeutic procedure. Both gonadal tissues and the genital tract were removed during surgery and brought to the laboratory for pathological examinations. Microscopic examinations showed that the right gonad had both seminiferous tubules like testis and primer follicles like ovarium. The concentrations of serum progesterone, testosterone and 17β-estradiol were 0,188 ng/ml, 1,008 ng/ml and 23,61 pg/ml respectively. We concluded that this is a true hermaphrodismus case due to the simultaneous presence of two sex gonads.

Keywords: Dog, Intersexuality, True hermaphroditism, Ovotestis, Hormone concentrations

***

Bir Köpekte Gerçek Hermafrodismus: Olgu Sunumu

ÖZ

Bu çalışmada, gerçek hermafrodit bir köpek anlatılmıştır. Vulvasından dışarıya doğru çıkan büyük bir kitle olan 8 aylık melez bir köpek kısırlaştırma operasyonu için araştırma merkezimize getirildi. Fiziksel muayenelerde os klitorisin varlığı ve bunun bazalinde uretral deliğin olduğu saptandı. Teşhis ve tedavi amacıyla hayvana ovariohysterectomie operasyonu uygulandı. Her iki gonadal doku ve uterus patolojik incelemeler için laboratuvara gönderildi. Mikroskobik incelemeler, sağ gonadın hem testis gibi seminifer tubuller hem de ovaryum gibi primer foliküller içerdiğini gösterdi. Serum progesteron, 17β-östradiol ve testosteron düzeyleri sırasıyla 0,188 ng/ml, 23,61 pg/ml, 1,008 ng/ml olarak ölçüldü. Köpekte her iki gonadal dokunun aynı anda bulunması nedeniyle olgunun bir gerçek hermafrodismus olgusu olduğu kanısına varıldı.

Anahtar Kelimeler: Köpek, İnterseksüalite, Gerçek hermafroditizm, Ovotestis, Hormon seviyeleri

To cite this article: Yazıcı E. Bozkurt M.F. Yılmaz O. True Hermaphroditism in a Dog: A Case Report. Kocatepe Vet J. (2019) 12(2):221-225.

Submission: 12.07.2018 **Accepted**: 09.03.2019 **Published Online**: 16.05.2019
ORCID ID; EY: 0000-0002-1219-4370, MFB: 0000-0002-1669-0988, OY: 0000-0002-9722-5155
*Corresponding author e-mail: oktayyilmaz@aku.edu.tr

INTRODUCTION

The chromosomal sex of zygote resulting in XY or XX is determined after fertilization. During the normal sex development of males, the SRY gene in the Y chromosome causes differentiation of primordial follicles in the testes at the sexual differentiation stage of the embryo. Secretion of testosterone and Mullerian Inhibiting Substance (MIS) results in the development of male genital organs. The absence of the Y chromosome and the SRY gene leads to the development of ovaries and female genitalia (Hare 1976, Poth at al. 2010). Any defect at this progression stage may cause the development of sexual disorders such as hermaphrodismus. An animal that has genitalia with a part or all of the genital organs of both sexes is called a 'Hermaphrodite' (Alam et al. 2007). Male pseudohermaphrodites have testicular gonadal tissue with female genital organs, while female pseudohermaphrodites have ovarian gonadal tissue with male genital organs. As for true hermaphrodites, they have the gonadal tissues of both sexes (Hare 1976). There are various combinations involving the presence of both ovarian and testicular tissues in true hermaphrodites. A testis and ovary may be found in contralateral sides, ovotestis may be found in both sides or the ovotestis may be paired with one ovary or testis (Alam at al. 2007). Poth et al. (2010) classified the reproductive tract abnormalities into three categories as sex chromosomes, gonadal sex development and phenotypic sex development disorders. Developmental disorders like these are caused by genetic or chromosomal abnormalities and iatrogenic hormonal or chemical exposure (Pasello-Legrand and Mowat 2004). Hermaphroditism is generally reported to be rare in dogs and it is often associated with infertility (Hare 1976, Kim and Kim 2006). In this paper, we report a case of true hermaphroditism in a dog.

CASE HISTORY

A cross bred dog aged 8 months was brought to the Veterinary Health, Practice and Research Centre of Afyon Kocatepe University for an ovariohysterectomie. The owner noted that the dog was classified as a female at birth and no abnormalities were reported except the absence of estrous behavior and an enlarged clitoris. The initial examination of the external genital organs revealed that an enlarged clitoris protruded from the vulva (Fig. 1). It was approximately 4 cm in length and 0,5 cm wide. Palpation of the enlarged tissue indicated the presence of an urethral orifice at the base of the os clitoris. During transabdominal ultrasonography, neither follicles nor pathological structures were observed on the ovaries. Blood samples were collected for sex hormone analyses and it was decided to perform exploratory laparotomy for diagnosis and treatment.

The dog was administered 0,04 mg/kg atropine sulphate (Atropine sulphate inj®, Vetaş, Turkey) subcutaneously 30 min before the surgery. Intra catch i.v. cannula (18 G) was placed into the v. cephalica antebrachii for applying the anesthetics and intravenous solutions. The animal was premedicated with 0,3 mg/kg midazolam (Dormicum, Roche®, Turkey) given intravenously. Induction of anesthesia was performed with 6 mg/kg propofol (Propofol®, Abbott, Turkey) via IV bolus. Following endotracheal intubation, general anesthesia was maintained with 2% isoflurane (Forane®, Abbott, Turkey). The surgical incision was made on the abdominal midline about 2-3 cm in length. The reproductive internal organs were similar to those of a female dog in the inspection of the abdomen. After two ligations of the ovarian pedicles and the uterine body, both gonadal tissues and the genital tract were removed and brought to the laboratory for pathological examinations. The surgery was concluded in a routine manner by closing the abdominal wall. Additionally the enlarged clitoris protruding from the vulva was removed (clitorisectomy). No complication occurred after the operation.

Uterine horns were 17 cm in length, left and right gonads were 1 cm and 2,5 cm in diameter respectively. Gonadal tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin wax, sectioned at 5-6 μm and stained with hematoxylin and eosin. In the microscopic examination, there were numerous structures such as degenerative seminiferous tubules containing 2-3 layer cells on basal and primer follicles close to the cortex in the right gonad (Fig. 2) and numerous wide vessels filled with erythrocytes in the left gonad (Fig. 3). Microscopy of the uterine mucosa revealed wide hemorrhages between glands in the lamina propria (Fig. 4). Blood samples were transported to the Faculty of Medicine of Afyon Kocatepe University for measuring serum progesterone, testosterone and 17β-estradiol concentrations. Hormone levels were determined by electrochemiluminescence immunoassay (ECLIA, cobas e, Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. The concentrations of serum progesterone, testosterone and 17β-estradiol were 0,188 ng/ml, 1,008 ng/ml and 23,61 pg/ml respectively.

Fig. 1. Enlarged structure protruding from the vulva.

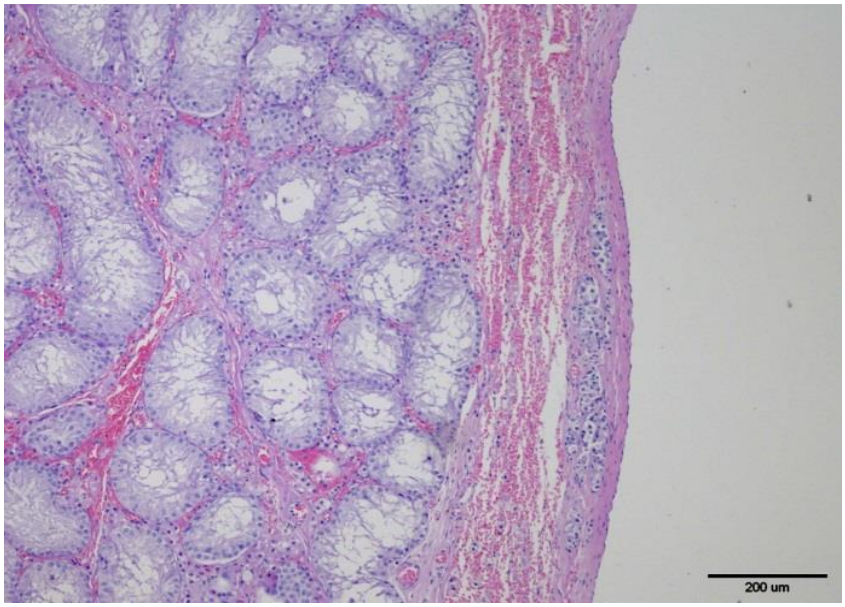


Fig. 2. Right gonad with degenerative seminiferous tubules and primer follicles.

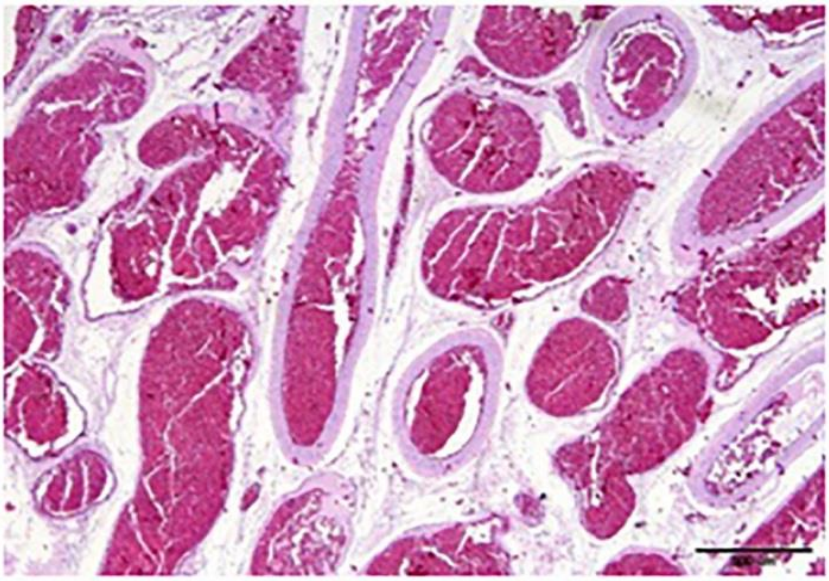


Fig. 3. Left gonad containing wide vessels filled with erythrocytes.

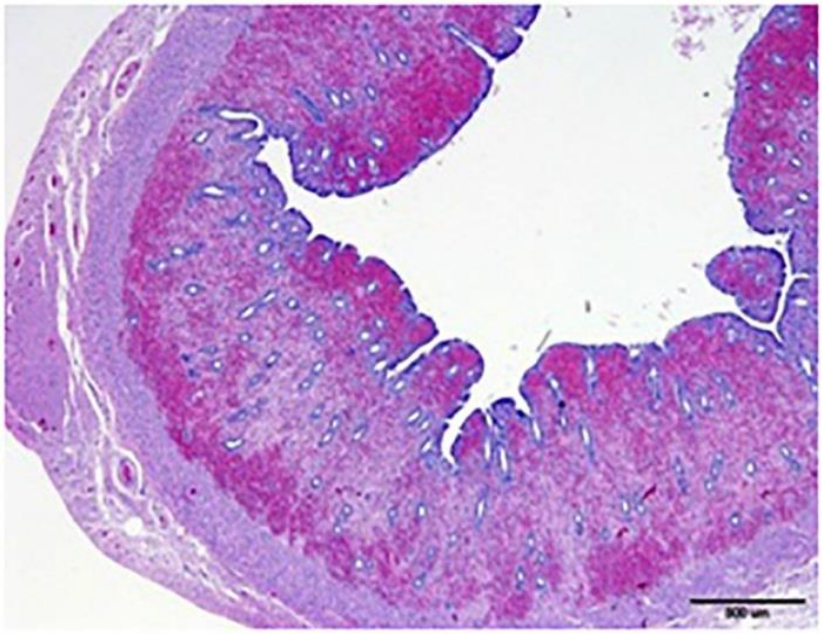


Fig. 4. Uterus

DISCUSSION

The intersexuality (true hermaphrodismus or pseudohermaphrodismus) in mammals has been described in numerous species including humans, cattle, horses, dogs and monkeys (Atakam 1954, Dunn et al. 1968, Meyers-Wallen et al. 1997, Del Amo et al. 2001, Pasello-Legrand and Mowat 2004). A defect at any step of sexual development, the chromosomal, gonadal or phenotypic level of differentiation can lead to sexual abnormalities (Kim and Kim 2006). Incorrect timing of releasing the Mullerian Inhibiting Factor and inadequate secretion of testosterone by leydig cells can cause the presence of Mullerian and Wolffian structures together in true hermaphrodites (Vani 2008). In this case, a rare condition, true hermaphrodismus, has been reported with the findings of macroscopic, histological and the hormonal profile of a dog.

Serum levels of testosterone, estradiol and progesterone were reported as 0,01-41,5 ng/ml, 33,6-66,6 pg/ml and 0,02-0,5 ng/ml in healthy male dogs and 0,01-0,3 ng/ml, 31,5-69,0 pg/ml and 0,01-0,65 ng/ml in healthy female dogs, respectively (Concannon and Castracane 1985, Frank et al. 2003). However, we were unable to find any research that reported steroid levels of true hermaphrodite dogs, Del Amo et al. (2001) and Alam et al. (2007) reported hyperestrogenemia and low serum concentrations of testosterone in male pseudohermaphrodite dogs. In this case, the concentrations of serum testosterone were found to be 1,008 ng/ml. The level of testosterone is similar to the basal levels of male dogs but higher than females and male pseudohermaphrodites. It is suggested that the normal testosterone concentrations may be due to true hermaphroditism and the presence of seminiferous tubules. The estrogen level (23,61 pg/ml) was lower than that of healthy males, females and male pseudohermaphrodites. Del Amo et al. (2001) suggested that the hyperestrogenemia and low testosterone serum concentrations in male pseudohermaphrodite dogs may be due to the sertoli cell tumors. In the present study, no evidence of sertoli cell tumors has been observed. The serum progesterone level of the patient was similar to that of healthy male and female dogs. Since there were no clear discrepancies regarding the concentrations of progesterone, it might be suggested that there is no association between the concentrations of progesterone and hermaphroditism.

True hermaphrodites have the gonads of both sexes (Alam et al. 2007). Although it is very rare; they can have one testis on one side and one ovary on the other (Atakam, 1954) or ovotestis may be paired with one ovary or testis (Alam at al. 2007). In our case, pathological examinations revealed that there are degenerative seminiferous tubules similar to testis tissue and primer follicles close to the cortex on the right gonad and the other gonad has numerous blood vessels filled erythrocyte. It is suggested that this is a true hermaphrodite dog because of the pathological findings.

Pasello-Legrand and Mowat (2004) reported that hermaphrodismus is caused by genetic or chromosomal abnormalities and incorrect hormonal or chemical exposure. According to the anamnesis,

no hormonal or chemical treatment was applied to the patient. Therefore, it is suggested that the abnormality originates either from genetic or chromosomal defects. This report describes the first true hermaphrodite case in a cross-bred dog in Turkey.

REFERENCES

Alam MR, Cho YG, Cho SJ, Lee JI, Lee HB, Tae HJ, Kim IS, Kim NS. Male pseudohermaphroditism in dogs: three case report. Veterinarni Med. 2007; 52(2): 74-78.

Atakam AM. A case of hermaphrodismus masculinus internus. Annals in Surgery 1954; 140(2): 216-222.

Concannon PW, Castracane VD. Serum androstenedione and testosterone concentrations during pregnancy and nonpregnant cycle in dogs. Biology of Rep. 1985; 33(5): 1078-1083.

Del Amo AN, Luca JD, Zufriategui L, Armocida A, Barbeito CG, Gobello C. Male pseudohermaphroditism in a dog: A case report. Communications in Theriogenology 2001; 1(1): 1-11.

Dunn HO, Kenney RM, Lein DH. XX/XY chimerism in a bovine true hermaphrodite: an insight into the understanding of freemartinism. Cytogenetics 1968; 7(5): 390-402.

Frank LA, Rohrbach BW, Bailey EM, West JR, Oliver JW. Steroid hormone concentration in healthy intact neutered dogs before and after cosyntropin administration. Dom Anim Endoc. 2003; 24(1): 43-57.

Hare WCD. Intersexuality in the dog. Can Vet J. 1976; 17(1): 7-15.

Kim KS, Kim O. A hermaphrodite dog with bilateral ovotestes and pyometra. J Vet Sci. 2006; 7(1): 87-88.

Meyers-Wallen VN, Hurtgen J, Schlafer D, Tulleners E, Cleland WR, Ruth GR, Acland GM. Sry-negative XX true hermaphroditism in a pasa fino horse. Equine Vet J. 1997; 29(5): 404-408.

Pasello-Legrand F, Mowat V. Two cases of spontaneous pseudohermaphroditism in cynomolgus monkey (Macaca fascularis). J Vet Med A Phys Path Cli Med. 2004; 51: 344-347.

Poth T, Breuer W, Walter B, Hecht W, Hermanns W. Disorders of sex development in dog – Adoption of a new nomenclature and reclassification of reported case. Anim Rep Sci. 2010; 121: 197-207.

Vani IA. True Hermaphroditism: Review Article. The Internet J. Pediatrics and Neonatology 2008; 9 (1).

Instruction for Authors

Kocatepe Veterinary Journal (KVJ)has the policy with One Medicine OneHealth. Research article, reviews, brief communication and case reports, lettersto editor and book reviews are also welcome for consideration to publisharticles of high scientific and ethical standards.
The journal is published four times a year. The publication of the text andfigures is **free** of change.
Acceptance of papers for the KVJ is undertaken by Editors. Editorial Board members adjudicate in the case of conflicting or adverse reports.
Manuscripts are accepted for consideration on the understanding thatthey are for publication solely in KVJ and that they neither have beenpublished nor are under consideration for publication elsewhere. Submissionalso implies that all authors have approved the paper for release and are inagreement with its content. Upon acceptance of the article by the journal, theauthor(s) will be asked to transfer the copyright of the article to thePublisher.
Each author accepts all ethical responsibility of the article and all authors agree with the content of the study. After article is checked by Professional Plagiarism Prevention program, article will be sent to authors. Articles are checked by iThenticate® program, when plagiarism or self-plagiarism are detected, they will not be evaluated for publication.
If animals are used in the studies, study should be approved by an Ethical Committee, Name of Ethical Committee and Approved Number should be mentioned in the Material and Method section. Editor may be reject directly the article, if animal is exposed to stressful or painful conditions.
Authors accept ethical rules when article is sent for publication. Author(s) should send CopyrightTransfer Agreement, after acceptance of article.
Each author accepts all ethical responsibility of the article and allauthors agree with the content of the study.
Article should be written using Garamond, font of 11 point, with 1.5 line spacing, margins of the A4 paper should be 2.5 cm from all edges (Word97-2010.doc). Abbreviations should be written in SI. Research article submittedto Kocatepe Veterinary Journal should be divided into the following sections:
Title page (Abstract, Key words without authors name and adress)**, Materials and Methods, Results, Discussion, Conclusions, References,Tables, Graphics, Figures.**
Title page: Papers should be headed with the full title, the initial letters ofname and surnames of the authors, the name and address of the institution wherethe work is carried out. The telephone number, fax number and e-mail address ofthe corresponding author should also be provided. The title should be short,specific and informative.

Abstract Should be no more than 200words, outlining in a single paragraph.

Keywords, 5 keywords that describe the crucial points of the paper should beprovided. Keywords should be chosen fromTurkey Science Term (www.bilimterimleri.com)

Introduction, an updated literature related to paper and aim(s) of the study shouldbe clearly given in this section.
Materials and methods, a clear account of materialsused and methods employed should be given and it should beapplicaple/repeatable by other researhers.
Results, as concise as possible. Text, tables and figures illustrating the samedata should be limited and succinctly outline the pertinent outcomes of thestudy.
Discussion: Results of the study should be discussed with directly relevantreferences. This section may also be divided into subsections.
Conclusions: This section should state clearly the main conclusions of theresearch. Results should not be repeated.
Acknowledgements, it is adviced to acknowledge persons or institutions directly orindirectly involved in the study.

References
References in the text should be made as follows: **Kara (2012)** described. / . was reported (**Zemheri 2015, Eryavuz and Yeni, Eryavuz et al. 2015**). List of references should be given alphabetically in the reference list. Different publications having the same author(s) of same year should be writtenas **2011a, 2011b.** Web address should be referenced as anonim for example **Anonim 2015**. Only official web pages should be used. Author name(s) and date sould be written bold. The reference list at the end of the paper should be written asbelow.

Journal:
Ince S, Kucukkurt I, Cigerci IH, Fidan AF, Eryavuz A. The effects of dietary boric acid and borax supplementation on lipid peroxidation, antioxidant activity, and DNA damage in rats. J Trace Elem Med Biol. 2010; 24(3):161-164.
Book section:
Juneja R, Koide SS. Molecular Biology of Reproduction, In: Reproduction in Farm Animals, Ed; HafezB, Hafez ESE, 7th Ed., LippincottWilliams and Wilkins, Philadelphia, USA. 2000; pp. 354-361.
Web page:
Anonymous. http://www.tuik.gov.tr/VeriBilgi.do?tb_id=46&ust_id=13;Accessien date: 02.01.2012.
Thesis:
Yeni D. Some andrological parameters and biochemical properties in relation toseason in rams. PhD thesis, Afyon Kocatepe University Health Science Institute, Afyonkarahisar,2010.

Tables: Tables should be presented in a separate page atthe end of manuscript.
Graphics: Figures should be presented in a separate page atthe end of manuscript.
Figures : Figures should be presented in a separate page atthe end of manuscript. Figures should be 80 or 160 mm, minimum 300 dpi.
Titles of tables, graphics and figures should be both Turkish and English.

Brief Communications: Briefcommunications should be consise but complete description of a limitedivestigation, which will not be included in a later publication. They shouldnot exceed 1600 words. They should bear no more than two tables or figures. AnABSTRACT should be given but no other sections. Typescripts should be clearlymarked Brief Communication.

Review Articles: Review articles related to allmedical topics are welcome for publication. They should give an update onrecent advances in a particular field and be targeted at research veterinariansor clinicians who are not necessarily working in the same field. The lengthshould not exceed 4500 words. It should have a precise abstract. Author ofreview should have at least two citations. For each issue maximum 2 reviews arepublished.

Case Reports: Reports of SINGLE or small numbers of cases willbe considered for publication in KVJ if the case(s) are particularlyunusual/rare or the report contributes materially to the literature. A casereport should not exceed 1500 words and must comprise a Summary (maximum 150words), Introduction, Case History and Discussion. The report should accomplishone of the followings:

- To be a substantially novel presentation
- To be a technique or treatment that wouldsubstantially alter management and prognosis of the described condition
- The first clinical report or first case(s) ofdiseases in a particular location where epidemiology is an important factor

– To exemplify bestpractice in medical science.

Letters to The Editor: Letters describingcase reports or original material may be published in the KVJ and will bepeer-reviewed prior to publication. Letters making critisisms on recentlypublished papers in the KVJ will also be considered and the correspondingauthors of the original paper will be invited to respond accordingly.

All articles sent to KVJ (Kocatepe Veterinary Journal) ONLINE submition only.

During submition documentswhish are listed below, have to instal to the system;

1. **Title Page:** Author and institution names
2. **Main text:** Author and institution names should NOT be.Tables(s), graphic(s) and figure(s) etc. Should be an the last page of article,olso title of them both in Turkish and English.
3. **Article addition**: Table(s), graphic(s) andfigure(s) should have been installed to the system separately.
4. **Author Approval Form (Cover Letter):** All authorsneed to sing it and install to the system. Signatures should be wet signuturesand send to the Editorial Board of Kocatepe Veterinary Journal.
5. **Copyright:** All authors need to sing it and install to thesystem. Signatures should be wet signutures and send to the Editorial Board ofKocatepe Veterinary Journal.