Official Publication of
The Afyon Kocatepe University
Faculty of Veterinary Medicine

Kocatepe Veterinary Journal

2022, 15 (2) June

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

https://dergipark.org.tr/kvj

DergiPark AKADEMİK

Kocatepe Veterinary Journal
2022 June 15 / 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, CrossRef, Google Scholar*

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****

Kocatepe Veterinary Journal
2022 June 15 / 2

Official Publication of
The Afyon Kocatepe University

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

The Reviewer List (in alphabetical order)

Ahmet Ceylan	Ankara University
Akın Yakan	Mustafa Kemal University
Arzu Funda Bağcıgil	İstanbul University
Asm Golam Kibria	Chittagong Veterinary and Animal Sciences University
Aydın Vural	Dicle University
Bengi Çınar Kul	Ankara University
Buğrahan Bekir Yağcı	Kırıkkale University
Cüneyt Çağlayan	Bingöl University
Erhan Keyvan	Burdur Mehmet Akif Ersoy University
Fatih Yıldırım	Atatürk University
Harun Çınar	Burdur Mehmet Akif Ersoy University
Hasan Erdoğan	Aydın Adnan Menderes University
Hasan Güzelbekteş	Selcuk University
Hatice Ahu Kahraman	Burdur Mehmet Akif Ersoy University
Hüseyin Serkan Erol	Kastamonu University
İbrahim Demirkıran	Afyon Kocatepe University
Kemal Varol	Burdur Mehmet Akif Ersoy University
Mehmet Aykur	Tokat Gaziosmanpaşa University
Mehmet Başeğmez	Pamukkale University
Mehmet Çağrı Karakurum	Burdur Mehmet Akif Ersoy University
Mehmet Fatih Aydın	Karamanoğlu Mehmetbey University
Mesut Bünyami Halıcı	Atatürk University
Mustafa Cellat	Mustafa Kemal University
Mustafa Kabu	Afyon Kocatepe University
Nalan Kaya Tektemur	Fırat University
Ö. Faruk Lenger	Afyon Kocatepe University
Özlem Yıldız Gülay	Burdur Mehmet Akif Ersoy University
Pınar Karatepe	Fırat University
Servet Kılıç	Namık Kemal University
Tayfun Dikmen	Afyon Kocatepe University
Yunus Emre Özkanlar	Ondokuz Mayıs University
Zafer Sayın	Selçuk University
Zeki Aras	Aksaray University

KOCATEPE VETERINARY JOURNAL 2022 15 (2) JUNE

RESEARCH ARTICLES

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022) 15(2): 139-143

DOI: 10.30607/kvj.1051124

RESEARCH ARTICLE

Investigation of *Pseudomonas* Species in Chicken Drumstick Samples

Hayriye Yeşim CAN[1*]

[1]Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Hatay, Turkey

ABSTRACT

This study aimed to detect the contamination of refrigerated poultry meat with psychrotrophic bacteria, such as *Pseudomonas* species. *Pseudomonas* spp. can grow well in the skin and muscle of poultry meat by using carbonhydrates and amino acids at refrigeration temperature (4 °C). They are mainly responsible for the spoilage of poultry meat with their enzymatic activiy. For this purpose, a total of 107 chicken drumstick samples were analyzed for the presence of *Pseudomonas* species (*Pseudomonas fragi, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas lundensis, Pseudomonas aeruginosa*). Of the samples, 92.5% found to be contaminated with *Pseudomonas* spp. A total of 99 isolates were confirmed as *Pseudomonas* spp. by PCR, targeting the *16S rDNA* gene. Among the 99 isolates, 78.7% were identified as *P. fluorescens*, whereas *P. fragi*, *P. putida*, *P. lundensis*, and *P. aeruginosa* were not detected in the study. The findings show that *P. fluorescens* is the most prevalent species in refrigerated poultry meat, thus posing a potential risk for the spoilage of poultry meat.

Keywords: PCR, Poultry Meat, *Pseudomonas*, Spoilage

***

Piliç Baget Örneklerinde *Pseudomonas* Türlerinin Araştırılması

ÖZ

Bu çalışmada, soğukta muhafaza edilen kanatlı etlerinin *Pseudomonas* türleri gibi psikrotrofik bakterilerle kontaminasyonunun belirlenmesi amaçlandı. *Pseudomonas*'lar soğuk muhafaza şartlarında (4 °C'de) kanatlı etindeki karbonhidratlar ile amino asitleri kullanarak deri ve kasta kolaylıkla gelişebilir. Enzimatik aktiviteleri ile kanatlı etlerinin bozulmasından esas olarak sorumludurlar. Bu amaçla, toplam 107 adet tavuk baget örneğinde *Pseudomonas* türlerinin (*Pseudomonas fragi, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas lundensis, Pseudomonas aeruginosa*) varlığı analiz edildi. Örneklerin % 92.5'inin *Pseudomonas* spp. ile kontamine olduğu bulundu. Toplam 99 izolat, *16S rDNA* geni hedef alınarak *Pseudomonas* spp. yönünden PCR ile doğrulandı. Çalışmada 99 izolatın %78,7'si *P. fluorescens* olarak identifiye edilirken, *P. fragi*, *P. putida*, *P. lundensis* ve *P. aeruginosa* tespit edilemedi. Bulgular, soğukta muhafaza edilen kanatlı etlerinde *P. fluorescens*'in en yaygın tür olduğunu ve dolayısıyla, kanatlı etinin bozulması için potansiyel bir risk oluşturduğunu göstermektedir.

Anahtar kelimeler: Bozulma, kanatlı eti, PCR, *Pseudomonas*

To cite this article: Can HY, Investigation of Pseudomonas Species in Chicken Drumstick Samples Kocatepe Vet J. (2022) 15(2): 139-143

Submission: 30.12.2021 **Accepted**: 16.03.2022 **Published Online**: 04.04.2022
ORCID ID; *HYC: 0000-0002-5191-6268.*
*Corresponding author e-mail: yesimcan@mku.edu.tr

INTRODUCTION

The people widely consume chicken meat due to being rich from essential amino acids, zinc, iron and group B vitamins, easily digestible besides being an economic animal protein source. Many factors beginning from production to consumption (the infections in the live birds, breeding, transporting, slaughtering, packaging, storage, and distribution conditions) affect the microbial flora of poultry meat (Okolocha and Ellerbroek 2005).

High post-rigor pH and water activity (a_w) values of poultry meat are risk factors for microbial spoilage (Allen et al. 1997). The competitive ability of the microorganisms that lead to food spoilage is good, and they cause structural changes in the food with their proteolytic and lipolytic enzymes. *Pseudomonas* spp. are the most commonly isolated psychrotrophic bacteria from food, and they lead to spoilage in foods stored at refrigeration temperatures by growing under aerobic conditions (Salvat et al. 1997, Geornaras et al. 1999, Mead 2004, Ercolini et al. 2007, Al-Rodhan and Nasear 2016, Caldera et al. 2016).

Poultry meat is commonly consumed with the skin, and the initial microbial load of the skin is generally high. Although the initial microbial load in poultry meat varies depending on sanitary and hygienic practices, technical design, and cross-contaminations in the slaughterhouse, its high shortens the product's shelf life (Höll et al. 2016, Morales et al. 2016). While the initial microbial flora originating from live birds usually includes Gram-positive bacteria, they leave their place in Gram-negative bacteriae (*Pseudomonas*, *Flavobacteria, Acinetobacter, Moraxella*, and *Enterobacteriaceae*) in the final product over time. So, the final product may carry a risk for the presence of the microorganisms that cause spoilage like *Pseudomonas* spp. and pathogens such as *Salmonella, Campylobacter, Clostridium perfringens, Escherichia coli* O157, *Listeria monocytogenes*, and *Staphylococcus aureus*. The undesired levels of the abovementioned microorganisms in the final product indicate the insufficient sanitisation in the slaughterhouse, that hygienic practices are not cared during or after slaughter process, cross-contamination and recontamination in the plant. Because *Pseudomonas* spp. are usually destroyed at the scalding stage of the poultry slaughtering process (Mead 2004, Okolocha and Ellerbroek 2005, Wang et al. 2018). So, although the systems like HACCP that aims food safety in poultry production primarily target pathogen bacteria control, they also make a certain reduction in the level of spoilage-causing bacteriae. Thereby, the HACCP system positively contributes to shelf life of the product and public health (Mead 2004, Morales et al. 2016).

Hygienic practices during slaughtering, cold storage conditions, packaging techniques, post-rigor pH value of the product, species, and level of the psychrotrophic bacteria in the flora may be effective in spoilage of the poultry meat. The post-rigor pH value of the chicken leg is higher than that of the chicken breast, so spoilage can be faster here. *Pseudomonas* species may become dominant in the flora by quickly growing in chicken leg and breast meat. When the level of *Pseudomonas* reaches 10^7-10^8 cells per square centimeter, color change, off-odor, and slime formation occur. The unwanted metabolites that arise from protein degradation are reported to cause these changes (Allen et al. 1997, Nychas et al. 2008).

Usually, in fresh meat, the number of *Pseudomonas* is low and they grow during storage in cold and the number gradually increases. They easily compete with the other spoiler microorganisms in the flora as they can grow in cold and normal atmospheric conditions due to their oxidative properties. So, the spoilage in storage in the cold usually occurs from *Pseudomonas*. The frequently isolated *Pseudomonas* species in poultry stored in cold are reported *P. fragi, P. fluorescens, P. putida,* and *P. lundensis* (Geornaras et al. 1999, Ercolini et al. 2007, Nychas et al. 2008, Morales et al. 2016, Kumar et al. 2019).

Cold storage is the most commonly applied procedure for prolonging the shelf life of poultry meat. So, the presence and the level of psychrophile/psychrotrophic microorganisms that lead to spoilage in poultry meat have critical significance for determining shelf life. The present study was conducted to investigate *Pseudomonas* species (*P. fragi, P. fluorescens, P. putida, P. lundensis, P. aeruginosa*) in chicken drumsticks stored at refrigeration temperature (4°C) by using PCR method.

MATERIAL and METHODS

Sampling, Isolation and Identification of *Pseudomonas*

In the present study, 107 packaged chicken drumsticks belonging to different companies and sold in Hatay province consisted of the study sample. *Pseudomonas* isolation was done with the rinse method in the samples taken under aseptic conditions and brought to the laboratory under a cold chain. For pre-enrichment, each sample was put into sterile plastic bags and 100 ml Tryptic Soy Broth (Merck, 105459) was added. After the samples were shaken and washed during 1 min within the bag, the broth was closed and incubated at 30°C for 24 hours. One loop of culture suspension was taken from pre-enrichment broth and streaked on *Pseudomonas* Agar Base (Oxoid, CM0559) containing *Pseudomonas* CFC supplement (Cetrimide, Fucidin, Cephalosporin, Oxoid, SR0103).

Plates were incubated at 30°C for 24 hours. The oxidase positive, straw-coloured colonies were selected and suspended into tubes containing 4-5 ml of Brain Hearth Infusion broth (Oxoid, CMO225). The broth culture was incubated at 30°C for 18-24 hours under aerobic conditions.

For DNA extraction, 1 ml of broth culture was taken to eppendorf tube and centrifuged at 6000xg for 2 min. Supernatant was thrown and the following procedures were applied in order as instructed in the commercial kit (GF-1 Bacterial DNA Extraction, GF-BA-100, Vivantis). The obtained DNAs were stored at -20°C.

Verification of Isolates as *Pseudomonas* spp., and *Pseudomonas aeruginosa*

According to the amplification protocol described by Spilker et al. (2004), PCR was done by using primer pairs for amplifying the 618 bp (genus- specific; *Pseudomonas* spp.) and 956 bp (species-specific; *P. aeruginosa*) 16S rDNA gene sequence (Table 1). Two µl of DNA extract was taken and added to 23µl PCR mixture (2.5µl of 10xPCR buffer, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 1 U *Taq* polymerase, 1 µl of each primer at 10 µM concentration, and 2 mM $MgCI_2$) and a total of 25 µl mixture was subjected to amplification.
Amplification conditions included initial denaturation at 95°C for 2 min, 25 cycles of denaturation at 94°C for 20 sec, primer annealing at 58°C for 20 sec, and primer extension at 72°C for 40 sec in a thermal cycler (Boeco, Germany), and final extension at 72°C for 1 min.

Molecular Identification of *Pseudomonas* Species

The obtained isolates were identified by PCR with regard to *Pseudomonas fluorescens, Pseudomonas fragi*, *Pseudomonas putida*, *Pseudomonas lundensis* by using specific primer pairs described by Al-Rodhan and Nasear (2016), Ercolini et al. (2007), Morales et al. (2016). For the verification of isolates for *P. fluorescens*, a total of 25 µl of PCR mix (1 µl of each primer, 12.5 µl DreamTaq Green Master Mix (K1081, Thermo Scientific), and 5 µl of template DNA) was subjected to amplification. Amplification conditions included initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 45 sec, primer annealing at 59°C for 45 sec, primer extension at 68°C for 2 min, and final extension at 72°C for 5 min (Al-Rodhan and Nasear, 2016). Isolates were analyzed with multiplex PCR for *P. fragi, P. putida* and *P. lundensis*. For this purpose, a total of 20 µl PCR mix (0.4 µl of each primer, 1.2 µl reverse primer, 10 µl DreamTaq Green Master Mix (Thermo Scientific), 1 µl of template DNA) was prepared. For multiplex PCR, amplification conditions included initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, primer extension at 72°C for 1 min, and final extension at 72°C for 7 min (Morales et al. 2016).

Positive Control

Pseudomonas fluorescens ATCC 13525 was used as positive control in the study.

Electrophoresis and Imaging Procedure

The amplified DNAs were loaded to 1.5% agarose gel and subjected to electrophoresis procedure at 100 V for 50 min (CS-300V, England). At the end of this procedure, specific amplicons for *Pseudomonas* were analyzed at UV-transulliminator (UVP, USA) with the aid of positive control and DNA Ladder (Bioatlas, Estonia).

Table 1. Primers used in this study

Gene	Primer	Sequence (5'- 3')	Target	Annealing temp (°C)	Size (bp)	Reference
16S rDNA	F1	GACGGGTGAGTAATGCCTA	*Pseudomonas spp.*	54	618	Spilker et al. (2004)
	R1	CACTGGTGTTCCTTCCTATA				
	F2	GGGGGATCTTCGGACCTCA	*P. aeruginosa*	58	956	
	R2	TCCTTAGAGTGCCCACCCG				
16S rDNA	F3	TGCATTCAAAACTGACTG	*P. fluorescens*	59	850	Al-Rodhan and Nasear (2016)
	R3	AATCACACCGTGGTAACCG				
carA	F4	CGTCAGCACCGAAAAAGCC	*P. fragi*	60	370	Ercolini et al. (2007), Morales et al. (2016)
	R	TGATGRCCSAGGCAGATRCC				
	F5	ATGCTGGTTGCYCGTGGC	*P. putida*	60	230	
	R	TGATGRCCSAGGCAGATRCC				
	F6	TGTGGCGATTGCAGGCATT	*P. lundensis*	60	530	
	R	TGATGRCCSAGGCAGATRCC				

RESULTS

In the study, *Pseudomonas* spp. were detected in 99 out of 107 chicken drumstick samples (92.5%) (Figure 1). In total, *P. fluorescens* was isolated from 78 samples (72.8%), and it was found to that it constituted the dominant flora for *Pseudomonas* (Figure 2). The other *Pseudomonas* species (*P. aeruginosa, P. fragi, P. putida,* and *P. lundensis*) were not detected in the samples.

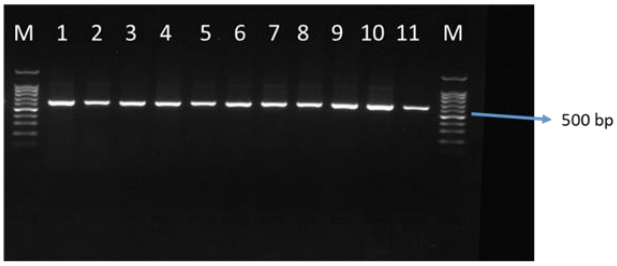


Figure 1. PCR analysis of *Pseudomonas* spp. isolates [M: 100 bp DNA marker, 1-11: *Pseudomonas* spp. Positive isolates (618 bp)]

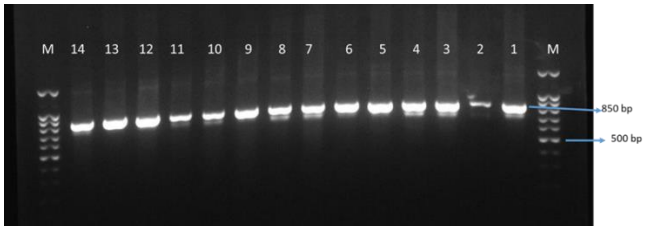


Figure 2. PCR analysis of *Pseudomonas fluorescens* isolates [M: 100 bp DNA marker, 1: Positive control (*Pseudomonas fluorescens* ATCC 13525), 2-14: Some of *P.fluorescens* positive isolates (850 bp)]

DISCUSSION and CONCLUSION

Globally, it is estimated that about 1/3 of the food produced for human consumption is lost or wasted each year (FAO, 2011). Food's becoming unavailable to introduce to consumers due to microbial or chemical spoilage is an important food loss (Morales et al., 2016). Meat and meat products account for approximately 21% of food losses in Europe and North America (Nychas et al., 2008). According to the world meat projection, poultry is the second most consumed meat worldwide and is expected to rank first by 2022 (OECD-FAO, 2013). In this context, the fact that chicken meat is generally consumed with its skin, mainly due to its nutritional content, and that the post-rigor pH and aw value are suitable for microbial growth limits the shelf life of poultry even under cold storage conditions. Poultry is susceptible to spoilage, and storage temperature is critical for spoilage. However, cold storage conditions of poultry create the typical microflora of poultry by bringing the microorganisms that grow in this condition in the foreground (Höll et al., 2016; Morales et al., 2016).
P. fluorescens was predominantly isolated from the chicken drumstick samples in the present study. Similarly, Morales et al.(2016) detected *P. fragi* and *P. fluorescens* most in chicken breast meat. Phenotypically, *P. fluorescens* was found to show proteolytic, lipolytic and lecithinase activity, while *P. fragi* was found to produce mainly proteolytic enzymes. As the result of the study, the researchers reported that there are important phenotypical and genotypical differences among *Pseudomonas* species from poultry meat. Differently, Caldera et al. (2016) isolated *P. fragi* and *P. putida* most from meat products. Also, when they have investigated the enzymatic activities of their isolates at 5°C and 25°C, they have detected that 30% of them showed proteolytic activity at 5°C, whereas 10% showed proteolytic activity at 25°C.

In addition, the packaging method also determines the spoilage of poultry meat. Höll et al. (2016) used a modified atmosphere packaging (MAP) method with two different gas mixtures (65% N_2 + 35% CO_2; 80% O_2 + 20% CO_2) for the packaging of chicken breast meats. The samples were packaged in both atmospheric conditions and incubated for 14 days at 4°C and 10°C. At the end of the eighth day, *Pseudomonas*, *Carnobacterium* and *Brochothrix thermosphacta* were found to form the dominant flora in the samples packed with the MAP method with high O_2 content. However, in the MAP method with low O_2 content, the dominant species responsible for spoilage were found to be *Carnobacterium, Serratia* and *Yersinia* at 4°C, while it was found to be *Hafnia alvei* at 10°C. Consequently, researchers recommend packaging poultry with MAP with a high O_2 content, due to its being less harmful and as pathogenic bacteria such as *Yersinia* cannot grow. Another advantage of packaging poultry with MAP with a high O_2 content is it inhibits the growth of an important food pathogen, *Campylobacter jejuni*, which poses a risk for poultry meat (Rajkovic et al., 2010). Usually, poultry is packaged in conditions where the CO_2/N_2 concentration is high and the O_2 concentration is reduced. Because oxymyoglobin formation and preservation of bright red color are not required in poultry meat as it is required for red meat (Sante et al., 1994). Besides, *Pseudomonas*, which is one of the most important spoilage factors in poultry meat is very sensitive to high CO_2 concentration, thus inhibiting their growth (Höll et al., 2016).

Different decontamination practices in poultry can increase the microbiological quality and shelf life of products by creating different levels of inhibition on both pathogenic and spoilage bacteria (Mead, 2004). In this context, Okolocha and Ellerbroek (2005) applied decontamination by spray or dip method with acid and alkaline agents (1% lactic acid and 10% trisodium phosphate) in poultry carcasses. The authors have reported that decontamination with the immersion method was more effective, and an average of 0.2-2.4 logarithmic reduction in *Pseudomonas* number was obtained as a result of

storage at 4°C for 6 days. They also stated that the sensoric evaluations were also positive by the consumers, and accordingly, with acid or alkali applications within the legal limits, poultry can be stored at 4°C for at least 6 days, preserving its quality.

Biofilm formation by *P. fluorescens* is also an important problem for the poultry industry, and this event can cause cross-contamination throughout the production process. Next time, this bacterium can also get resistance against disinfectants used in the poultry plant (Geornaras et al., 1999; Wang et al., 2018; Kumar et al., 2019).

In conclusion, the present study has revealed that the prevalence of *Pseudomonas* spp. is quite high in chicken drumstick samples stored at refrigeration temperature (4°C) and *P. fluorescens* was found to be the dominant species. *Pseudomonas* species except for *Pseudomonas aeruginosa* have not been included in food safety management systems as they do not cause any infection in humans. However, *Pseudomonas* spp. are primarily responsible for microbial spoilage in poultry samples kept in cold and distributed under cold chain, which is extremely important in terms of the quality and shelf life of poultry in today's global conditions. On the other hand, the presence of *Pseudomonas* in poultry may facilitate the growth of *Campylobacter jejuni* due to the microaerophilic environment that they create. This is an adverse event and thereby, *Pseudomonas* as an important spoilage bacterium that should not be overlooked by focusing only on pathogen bacteriae in the poultry production chain.

Conflict of interest: --

Ethical Approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k).

Financial support: --

Acknowledgment: --

Description: --

REFERENCES

Al–Rodhan AM, Nasear HA. PCR-based detection of *Pseudomonas fluorescens* in cows and buffalos raw milk. Bas J Vet Res. 2016; 15(1): 194-208.

Allen CD, Russell SM, Fletcher DL. The relationship of broiler breast meat color and pH to shelf-life and odor development. Poultry Sci. 1997; 76(7): 1042-1046.

Caldera L, Franzetti L, Van Coillie E, De Vos P, Stragier P, De Block J, Heyndrickx M. Identification, enzymatic spoilage characterization and proteolytic activity quantification of *Pseudomonas* spp. isolated from different foods. Food Microbiol. 2016; 54: 142-153.

Ercolini D, Russo F, Blaiotta G, Pepe O, Mauriello G, Villani F. Simultaneous detection of *Pseudomonas fragi*, *P. lundensis*, and *P. putida* from meat by use of a multiplex PCR assay targeting the carA gene. Appl Environ Microbiol. 2007; 73(7): 2354-2359.

FAO. What is food loss and food waste? Available at: https://www.fao.org/food-loss-and-food-waste/flw-data. 2011.

Geornaras I, Kunene NF, von Holy A, Hastings JW. Amplified fragment length polymorphism fingerprinting of *Pseudomonas* strains from a poultry processing plant. Appl Environ Microbiol. 1999; 65(9): 3828-3833.

Höll L, Behr J, Vogel RF. Identification and growth dynamics of meat spoilage microorganisms in modified atmosphere packaged poultry meat by MALDI-TOF MS. Food Microbiol. 2016; 60: 84-91.

Kumar H, Franzetti L, Kaushal A, Kumar D. *Pseudomonas fluorescens:* a potential food spoiler and challenges and advances in its detection. Ann Microbiol. 2019; 69: 873-883.

Mead GC. Microbiological quality of poultry meat: a review. Braz J Poult Sci. 2004; 6(3): 135-142.

Morales PA, Aguirre J, Troncoso M, Figueroa G. Phenotypic and genotypic characterization of *Pseudomonas* spp. present in spoiled poultry fillets sold in retail settings. LWT-Food Sci Technol. 2016; 73(22): 609-614.

Nychas GJE, Skandamis PN, Tassou CC, Koutsoumanis KP. Meat spoilage during distribution. Meat Sci. 2008; 78(1-2): 77-89.

OECD-FAO. OECD-FAO agricultural outlook 2013-2022 highlights. World meat projections. Available at: http://www.agri-outlook.org/highlights-2013-EN.pdf. 2013.

Okolocha EC, Ellerbroek L. The influence of acid and alkaline treatments on pathogens and the shelf life of poultry meat. Food Control. 2005; 16(3): 217-225.

Rajkovic A, Tomic N, Smigic N, Uyttendaele M, Ragaert P, Devlieghere F. Survival of *Campylobacter jejuni* on raw chicken legs packed in high-oxygen or high-carbon dioxide atmosphere after the decontamination with lactic acid/sodium lactate buffer. Int J Food Microbiol. 2010; 140(2-3): 201-6.

Salvat G, Rudelle S, Humbert F, Colin P, Lahellec C. A selective medium for the rapid detection by an impedance technique of *Pseudomonas* spp. associated with poultry meat. J Appl Microbiol. 1997; 83(4): 456-63.

Sante V, Renerre M, Lacourt A. Effect of modified atmosphere packaging on color stability and on microbiology of turkey breast meat. J Food Qual.1994; 17(3): 177-195.

Spilker T, Coenye T, Vandamme P, LiPuma JJ. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. J Clin Microbiol. 2004; 42(5): 2074-9.

Wang H, Cai L, Li Y, Xu X, Zhou G. Biofilm formation by meat-borne *Pseudomonas fluorescens* on stainless steel and its resistance to disinfectants. Food Control. 2018; 91: 397-403.

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022):15(2):144-150
DOI: 10.30607/kvj.1058247

RESEARCH ARTICLE

Seroprevalence of *Neospora Caninum* and *Toxoplasma Gondii* in Honamlı Goats in Burdur Province

Mustafa ESER[1]*, Kemal VAROL[2]

[1]Anadolu University Open Education Faculty Health Programs, Yunusemre Campus, TR-26470 Eskisehir, Turkey
[2]Burdur Mehmet Akif Ersoy University, Food, Agriculture and Livestock Vocational College of Burdur, Department of Veterinary, 15030, Burdur, Turkey

ABSTRACT

Neospora caninum and *Toxoplasma gondii* are intracellular protozoan parasites that cause economic losses in goats. Both parasites are similar in features and cause abortions in goats. For this reaon, in this study, it was aimed to determine the seroprevalence of *N. caninum* and *T. gondii* in Honamlı goats bred in Burdur province. The study material consisted of 273 Honamlı goats aged between 2-7 years in 11 different goat herds. While 69 of these goats were aborted in the 5th month of pregnancy, the remaining 189 were normal goats that gave birth to healthy kid. In the findings; *N. caninum* specific antibodies were determined in eight (2.9 %) blood serum taken from the goats included in the study. In the blood serum of three goats (1.1 %), *N. caninum* suspectable antibody positivity was detected. *Toxoplasma gondii* specific antibodies were determined in seven (2.6 %) blood serum taken from the goats included in the study. In the blood serum of three goats (1.1 %), *T. gondii* suspectable antibody positivity was detected. As a result, it was observed that *N. caninum* and *T. gondii* had low serprevalence levels in Honamlı goats bred in Burdur province.

Keywords: ELISA, Goat, Honamlı Goat, *Neospora caninum*, *Toxoplasma gondii*, Seroprevalence.

****

Burdur İlinde Honamlı Keçilerinde *Neospora Caninum* ve *Toxoplasma Gondii*'nin Seroprevalansı

ÖZ

Neospora caninum ve *Toxoplasma gondii* keçilerde ekonomik kayıplara neden olan hücre içi yerleşim gösteren protozoer parazitlerdir. Her iki parazitte özellik olarak benzerlik göstermekte ve keçilerde abortlara yol açmaktadır. Bu nedenle bu çalışmada, Burdur ilinde yetiştirilen Honamlı ırkı keçilerde *N. caninum* ve *T. gondii*'nin seroprevalansının belirlenmesi amaçlanmıştır. Çalışma materyalini 11 farklı keçi sürüsünde, yaşları 2-7 yaş arasında değişen toplam 273 adet Honamlı ırkı keçi oluşturmuştur. Bu keçilerin 69 tanesini gebeliğin beş aylık döneminde abort yapan keçiler oluştururken, kalan 189 tanesini normal sağlıklı yavru doğuran keçiler oluşturmaktadır. Bulgularda; çalışmaya dahil edilen keçilerden alınan kan serumlarının sadece (% 2.9) 8' inde *N. caninum* spesifik antikor varlığı saptanmıştır. Üç keçinin kan serumda (% 1.1) ise, *N. caninum* şüpheli antikor pozitiflik belirlenmiştir. Çalışmaya dahil edilen keçilerden alınan kan serumlarının, sadece (% 2.6) 7' sinde *T. gondii* spesifik antikor varlığı saptanmıştır, Üç keçinin kan serumda (% 1.1) ise, *T. gondii* şüpheli antikor pozitiflik belirlenmiştir. Sonuç olarak, Burdur ilinde yetiştirilen Honamlı ırkı keçilerde *N. caninum* ve *T. gondii*'nin düşük serprevalans düzeyine sahip olduğu görülmüştür.

Anahtar kelimeler: ELISA, Keçi, Honamlı Keçisi, *Neospora caninum*, *Toxoplasma gondii*, Seroprevalans.

To cite this article: Eser M., Varol K.,. Seroprevalence of Neospora caninum and Toxoplasma gondii in Honamlı Goats in Burdur Province Kocatepe Vet J. (2022):15(2):144-150

Submission: 17.01.2022 **Accepted**: 21.03.2022 **Published Online**: 13.04.2022
ORCID ID; *ME: 0000-0003-1542-2989, KV: 0000-0002-3057-2865.*
*Corresponding author e-mail: meser961@anadolu.edu.tr

INTRODUCTION

Neosporosis is a protozoan disease caused by *Neospora caninum*. It causes cyst formation in host tissues (Dubey 1999). This disease causes abortions in domestic livestock and deer. It also causes infections in the nervous system in horses and dogs. Dogs are the last hosts of *N. caninum*. It is also known that wolves (gray and coyote worms) are the last host of the disease. Although neosporosis does not show any specific clinical signs in adult cattle, it causes abortions in sheep, goats and young pregnant cattle. In addition, calves born from cattle infected with *N. caninum* are weak and in poor condition. Neuromuscular anomalies can be observed in calves born this way in a few days after birth (Aktaş et al. 2005). Dogs play an important role in the spread of the disease. Cattle and goats should not be grazed on pastures where dogs are overpopulated (Dubey and Lindsay 1996). Today, vaccine development studies are carried out to prevent the disease. Vaccines prepared from tachyzoites of the agent against neosporosis are used in regions where the disease is common (Andrianarivo et al. 1999).

Toxoplasmosis is one of the most common parasitic zoonotic diseases worldwide caused by *Toxoplasma gondii* (Tenter et al. 2000). *T. gondii* was first detected in the tissues of a hamster-like wild rodent named *Ctenodactylus gundi* by Nicolle and Manceaux (1908) in the laboratories of the Pasteur Institute in Tunisia in 1908 (Dubey 2008, Dubey 2009). It is an obligate intracellular protozoan belonging to the class Coccidea of the Apicomplexa subphylum. They cause toxoplasmosis infection by localizing in all cells except erythrocytes of humans and many animals. Cats are the last host of the parasite. Its intermediate hosts are poultry, mammals and humans (Kolören and Dubey 2020). Toxoplasmosis causes abortions in cattle, sheep and goats. The disease is transmitted to humans by consumption of Toxoplasma infected meats and food and beverages contaminated with sporulated oocysts from the final host, and causes an important public health problem (Dubey and Beattie 1988, Tenter et al. 2000, Aköz et al. 2009).

N. caninum and *T. gondii* are intracellular protozoan parasites that cause economic losses in goats. Both parasites are similar in features and cause abortions in goats (Dubey and Beattie 1988, Dubey and Lindsay 1996, Tenter et al. 2000, Aköz et al. 2009). *Toxoplasma gondii* has zoonotic properties. It causes serious health problems that threaten public health. Developing an effective treatment method against diseases and performing preventive medicine in a healthy way will be possible by determining the epidemiological spread of these diseases. The aim of this study was to evaluate the seroprevalence of *N. caninum* and *T. gondii* in Honamlı goats bred in Burdur province, Turkey.

MATERIAL and METHODS

Study Area

The research was evaluated at the meeting of Anadolu University Animal Experiments Local Ethics Committee, dated 04.06.2018, with registration number 18-37. According to the decision numbered 825172047.09100/2018-4, there was no ethical objection. In addition, this research was supported by Anadolu University Scientific Research Projects Commission as project number 1805S215.

Animals and Serum

This study was carried out on Honamlı goats, which are one of our domestic goat breeds, bred in Burdur province, Turkey. The study material consisted of 273 Honamlı goats aged between 2-7 years in 11 different goat herds. While 69 of these goats were aborted in the 5th month of pregnancy, the remaining 189 were goats that gave birth to normal healthy kid.

Eight milliliter of blood was taken from the vena jugularis of goats into vacuum tubes with coagulation factor. Collected blood was brought to the laboratory and centrifuged at 3500 rpm for 10 minutes. Extracted serums were placed in capped plastic tubes and stored at -20 °C until testing.

Detection of *N. caninum* and *T. Gondii* antibodies

In order to investigate *N. caninum* and *T. gondii* antigens from the serums, noncompetitive Enzyme Linked Immunosorbent Assay (ELISA) kits (Sinogeneclon ELİSA Kit) were used in accordance with the procedure (Sandwic ELISA Method-Noncompetitive). As a result of the test, the plates were read in an ELISA Microplate reader (Biotek Synergy/H1/USA) at a wavelength of 450 nm. As a result of the test, the % inhibition value was obtained. It was calculated according to the specific procedure of the kit with the specified formula. In the tests applied for both parasites, positivity and negativity were determined according to the following criteria;
Test validity: Positive control mean ≥1.00; Negative control mean ≤0.10.
Critical Calculation (CUT OFF): Critical = Average of Negative control well + 0.15.
Negative verdict: Example OD <Critical Calculation (CUT OFF) TG and NC are Negative.
Positive decision: Example OD≥ Critical Calculation (CUT OFF) TG and NC are Positive.

Statistical analysis

The findings were evaluated using the IBM SPSS 22.0 for Windows package program. Cross-tabs were used to determine the compositional distribution of the variables. Spearman Correlation analysis was used to determine the relationship between variables.

RESULTS

In the findings; *N. caninum* specific antibodies were determined in only eight (2.9 %) blood serum taken from the goats included in the study. In the blood serum of three goats (1.1 %), *N. caninum* suspectable antibody positivity was detected. *T. gondii* specific antibodies were determined in only seven (2.6 %) blood serum taken from the goats included in the study. In the blood serum of three goats (1.1 %), *T. gondii* suspectable antibody positivity was detected (Table 1 and Table 2).

When the aborted goats are examined in terms of *N. caninum*; two goats were determined as *N. caninum* seropositive and one goats were determined as *N. caninum* suspectable. The distribution of *N. caninum* according to herds; one goat in Ovacık 1, and one goat in Ovacık 2 were determined as seropositive. One goat in Kuzköy 1 were determined as suspectable (Table 1).

When the non-aborted goats are examined in terms of *N. caninum*; six goats were determined as *N. caninum* seropositive and two goats were determined as *N. caninum* suspectable. The distribution of *N. caninum* according to herds; one goat in Bağsaray 1, two goat in Bağsaray 3, one goat in Bağsaray 4, one goat in Kuzköy 2, and one goat in Ovacık 2 were determined as seropositive. One goat in Bağsaray 1, and one goats in Bağsaray 2 were determined as suspectable (Table 1).

When the aborted goats are examined in terms of *T. gondii*; one goat was determined as *T. gondii* seropositive. The distribution of *T. gondii* according to herds; positivity was determined in Ovacık 2 (Table 2).

When the non-aborted goats are examined in terms of *T. gondii*; six goats were determined as *T. gondii* seropositive and two goats were determined as *T. gondii* suspectable. The distribution of *T. gondii* according to herds; one goat in Bağsaray 1, one goat in Bağsaray 2, one goat in Bağsaray 3, one goat in Bağsaray 4, one goat in Çeltikçi 1, and one goat in Ovacık 2 were determined as seropositive. One goat in Bağsaray 1, and two goats in Bağsaray 3 were determined as suspectable (Table 2).

Table 1. Distribution of *N. caninum* in aborted and non-aborted goats by herds.

N. caninum		**Aborted goats**				**Non-aborted goats**			
Village		**Negative**	**Positive**	**Suspectable**	**Total**	**Negative**	**Positive**	**Suspectable**	**Total**
Bağsaray 1	Count	6	0	0	6	28	0	1	29
	% of Total	8.3%	0.0%	0.0%	8.3%	13.9%	0.0%	0.5%	14.4%
Bağsaray 2	Count	8	0	0	8	27	1	1	29
	% of Total	11.1%	0.0%	0.0%	11.1%	13.4%	0.5%	0.5%	14.4%
Bağsaray 3	Count	7	0	0	7	25	2	0	27
	% of Total	9.7%	0.0%	0.0%	9.7%	12.4%	1.0%	0.0%	13.4%
Bağsaray 4	Count	9	0	0	9	30	1	0	31
	% of Total	12.5%	0.0%	0.0%	12.5%	14.9%	0.5%	0.0%	15.4%
Bağsaray 5	Count	3	0	0	3	7	0	0	7
	% of Total	4.2%	0.0%	0.0%	4.2%	3.5%	0.0%	0.0%	3.5%
Çeltikçi 1	Count	7	0	0	7	21	0	0	21
	% of Total	9.7%	0.0%	0.0%	9.7%	10.4%	0.0%	0.0%	10.4%
Çeltikçi 2	Count	1	0	0	1	9	0	0	9
	% of Total	1.4%	0.0%	0.0%	1.4%	4.5%	0.0%	0.0%	4.5%
Kuzköy 1	Count	6	0	0	6	13	0	0	13
	% of Total	8.3%	0.0%	0.0%	8.3%	6.5%	0.0%	0.0%	6.5%
Kuzköy 2	Count	10	0	1	11	8	1	0	9
	% of Total	13.9%	0.0%	1.4%	15.3%	4.0%	0.5%	0.0%	4.5%
Ovacık 1	Count	7	1	0	8	13	0	0	13
	% of Total	9.7%	1.4%	0.0%	11.1%	6.5%	0.0%	0.0%	6.5%
Ovacık 2	Count	5	1	0	6	12	1	0	13
	% of Total	6.9%	1.4%	0.0%	8.3%	6.0%	0.5%	0.0%	6.5%
Total	Count	69	2	1	72	193	6	2	201
	% of Total	95.8%	2.8%	1.4%	100.0%	96.0%	3.0%	1.0%	100.0%

Table 2. Distribution of *T. gondii* in aborted and non-aborted goats by herds.

T. gondii		**Aborted goats**				**Non-aborted goats**			
Village		**Negative**	**Positive**	**Suspectable**	**Total**	**Negative**	**Positive**	**Suspectable**	**Total**
Bağsaray 1	Count	6	0		6	27	1	1	29
	% of Total	8.3%	0.0%		8.3%	13.4%	0.5%	0.5%	14.4%
Bağsaray 2	Count	8	0		8	28	1	0	29
	% of Total	11.1%	0.0%		11.1%	13.9%	0.5%	0.0%	14.4%
Bağsaray 3	Count	7	0		7	24	1	2	27
	% of Total	9.7%	0.0%		9.7%	11.9%	0.5%	1.0%	13.4%
Bağsaray 4	Count	9	0		9	30	1	0	31
	% of Total	12.5%	0.0%		12.5%	14.9%	0.5%	0.0%	15.4%
Bağsaray 5	Count	3	0		3	7	0	0	7
	% of Total	4.2%	0.0%		4.2%	3.5%	0.0%	0.0%	3.5%
Çeltikçi 1	Count	7	0		7	20	1	0	21
	% of Total	9.7%	0.0%		9.7%	10.0%	0.5%	0.0%	10.4%
Çeltikçi 2	Count	1	0		1	9	0	0	9
	% of Total	1.4%	0.0%		1.4%	4.5%	0.0%	0.0%	4.5%
Kuzköy 1	Count	6	0		6	13	0	0	13
	% of Total	8.3%	0.0%		8.3%	6.5%	0.0%	0.0%	6.5%
Kuzköy 2	Count	11	0		11	9	0	0	9
	% of Total	15.3%	0.0%		15.3%	4.5%	0.0%	0.0%	4.5%
Ovacık 1	Count	8	0		8	13	0	0	13
	% of Total	11.1%	0.0%		11.1%	6.5%	0.0%	0.0%	6.5%
Ovacık 2	Count	5	1		6	12	1	0	13
	% of Total	6.9%	1.4%		8.3%	6.0%	0.5%	0.0%	6.5%
Total	Count	71	1		72	192	6	3	201
	% of Total	98.6%	1.4%		100.0%	95.5%	3.0%	1.5%	100.0%

In correlation findings; while there was a low and statistically insignificant positive correlation between abortion and *T. gondii* (r=0.073; p=0.230); a low and statistically insignificant negative correlation was found between abortion and *N. caninum* (r= - 0.004; p=0.941). A moderate and statistically significant positive correlation was found between *T. gondii* and *N. caninum* (r=0.615; p<0.001) (Table 3).

Table 3. Correlation findings between *T. gondii*, *N. caninum* and abortion.

Spearman's rho		**Abortion**	***T. gondii***	***N. caninum***
Abortion	Correlation Coefficient	1.000	.073	-.004
	Sig. (2-tailed)	.	.230	.941
	N	273	273	273
T. gondii	Correlation Coefficient	.073	1.000	.549**
	Sig. (2-tailed)	.230	.	.000
	N	273	273	273
N. caninum	Correlation Coefficient	-.004	.549**	1.000
	Sig. (2-tailed)	.941	.000	.
	N	273	273	273

**Correlation is significant at the 0.01 level (2-tailed).

DISCUSSION

Neospora caninum, protozoan parasite of the phylum Apicomplexa that lives as obligate intracellular tissue cysts. It causes a disease called neosporosis, which affects many systems (Donahoe et al. 2015). In order to determine the seropositivity of *N. caninum* in goats, ELISA test, Indirect Fluorescent Antibody Test (IFAT) methods are used. In studies conducted according to these methods, the lowest rate of seropositivity was found to be 0.7 % and the highest 26.6 % in the world (Naguleswaran et al. 2004, Tembue et al. 2011). There are limited studies on the seroprevalence of *N. caninum* on goats in Turkey. For the first time in Turkey, Sevgili et al. (2003) determined the *N. caninum* antibody level as 5 % in goats in Şanlıurfa by ELISA test. In addition, Sevgili et al. (2003) reported that this rate did not show a significant difference on race and age. Afterwards, Ütük et al. (2011) determined 13.8 % positivity in Saanen goats and 2.4 % positivity in Hair goats with ELISA test in Elazig, Erzurum and Kırşehir provinces. Cayvaz et al. (2011) obtained a positivity rate of 25.9 % with ELISA test in goats in the Niğde region. Özdamar et al. (2021) determined seropositivity in 8.69% of goats with ELISA test in 5 different study centers in Ordu's Mesudiye district. According to these studies, the lowest rate of seropositivity in goats in Turkey was 2.4 % and the highest was 25.9 %. In parallel with this information, in our study; *N. caninum* specific antibodies were determined in only 8 (2.9 %) blood serum taken from the 273 goats included in the study. In the blood serum of three goats (1.1 %), *N. caninum* suspectable antibody positivity was detected (Table 1). When the findings are compared with the results of other studies; it showed similarity to the values determined in hair goats in Elazig, Erzurum and Kirsehir provinces. It showed a lower course than the values detected in other studies.

Toxoplasma gondii, which has zoonotic importance, causes important reproductive system diseases in both humans and animals (Ataseven et al. 2006). Its distribution in the world varies. Age, education, lifestyle, nutrition and cleanliness play an important role in this difference (Dubey and Battie, 1988). In order to determine *T. gondii* seropositivity in goats, ELISA test, IFAT test, Sabin-Feldman Dye Test (SFDT), Indirect Hemagglutination (IHA) Complement Fixation Test (CFT), Modified Agglutination Test (MAT), Direct Agglutination Test (DAT) and Latex Agglutination Test (LAT) methods have been used (Karaca et al. 2007, Czopowicz et al. 2011, Almería et al. 2018, Bozukluhan et al. 2018). Many studies have been carried out using these methods in the world. According to these studies, the rate of *T. gondii* seropositivity in goats varies between 5.6 % and 100 %. *Toxoplasma gondii* seroprevalence in goats in Turkey was determined for the first time by Weiland and Dalchow (1970). According to this study, the seropositivity of *T. gondii* in goats in Turkey was reported as 51.6 % (Ataseven et al. 2006). Babür et al. (1997) determined the seroprevalence of *T. gondii* as 63.15 % by using SFDT test in goats in Çankırı province. Muz et al. (2013) determined 35.9 % positivity by using ELISA test in Hatay province. Ataseven et al. (2006) determined the seroprevalence of *T. gondii* as 72.7 % using SFDT test in local goat breeds in Eastern and Southeastern Anatolia Regions. Karaca et al. (2007) determined the seroprevalence of *T. gondii* as 80.61 % by using SFDT test in goats in Van province. In another study conducted in the same province, was reported that the seroprevalence of *T. gondii* was 33.3 % using the IHA test (Tütüncü et al. 2003). Ural et al. (2009) determined the seroprevalence of *T. gondii* as 81.08 % in SaneenxKilis goats and 82.53 % in Angora goats using the SFDT test in Ankara. Yağcı et al. (1997) reported that *T. gondii* seroprevalence was determined as 54 % using the SFDT test in goats in Ankara province. It has been reported that *T. gondii* seroprevalence was determined as 43.87 % using the SFDT test in Angora goats in Eskişehir (Babür et al. 1999). It has been reported that the seroprevalence of *T. gondii* was determined as 95.24 % using the SFDT test in Aleppo goats in Kilis province (Beyhan et al., 2013). According to studies conducted with different methods in goats in Turkey, *T. gondii* seropositivity rates vary between 12.1 % and 95.24 %. In parallel with this information, in our study; *T. gondii* specific antibodies were determined in only seven (2.6 %) blood serum taken from the goats included in the study. *T. gondii* suspectable antibody positivity was detected 1.1 % in goats (Table 2). When the findings are compared with the results of other studies; it was determined that *T. gondii* seroprevalence in Honamlı breed goats in Burdur province was lower than other studies. This result suggests that the goats are constantly fed on the rocky areas in the region with vegetation. In addition, it is known that the breeders in the region take serious control measures in their herds in order to ensure breeding and prevent diseases. Among these measures; it is thought that the selection of female progenies who have better condition and complete their development stages without any disease as breeding and the inclusion of breeding goats that did not abort in the previous pregnancy periods in the production contribute to this result.

Neosporosis progresses with non-specific symptoms such as abortion, congenital anomaly and stillbirth in domestic animals (Björkman and Uggla 1999). Cayvaz et al. (2011) stated that there was no statistically significant relationship between aborted goats and *N. caninum* seropositivity. Similarly, Özdamar et al. (2021) reported that there was no statistical relationship between aborted goats and *N. caninum* seropositivity. In this study, a low and statistically insignificant

negative correlation was found between abortion and *N. caninum* (r= - 0.004; p=0.941) (Table 3). Similarly, *T. gondii* is known to cause abortion in goats (Unzaga et al., 2014). It was determined that the seropositivity of *T. gondii* in aborted goats in Adana and Hatay provinces was 15.1 % by ELISA and 12.1 % by IHA. Öz et al. (1995) reported that there was no statistically significant difference (p>0.05) between aborted goats and non-aborted goats. In this study, a low and statistically insignificant positive correlation between abortion and *T. gondii* (r=0.073; p=0.230) (Table 3).

N. caninum is similar to *T. gondii* in many features (Björkman and Uggla 1999). Therefore, *N. caninum* was identified as *T. gondii* and misdiagnosed (Dubey 1999, Reichel 2000). It is stated that especially *T. gondii* and *N. caninum* give a cross-reaction in the diagnosis (Gondim et al. 2017). For this reason, research on *N. caninum* should also be investigated in terms of toxoplasmosis for a correct diagnosis. Bartova and Sedlak (2012) stated in their study conducted in the Czech Republic that all samples found positive for *N. caninum* were also positive for *T. gondii* antibodies.

In our study; seropositivity was determined for both *T. gondii* and *N. caninum*. And also there was a moderate and statistically significant positive correlation was found between *T. gondii* and *N. caninum* (r=0.615; p<0.001) (Table 3).

CONCLUSION

In this study, two important parasitic abortion factors, *N. caninum* and *T. gondii* were studied together in Honamlı goats reared in Burdur, Turkey. A new literature has been added to the very few literature, especially on the seroprevalence of *N. caninum* in Turkey. As a result of the study, the presence of both parasites, which cause abortion in goats in the region, was reported. It has been demonstrated once again that both diseases are extremely important for public health in the region with the consumption of raw or undercooked goat meat and goat milk.

Conflict of interest: The authors declared that there is no conflict of interest.

Ethical Approval: This study has received permission with, Anadolu University HADYEK number 825172047.09100/2018-4 and 04.06.2018 date. In addition, the authors declared that they comply with the Research and Publication Ethics.

Financial support: This research was supported by Anadolu University Scientific Research Projects Commission as project number 1805S215.

Acknowledgment: --

Description: --

REFERENCES

Aköz M, Aydın İ, Kamburgil K, Handemir E. Konya'nın Karapınar İlçesindeki Abort Yapan ve Yapmayan Koyunlarda *Toxoplasma Gondii*'nin Seroprevalansının İndirekt Fluoresan Antikor (IFA) Testi ile Belirlenmesi. Vet. Bil. Derg. 2009; 25, 1-2; 37-43.

Aktaş M, Şaki CE, Altay K, Şimşek S, Ütük AE, Köroğlu E, Dumanlı N. Doğu Anadolu Bölgesinin bazı illerinde bulunan sığırlarda *Neospora caninum*'un araştırılması. T Parazitol Derg. 2005; 29(1): 22-25.

Almería S, Cabezón O, Paniagua J, Cano-Terriza D, Jiménez-Ruiz S, Arenas-Montes A, Dubey JP, García Bocanegra I. *Toxoplasma Gondii* in sympatric domestic and wild ungulates in the Mediterranean ecosystem. Parasitol. Res. 2018; 117: 665-671

Andrianarivo AG, Choromanski L, McDonough SP, Packham, AE and Conrad, PA. Immunogenicity of a killed whole *Neospora caninum* tachyzoite preparation formulated with different adjuvants. International Journal for Parasitology. 1999; 29: 1613-1625.

Ataseven VS, Ataseven L, Tan T, Babür C, Oguzoglu TC. Seropositivity of agents causing abortion in local goat breeds in Eastern and South-eastern Anatolia, Turkey. Revue de médecine vétérinaire, 2006; 157(11): 545.

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, Pişkin FC, Bıyıkoğlu G, Dündar B, Yaralı C. Eskişehir Çifteler harası Ankara Keçilerinde Anti-*Toxoplasma Gondii* antikorlarının Sabin-Feldman Dye Test (SFDT) ile araştırılması. Türkiye Parazitol Derg. 1999; 23: 72-74.

Bartova E, Sedlak K. *Toxoplasma Gondii* and *Neospora caninum* antibodies in goats in the Czech Republic. Vet Med. 2012; 57(3): 111-114.

Beyhan YE, Babür C, Pekkaya S, Dalkiliç B. Investigation of anti-*Toxoplasma Gondii* antibodies in goats in Kilis province. Etlik Vet Mikrobiyol Derg. 2013; 24: 17-19.

Björkman C, Uggla A. Serological diagnosis of *Neospora caninum* infection. Int Parasitol. 1999; 2: 1497-1507

Bozukluhan K, Gökçe G, Uzlu E, Mor N, Erkılıç EE, Kızıltepe Ş. Kars Yöresindeki Koyun ve Keçilerde *Toxoplasma Gondii* Seroprevalansının Araştırılması. FÜ Sağ Bil Derg. 2018; 32(3): 169-172.

Cayvaz M, Karatepe M. Niğde Yöresi Keçilerinde *Neospora caninum*'un Seroprevalansı. Kafkas Univ Vet Fak Derg. 2011; 17: 935-939.

Czopowicz M, Kaba J, Szalu´s-Jordanow O, Nowicki M, Witkowski L, Frymus T. Seroprevalence of *Toxoplasma Gondii* and *Neospora caninum* infections in goats in Poland. Vet Parasitol. 2011; 178: 339-341

Donahoe S, Lindsay S, Krockenberger M, Phalen D, Šlapeta J: A review of neosporosis and pathologic findings of *Neospora caninum* infection in wildlife. International Journal for Parasitology: Parasites and Wildlife. 2015; 4: 216-238.

Dubey JP. Recent advances in Neospora and neosporosis. Vet Parasitol. 1999; 84: 349-367.

Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis Vet Parasitol. 1996; 67: 1-59.

Dubey JP. The history of *Toxoplasma Gondii* the first 100 years, J Eukaryot Microbiol. 2008; 55(6): 467-75.

Dubey JP. History of the discovery of the life cycle of *Toxoplasma Gondii*, Int. Parasitol. 2009; 39(8): 877-82.

Dubey JP, Beattie CP. Toxoplasmosis of Animal and Man. CRC Press Inc. Boca Raton. Florida. 1988: 1-220.

Gondim LF, Mineo JR, Schares G. Importance of serological cross-reactivity among *Toxoplasma Gondii*, *Hammondia spp., Neospora spp., Sarcocystis spp*. and Besnoitia besnoiti. Parasitology. 2017; 144(7), 851-868.

Karaca M, Babür C, Çelebi B, Akkan HA, Tütüncü M, Keleş İ, Kiliç S. Investigation on the seroprevalence of toxoplasmosis, listeriosis and brucellosis in goats living in the region of Van, Turkey. Van Vet J. 2007; 18(1): 45-49.

Kolören Z, Dubey JP. A review of toxoplasmosis in humans and animals in Turkey. Parasitology. 2020; 147(1): 12-28.

Muz MN, Altuğ N, Karakavuk M. Hatay yöresi süt işletmelerindeki ruminantlar ve çoban köpeklerinde *Toxoplasma Gondii* seroprevalansı ile kedi dışkılarında T. gondii benzeri Ookist tespiti. AVKAE Derg. 2013; 3(1): 38-45.

Naguleswaran A, Hemphill A, Rajapakse RPVJ, Sager H. Elaboration of a crude antigen ELISA for serodiagnosis of caprine neosporosis: validation of the test by detection of *Neospora caninum*-specific antibodies in goats from Sri Lanka. Vet Parasitol. 2004; 126: 257-262.

Nicolle C, Manceaux L. Sur une infection a corps de Leishman (ou organismes voisons) du gondi. C R Acad Sci 1908; 147: 736.

Öz İ, Özyer M, Çorak R. Adana Yöresi Sığır Koyun ve Keçilerinden ELISA ve IHA Testleri ile Toxoplasmosis' in Yaygınlığının Araştırılması. Etlik Veteriner Mikrobiyoloji Dergisi. 1995; 8(1): 87-99.

Özdamar D, KARATEPE B, YILDIRIM A. Ordu'nun Mesudiye İlçesi Keçilerinde anti-*Neospora caninum* Antikorlarının ELISA Testi ile Araştırılması. Kocatepe Vet J. 2021; 14(1): 1-5.

Reichel MP. *Neospora caninum* infections in Australia and New Zealand. Australian Vet J. 2000; 78: 258-261.

Sevgili M, Çimtay İ, Keskin O. Şanlıurfa Yöresindeki Keçilerde *Neospora caninum* Enfeksiyonunun Seroprevalansı. Türkiye Parazitol Derg. 2003; 27: 249-251

Tembue AASM, Ramos RAN, Sousa TR, Albuquerque AR, Costa AJ, Meunier IMJ, Faustino MAG, Alves LC. Serological survey of *Neospora caninum* in small ruminants from Pernambuco State, Brazil. Rev Bras Parasitol Vet. 2011; 20: 246-248.

Tenter AM, Heckeroth AR, Weiss LM. *Toxoplasma Gondii*: from animals to humans. Int J Parasitol. 2000; 30: 1217-1258.

Tütüncü M, Ayaz E, Yaman M, Akkan HA. The seroprevalence of *Toxoplasma Gondii* in sheep, goats and cattle detected by indirect haemaglutination (IHA) test in the region of Van, Turkey. Ind Vet J. 2003; 47: 401-403.

Ural K, Alıç Ural D, Çelebi B, Haydardedeoğlu AE, Babür C, Barıtcı I, Kılıç S. Seroprevalence of listeriosis, toxoplasmosis and brucellosis in Saanen x Kilis and Angora goats in Ankara. Fırat Üniv Sağ Bil Vet. 2009; 23: 79-82.

Unzaga JM, Moré G, Bacigalupe D, Rambeaud M, Pardini L, Dellarupe A, De Felice L, Gos ML, Venturini MC. *Toxoplasma Gondii* and *Neospora caninum* infections in goat abortions from Argentina. Parasitology International, 2014; 63: 865-867.

Ütük AE, Simsek S, Piskin FC, Balkaya I. Detection of *Neospora caninum* IgG Antibodies in Goats in Elazig, Erzurum and Kırsehir Provinces of Turkey. Isr J Vet Med. 2011; 66: 157-160.

Yağcı Ş, Babür C, Karaer Z, Çakmak A. Ankara yöresinde keçilerde toxoplasmosis. Etlik Veteriner Mikrobiyoloji Dergisi. 1997; 9(1): 94-98.

Weiland VG, Dalchow W. Toxoplasma Infektionen bei Haustieren in der Türkei (Serologische untersuchungen im SabinFeldman test). Berl. Münch. Tierärztl. Wschr., 1970; 83: 65-68.

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022):15(2):151-160

DOI: 10.30607/kvj. 1062346

RESEARCH ARTICLE

Evaluation of the Effects of Chrysin on Diclofenac-Induced Cardiotoxicity in Rats by the Markers of Oxidative Stress, Endoplasmic Reticulum Stress and Apoptosis

Cihan GÜR[1]*, Özge KANDEMİR[2], Fatih Mehmet KANDEMİR[1]

[1]Atatürk University, Veterinary Faculty, Biochemistry Department, 25050, Erzurum, Turkey
[2]Aksaray University, Aksaray Technical Sciences Vocational School, 68000, Aksaray, Turkey

ABSTRACT

Diclofenac (DF) is among non-steroidal anti-inflammatory drugs and in high doses it has toxic effects on various tissues, including the heart. Chrysin (CRS) has many beneficial effects such as antioxidant and anti-apoptotic. In the present study, the effects of CRS on DF-induced cardiotoxicity were investigated. For this purpose, after DF and/or CRS treatments were applied to Sprague Dawley rats, the markers of oxidative stress, endoplasmic reticulum stress and apoptosis in heart tissues and serum CK-MB levels were analyzed. The data obtained displayed that oxidative stress induced by DF was alleviated by triggering the expression of SOD, CAT and GPx enzymes and increasing GSH levels after CRS administration, and a decrease in MDA levels occurred. It was also determined that the expressions of ATF-6, PERK, IRE1 and GRP78, which are ER stress markers and induced by DF, were downregulated after CRS treatment. Additionally, CRS suppressed Bax and Caspase-3 expressions and increased Bcl-2 expression in connection with ER stress. Another finding of this study demonstrated that CK-MB levels, which are an important indicator of heart damage, decreased after CRS administration. As a result, CRS provided a significant protection against cardiotoxicity caused by DF.
Keywords: Cardiotoxicity, chrysin, diclofenac, endoplasmic reticulum stress, oxidative stress.

***

Ratlarda Diklofenak ile İndüklenen Kardiyotoksisite Üzerine Krisinin Etkilerinin Oksidatif Stres, Endoplazmik Retikulum Stresi ve Apoptoz Belirteçleri ile Değerlendirilmesi

ÖZ

Diklofenak (DF), steroid olmayan anti-inflamatuar ilaçlar arasındadır ve yüksek dozlarda kalp dahil çeşitli dokularda toksik etkiler göstermektedir. Krisin (KRS) antioksidan ve anti-apoptotik gibi birçok yararlı etkiye sahiptir. Sunulan çalışmada DF ile indüklenen kardiyotoksisite üzerine KRS'nin etkileri araştırılmıştır. Bu amaçla Sprague Dawley ratlara DF ve/veya KRS uygulamaları yapıldıktan sonra kalp dokularında oksidatif stres, endoplazmik retikulum stresi ve apoptoz belirteçleri ve serum CK-MB seviyeleri analiz edildi. Elde edilen veriler DF ile indüklenen oksidatif stresin KRS uygulamasından sonra SOD, CAT ve GPx enzimlerinin ekspresyonlarının tetiklenmesi ve GSH seviyelerinin artması ile hafiflediğini ve MDA seviyelerinde azalma meydana geldiğini göstermektedir. Ayrıca ER stres belirteçleri olan ve DF'nin indüklediği ATF-6, PERK, IRE1 ve GRP78 ekspresyonlarının KRS tedavisinden sonra aşağı yönlü düzenlendiği belirlendi. ER stresi ile bağlantılı olarak KRS'nin Bax ve Kaspaz-3 ekspresyonlarını baskıladığı ve Bcl-2 ekspresyonunu arttırdığı da elde edilen veriler arasındadır. Ayrıca kalp hasarının önemli bir göstergesi olan CK-MB seviyelerinin KRS uygulamasından sonra azaldığı görüldü. Sonuç olarak DF'nin neden olduğu kardiyotoksisiteye karşı KRS'nin önemli bir koruma sağladığı tespit edildi.
Anahtar Kelimeler: Diklofenak, endoplazmik retikulum stresi, kardiyotoksisite, krisin, oksidatif stres.

To cite this article: Gür C. Kandemir Ö. Kandemir F.M. Evaluation of the Effects of Chrysin on Diclofenac-Induced Cardiotoxicity in Rats by the Markers of Oxidative Stress, Endoplasmic Reticulum Stress and Apoptosis. Kocatepe Vet J. (2022):15(2):151-160

Submission: 24.01.2022 **Accepted**: 29.03.2022 **Published Online**: 13.04.2022
ORCID ID; *CG: 0000-0001-6775-7858, ÖK: 0000-0001-8884-4168, FMK: 0000-0002-8490-2479*
*Corresponding author e-mail: cihan.gur@atauni.edu.tr

GİRİŞ

Steroid olmayan anti-inflamatuar ilaçlar (NSAID), ağrı ve inflamasyonun kontrolü için birçok ülkede yaygın olarak kullanılmaktadır (Alabi and Akomolafe, 2020; Prince, 2018). ABD'de her yıl yaklaşık 70 milyon NSAID'nin reçete edildiği bilinmektedir (Prince, 2018). Bunların normal terapötik dozlarının hafif yan etki gösterdiği ancak aşırı dozlarda ciddi toksisite sergilediği bildirilmiştir. NSAID'lerin çoğu COX enzimlerini (COX-1 ve COX-2) inhibe etmektedir. COX-1 ve COX-2, gastrointestinal, renal ve kardiyovasküler homeostazın korunmasında önemli bir rol oynayan araşidonik asidin eikosanoidlere dönüşümünü katalize ederler (Abdulmajeed et al., 2015; Alabi and Akomolafe, 2020). Son yıllarda, klinik uygulamalarda NSAID'lerin kullanımının güvenliği sorgulanır hale gelmiştir. Öte yandan kardiyovasküler sonuçları değerlendirme kapasitesine sahip randomize ve kontrollü çalışmaların eksikliği nedeniyle, NSAID'lerin kullanımı ile kardiyovasküler riskte artışa ilişkin kanıtlar yetersiz kalmaktadır. Bununla birlikte, ileriye dönük klinik çalışmaların ve meta-analizlerin sonuçları, bu ilaçların, artan miyokard enfarktüsü, iskemik kalp hastalıkları, kalp yetmezliği ve arteriyel hipertansiyon riskini içeren önemli olumsuz kardiyovasküler etkilere neden olduğunu göstermektedir (Abdulmajeed et al., 2015).

Diklofenak (DF), fenil asetik asitten türetilen ve 4'hidroksidiklofenak şeklinde metabolize edilen seçici olmayan bir NSAID'dir (Prince, 2018). DF, anti-inflamatuar, analjezik, antinosiseptif özelliklerinden dolayı romatoid artrit ve ağrı tedavisinde yaygın olarak kullanılmaktadır. DF'nin terapötik faydalarına rağmen, kayda değer ciddi yan etkilere sahip olduğu bilinmektedir. Bunlar arasında gastrointestinal toksisite ve akciğerler, kalp, karaciğer ve böbrek dokularında meydana gelen hasar önemli bir yere sahiptir (Abdulmajeed et al., 2015). DF kalp krizi ve felç riskini yaklaşık %40 oranında arttırmaktadır (Ghosh et al., 2016). Yüksek dozlarda DF'nin mitokondriyal transmembranda hasara neden olan glutatyon konjugasyonunun azalmasına neden olduğu bilinmektedir. Bunlar toksik metabolitleri oluşturur ve hücresel membranlarda peroksidatif hasara neden olan antioksidan aktiviteyi azaltır ve böylece nekroz oluşumuna ve ATP üretiminin azalmasına neden olur (Prince, 2018). Bu nedenle doğal olarak oluşan ve antioksidan özelliklere sahip bileşiklerin kullanımının DF toksisitesine karşı önemli koruma sağlayacağı düşünülmektedir.

Çeşitli meyve ve sebzelerde bol miktarda bulunan flavonoidler, farklı farmakolojik özellikleri nedeniyle birçok sağlık sorununa karşı etkin bir şekilde kullanılmakta ve yeni araştırmalar için umut vermektedir (Ileriturk et al., 2021). Krisin (KRS), yaygın olarak kullanılan ve birçok bitki özü, bal ve propoliste bulunan flavonoid aile üyelerinden biridir (Kucukler et al., 2021). KRS'nin antioksidan, anti-inflamatuar, antikanser, antidiyabetik, antialerjik ve antiapoptotik özellikleri birçok çalışmanın odak noktası olmuş (Aksu et al., 2018; Taslimi et al., 2019; Temel et al., 2020) ancak DF'nin neden olduğu kardiyotoksisite üzerine etkileri araştırılmamıştır.

Sunulan çalışmada DF'nin kalp hasarına karşı KRS'nin potansiyel koruyucu etkileri oksidatif stres, endoplazmik retikulum stresi ve apoptozda rol oynayan bazı biyolojik belirteçler üzerinden araştırılmıştır.

MATERYAL VE METOT

Çalışmada kullanılan hayvanlar ve etik kurul onayı

Çalışmada 35 adet Sprague Dawley erkek rat kullanıldı. Ağırlıkları 250-300 g olan hayvanların yaşları 12-14 hafta arasındaydı. Barındırıldıkları ortam şartları 24 ± 1 °C sıcaklık, %45 ± %5 nem ve 12 saat aydınlık/karanlık döngüsüne sahipti. Ratlar standart laboratuvar yemi ve musluk suyu ile ad libitum olarak beslendiler. Çalışma için etik kurul onayı Atatürk Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'ndan alınmıştır (Onay no: 2021-10/248).

Deney tasarımı

DF ve KRS'nin dozları önceki çalışmalar referans alınarak belirlendi (Ileriturk et al., 2021; Prince, 2018). Çalışmaya başlamadan önce hayvanlar her grupta 7 adet olacak şekilde 5 farklı gruba ayrıldı ve 1 hafta boyunca ortama adaptasyonları sağlandı. Gruplar ise şu şekilde tasarlandı;

1. Grup (Kontrol); hayvanlara sadece 5 gün intaperitoneal (i.p.) yoldan 0,5 ml serum fizyolojik verildi.
2. Grup (KRS); hayvanlara 5 gün süre ile oral olarak 50 mg/kg/gün dozunda KRS verildi.
3. Grup (DF); Hayvanlara kalp toksisitesi oluşturmak için 4. ve 5. günlerde 50 mg/kg/vücut ağırlığı i.p. DF uygulandı.
4. Grup (DF + KRS 25 mg/kg); hayvanlara 5 gün ard arda oral olarak 25 mg/kg/gün KRS verildi ayrıca 4. ve 5. günlerde 50 mg/kg/vücut ağırlığı i.p. DF uygulandı.
5. Grup (DF + KRS 50 mg/kg); hayvanlara 5 gün ard arda oral olarak 50 mg/kg/gün KRS verildi ayrıca 4. ve 5. günler 50 mg/kg/vücut ağırlığı i.p. DF uygulandı.

Deney sonunda hayvanlar hafif sevofloran anestezisi altında dekapite edilerek kalp dokuları ve kanları alındı.

Serumda CK-MB seviyelerinin belirlenmesi

Ratlardan alınan kanlar sarı kapaklı serum tüplerine aktarıldı ve 4000 rpm'de +4 °C santrifüj edilerek

serumları ayrıldı. Serumlarda Mindray Perfect Plus 400 cihazı vasıtasıyla CK-MB seviyeleri analiz edildi.

Kalp dokusunda lipid peroksidasyon derecesinin belirlenmesi

Kalp dokusunda lipid peroksidasyon derecesi malondialdehit (MDA) seviyelerinin ölçümü ile belirlendi. Bunun için ratlardan alınan kalp dokuları sıvı azot ile dondurulduktan sonra homojenizatörde toz hale getirildi. Daha sonra dokular %1,15'lik KCl içerisinde homojenize edilerek +4 °C ve 3500 rpm'de 15 dakika boyunca santrifüj edildi. Elde edilen süpernatantta lipit peroksidasyon (LPO) derecesi MDA'nın tiyobarbitürik asit ile reaksiyonu sonucu oluşturduğu rengin 532 nm'deki absorbansının ölçümüne dayanan metot ile analiz edildi (Placer et al., 1966).

Kalp dokusunda glutatyon seviyelerinin belirlenmesi

Kalp dokusunda glutatyon (GSH) seviyelerinin analizi için homojenatlar 10000 rpm'de 20 dakika boyunca santrifüjlenerek süpernatantlar elde edildi. Daha sonra Sedlak and Lindsay (1968)'in yöntemi kullanılarak dokuda GSH seviyeleri analiz edildi.

Real-Time PCR analizleri

Toz hale getirilen kalp dokularından 100 mg alınarak steril ependorf tüplere aktarıldı. Daha sonra QIAzol Lysis Reagent (Qiagen, Cat: 79306, Germany) kullanılarak üreticinin verdiği talimatlar ile total RNA izolasyonu yapıldı. Elde edilen total RNA'ların konsantrasyonları NanoDrop cihazında belirlendi ve alınan sonuçlara göre total RNA eşitlemesi yapıldı. Total RNA'lardan cDNA sentezi High-Capacity cDNA Reverse Transcription Kiti (Applied Biosystems™ Cat: 4368814, USA) ile firmanın verdiği talimatlara göre gerçekleştirildi. cDNA'lar dizilimleri Tablo 1'de sunulan genlerin mRNA transkript seviyelerinin belirlenmesinde kullanıldı. RT-PCR aşamasında SOD, KAT, GPx, Bax, Bcl-2, Kaspaz-3, ATF-6, PERK, IRE1 ve GRP78 genlerine ait primerler, SYBR Green PCR Master Mix, RNaz free water ve cDNA'lar ile mix hazırlandı. Sonrasında ROTOR-GENE Q (Qiagen, Germany) ile üç tekrarlı olarak analiz işlemi yapıldı. □ -aktin housekeeping gen olarak kullanıldı ve elde edilen CT değerlerinden Livak and Schmittgen (2001)'in 2−DeltaDeltaCT metodu ile göreli mRNA transkript seviyeleri hesaplandı.

Tablo 1. Primer dizilimleri
Table 1. Primer sequences

Gene	Sequences (5'-3')	Length (bp)	Accession No
SOD	F: AATGTGGCTGCTGGAAAGGA	171	NM_017050.1
	R: GCTTCCAGCATTTCCAGTCT		
KAT	F: CTGAGAGAGTGGTACATGCA	130	NM_012520.2
	R: AATCGGACGGCAATAGGAGT		
GPx	F: CAAGGTGCTGCTCATTGAGA	139	NM_030826.4
	R: ATGTCCGAACTGATTGCACG		
Bcl-2	F: GACTTTGCAGAGATGTCCAG	214	NM_016993.2
	R: TCAGGTACTCAGTCATCCAC		
Bax	F: TTTCATCCAGGATCGAGCAG	154	NM_017059.2
	R: AATCATCCTCTGCAGCTCCA		
Kaspaz-3	F: ACTGGAATGTCAGCTCGCAA	270	NM_012922.2
	R: GCAGTAGTCGCCTCTGAAGA		
ATF-6	F: TCAACTCAGCACGTTCCTGA	130	NM_001107196.1
	R: GACCAGTGACAGGCTTCTCT		
PERK	F: GATGCCGAGAATCATGGGAA	198	NM_031599.2
	R: AGATTCGAGAAGGGACTCCA		

IRE1	F: GCAGTTCCAGTACATTGCCATTG R: CAGGTCTCTGTGAACAATGTTGA	163	NM_001191926.1
GRP78	F: CATGCAGTTGTGACTGTACCAG R: CTCTTATCCAGGCCATATGCAA	143	NM_013083.2
β-Aktin	F: CAGCCTTCCTTCTTGGGTATG R: AGCTCAGTAACAGTCCGCCT	360	NM_031144.3

SOD: Süperoksit dismutaz, KAT: Katalaz, GPx: Glutatyon peroksidaz, Bcl-2: B hücreli lenfoma 2, Bax: Bcl-2 ile ilişkili X proteini, ATF-6: aktive edilmiş transkripsiyon faktörü 6, PERK: çift sarmallı RNA ile aktive olan kinaz (PKR) benzeri ER kinaz, IRE1: enzim 1 gerektiren inositol, GRP78: glukozla düzenlenen protein 78.

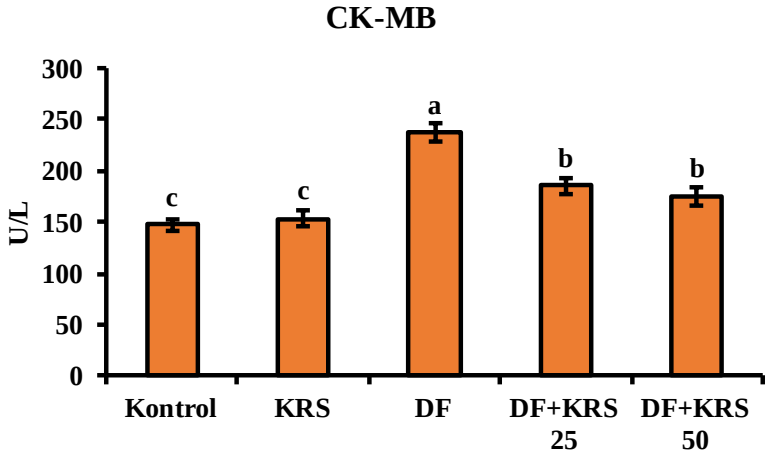
İstatistiksel Analizler

Kalp dokularında gerçekleştirilen analizlerin istatistiksel değerlendirmesi IBM SPSS programında yapıldı. Deney grupları arasında istatistiksel farkın olup olmadığı One-way ANOVA ve Tukey's post hoc tests ile belirlendi. Sonuçlar ortalama±standart sapma olarak sunuldu.

BULGULAR

Diklofenak ve krisin verilen ratların serum CK-MB seviyeleri

Ratlara DF ve KRS verildikten sonra kalp hasarının belirlenmesi için serumda CK-MB seviyeleri belirlendi ve sonuçlar Şekil 1'de sunuldu. Elde edilen verilere göre DF verilen hayvanların serum CK-MB seviyelerinde kontrol grubuna göre artış meydana geldiği görüldü ($p<0.05$). Bununla birlikte KRS tedavisinin kalp hasarına karşı koruma sağlayarak CK-MB seviyelerini DF grubuna göre önemli derecede azalttığı belirlendi ($p<0.05$). Öte yandan KRS tedavisinin dozları arasında anlamlı bir farkın olmadığı tespit edildi.



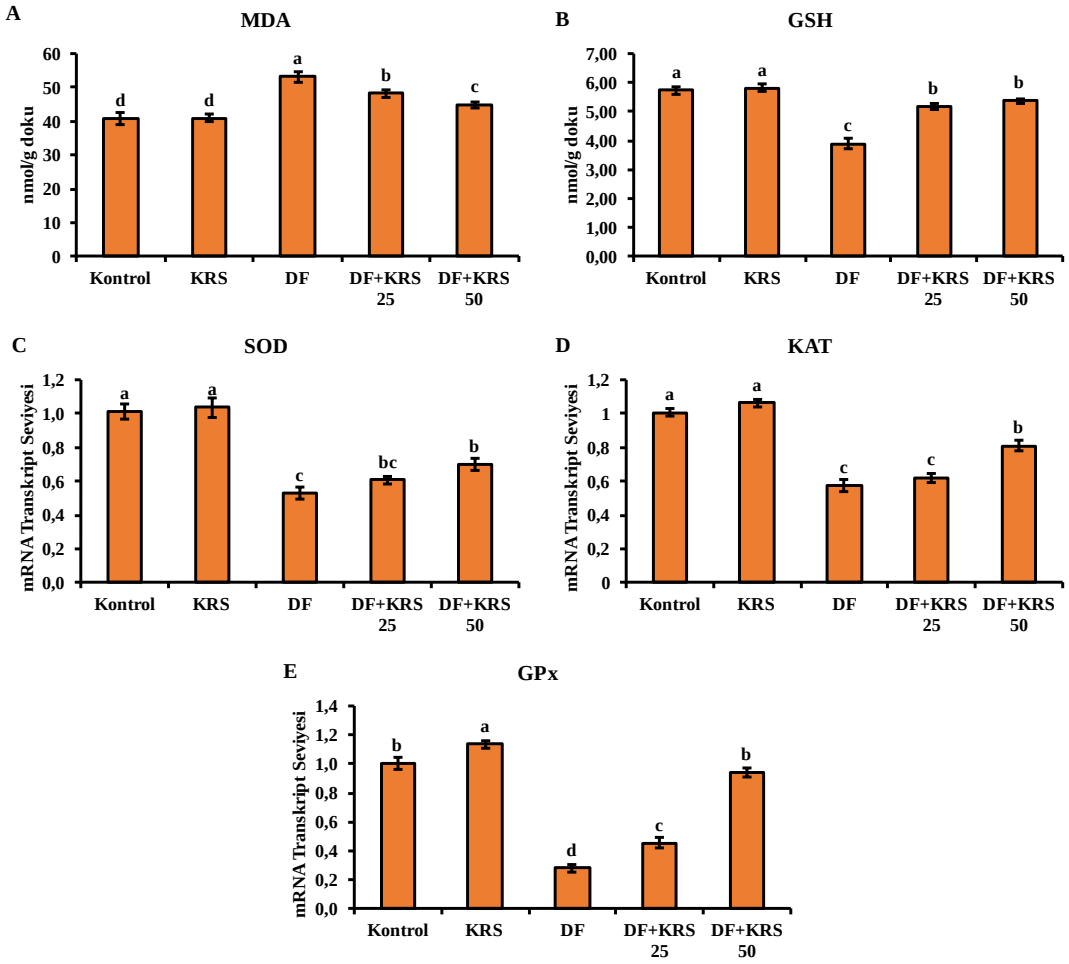
Şekil 1: Diklofenak ve krisin uygulamaları sonrasında ratların serum CK-MB seviyeleri. Tüm veriler ortalama±SD olarak ifade edildi (n=7). Sütunlardaki farklı harfler (a–c) gruplar arası istatistiksel farklılığı göstermektedir ($p<0.05$). (CK-MB: Kreatin kinaz-MB, DF: Diklofenak, KRS: Krisin)

Figure 1: Serum CK-MB levels of rats after diclofenac and chrysin administration. All data were expressed as mean±SD (n=7). Different letters (a–c) on the columns show a statistical difference ($p<0.05$).

Diklofenak ve krisin verilen ratların kalp dokularında oksidatif stres durumu

DF ve KRS uygulamalarından sonra kalp dokusunda oksidatif stres durumunu belirlemek için doku MDA ve GSH seviyeleri ile SOD, KAT ve GPx enzimlerinin mRNA transkript seviyeleri analiz edildi. Sonuçlar Şekil 2'de özetlenmiştir. DF uygulamasının kalp dokusunda GSH seviyeleri ile SOD, KAT ve GPx enzimlerinin mRNA transkript seviyelerini önemli derecede azalttığı belirlendi ($p<0.05$). Ayrıca MDA seviyelerinin DF grubunda kontrol grubuna göre artış gösterdiği ve oksidatif strese neden olduğu görüldü ($p<0.05$). KRS tedavisinden sonra GSH seviyelerinde artış olduğu ($p<0.05$) ancak dozlar arasında anlamlı bir fark oluşmadığı tespit edildi. KAT ve GPx enzimlerinin mRNA transkript seviyelerinin

özellikle 50 mg/kg verilen grupta DF grubuna göre arttığı belirlendi ($p<0.05$). DF ve DF+KRS 25 grupları arasında SOD enziminin ekpresyon seviyeleri anlamlı bir fark oluşturmazken, KRS'nin 50 mg/kg uygulamasının SOD ekspresyonunu yukarı yönlü düzenlediği görüldü ($p<0.05$). Ayrıca MDA seviyelerinin KRS tedavisi ile doz bağımlı olarak azaldığı ($p<0.05$) ve böylece DF ile tetiklenen oksidatif stresin hafiflediği belirlendi.

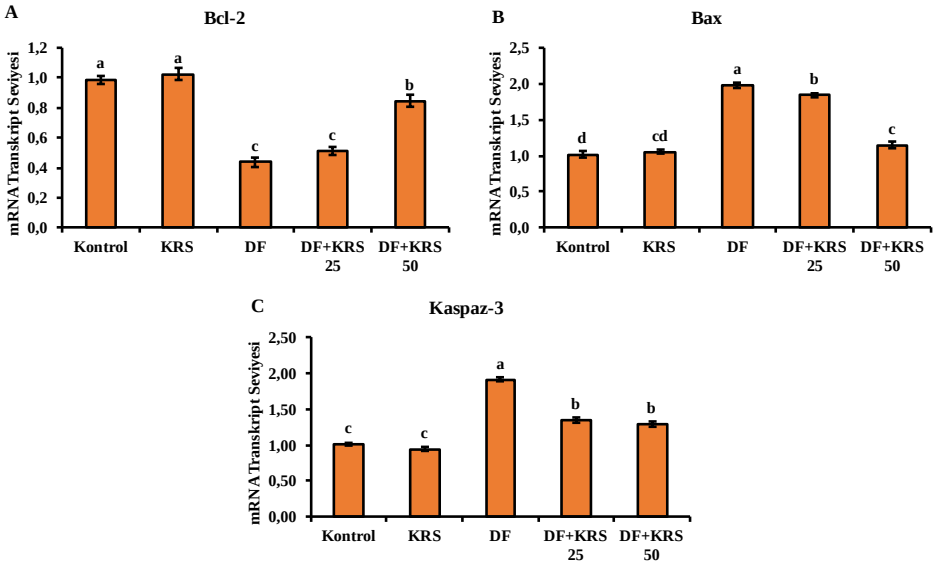


Şekil 2: Diklofenak ve krisin uygulamaları sonrasında ratların kalp dokularında oksidatif stres durumu. Tüm veriler ortalama±SD olarak ifade edildi (n=7). Sütunlardaki farklı harfler (a–d) gruplar arası istatistiksel farklılığı göstermektedir ($p<0.05$). (DF: Diklofenak, GSH: Glutatyon, GPx: Glutatyon peroksidaz, KAT: Katalaz, KRS: Krisin, MDA: Malondialdehit, SOD: Süperoksit dismutaz)

Figure 2: Oxidative stress status in heart tissues of rats after diclofenac and chrysin administration. All data were expressed as mean±SD (n=7). Different letters (a–d) on the columns show a statistical difference ($p<0.05$).

Diklofenak ve krisin verilen ratların kalp dokularında apoptoz durumu

Şekil 3'te verilen sonuçlara göre DF verilen ratların kalp dokularında anti-apoptotik protein olan Bcl-2 ekspresyon seviyelerinin azaldığı, apoptotik belirteçler olan Bax ve Kaspaz-3 ekspresyonlarının ise yukarı yönlü düzenlendiği belirlendi ($p<0.05$). Öte yandan 50 mg/kg KRS tedavisinin Bcl-2 mRNA transkript seviyelerini DF grubuna göre arttırdığı görüldü ($p<0.05$). 25 mg/kg KRS tedavisinin ise Bcl-2 ekspresyonunu etkilemediği belirlendi. Ayrıca Bax ve Kaspaz-3 mRNA transkript seviyelerinin her iki dozda da azaldığı tespit edildi ($p<0.05$). Kaspaz-3 ekspresyonu üzerinde dozlar arasında anlamlı bir farkın oluşmadığı da elde edilen veriler arasındadır.

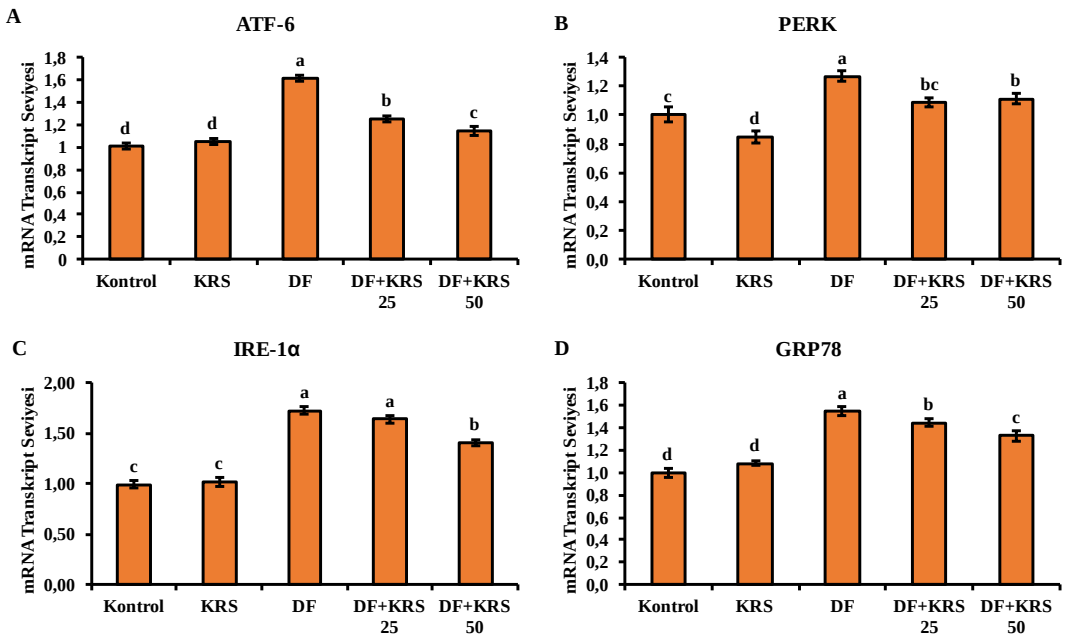


Şekil 3: Diklofenak ve krisin uygulamaları sonrasında ratların kalp dokularında apoptoz durumu. Tüm veriler ortalama±SD olarak ifade edildi (n=7). Sütunlardaki farklı harfler (a–d) gruplar arası istatistiksel farklılığı göstermektedir ($p<0.05$). (Bax: Bcl-2 ile ilişkili X proteini, Bcl-2: B hücreli lenfoma 2, DF: Diklofenak, KRS: Krisin)

Figure 3: Apoptosis status in heart tissues of rats after diclofenac and chrysin administration. All data were expressed as mean±SD (n=7). Different letters (a–d) on the columns show a statistical difference ($p<0.05$).

Diklofenak ve krisin verilen ratların kalp dokularında endoplazmik retikulum stres durumu

Kalp dokusunda ER stresinin belirlenmesi için ATF-6, PERK, IRE-1 ve GRP78 genlerinin ekspresyon seviyeleri RT-PCR analizi ile belirlendi. DF verilen ratların kalp dokularında ATF-6, PERK, IRE1 ve GRP78 mRNA transkript seviyelerinin artış göstererek ER stresine neden olduğu belirlendi ($p<0.05$). ATF-6 ve GRP78 ekspresyonlarının KRS tedavisinden sonra doz bağımlı olarak azaldığı görüldü ($p<0.05$). IRE-1 ekspresyonunun düşük doz verilen KRS tedavisinde DF grubu ile fark oluşturmadığı ancak yüksek doz verilen grupta önemli derece azaldığı tespit edildi ($p<0.05$). PERK ekspresyonunun ise DF grubuna göre hem düşük dozda hem de yüksek dozda azaldığı ($p<0.05$) ancak dozların kendi aralarında anlamlı bir fark oluşturmadığı görüldü.



Şekil 4: Diklofenak ve krisin uygulamaları sonrasında ratların kalp dokularında endoplazmik retikulum stres durumu. Tüm veriler ortalama±SD olarak ifade edildi (n=7). Sütunlardaki farklı harfler (a–d) gruplar arası istatistiksel farklılığı göstermektedir ($p<0.05$). (ATF-6: aktive edilmiş transkripsiyon faktörü 6, DF: Diklofenak, GRP78: glukozla düzenlenen protein 78, IRE1: enzim 1 gerektiren inositol, KRS: Krisin, PERK: çift sarmallı RNA ile aktive olan kinaz (PKR) benzeri ER kinaz)

Figure 4: Endoplasmic reticulum stress status in heart tissues of rats after diclofenac and chrysin administrations. All data were expressed as mean±SD (n=7). Different letters (a–d) on the columns show a statistical difference ($p<0.05$).

TARTIŞMA

Bazı çalışmalar, kardiyomiyositlerde COX-2'nin inhibisyonunun, NSAID alan hastalarda kalp yetmezliğine katkıda bulunabileceğini öne sürmektedir. Bunun nedeni kardiyomiyositlerde COX-2'nin seçici olarak silinmesinin kalp debisini baskılaması ve indüklenmiş aritmojeneze duyarlılığı arttırmasıdır (Ghosh et al., 2016). Bir NSAID olan DF'nin de yaygın kullanımı göz önüne alındığında DF kaynaklı kardiyotoksisite acil olarak çözülmesi gereken sağlık sorunlarından biridir. Bu nedenle sunulan çalışmada DF'nin kardiyotoksik etkisi üzerine KRS'nin potansiyel etkileri araştırılmıştır.

Diklofenak kaynaklı toksisite mekanizmasının mitokondriyal disfonksiyon ve artan oksidatif stresi içerdiği bilinmektedir (Aycan et al., 2018). Oksidatif stres hücrelerde aşırı miktarda reaktif oksijen türlerinin (ROT) üretildiği durumlarda meydana gelmektedir (Semis et al., 2021b) ve DF'nin in vivo metabolizması, ROT'ların aşırı üretimi ile ilişkilendirilmiştir. Bu da sonuç olarak oksidatif stres, genomik hasar ve hücresel apoptoz ile ölüme neden olur (Owumi et al., 2020). MDA, çoklu doymamış yağ asidi oksidasyonunun bir yan ürünüdür ve lipid oksidasyonunun ve oksidatif stresin bir göstergesidir (Küçükler et al., 2022). Sunulan çalışmada DF'nin kalp dokusunda oksidatif strese neden olarak MDA seviyelerini arttırdığı düşünülmektedir. Öte yandan antioksidan özelliği ile bilinen KRS'nin DF kaynaklı ROT'ların süpürülmesini sağlayarak oksidatif stresi hafiflettiği ve MDA seviyelerini azalttığı görüldü.

SOD, KAT ve GPx gibi enzimatik ve GSH gibi enzimatik olmayan antioksidanlar, oksidatif hasara maruz kalan hücrelerin korunmasında önemli bir rol oynar (Eldutar et al., 2017). SOD süperoksit radikalinin hidrojen peroksite dönüştürülmesinden sorumluyken, hücreler için oldukça toksik bir bileşik olan hidrojen peroksit KAT ve GPx tarafından su ve moleküler oksijene parçalanmaktadır. GSH ise oksidatif metabolizma sonucu meydana gelen serbest radikalleri bağlayarak okside forma (GSSG) dönüşmekte ve vücudu oksidatif hasardan korumaktadır (Gur et al., 2021b). Öte yandan çeşitli toksik ajanlar antioksidan enzimlerin aktivitelerinde azalmaya neden olmaktadır (Adeyemi and Olayaki, 2018; Gür et al.; Gur et al., 2021a; Kandemir et al., 2017). Ayrıca artan ROT'lar GSH'ı GSSG formuna dönüştürerek GSH depolarının tükenmesine sebep olmaktadır (Aja et al., 2020; Radwan et al., 2018). Tüm bunlar bir araya gelerek oksidatif strese neden olmakta ve canlı organizmalarda hayati rolleri bulunan makromoleküllere hasar vererek dokuların işlevlerinin yitirilmesine yol açmaktadır (İleritürk et al., 2021; Kuzu et al., 2021; Yardım et al., 2021). Sunulan çalışmada da DF'nin SOD, KAT ve GPx ekspresyonlarını baskıladığı ve muhtemel artan ROT seviyelerine bağlı olarak GSH depolarının azaldığı ve oksidatif stres meydana geldiği belirlendi. Öte yandan KRS'nin SOD, CAT ve GPx enzimlerinin gen ekspresyonlarını tetiklediği ve ROT'ların temizlenmesiyle GSH depolarının yenilendiği görüldü.

Endoplazmik retikulum (ER) ökaryotik hücrelerde hayati bir role sahiptir. ER'nin görevleri arasında transmembran proteinlerin biyosentezi, proteinlerin üç boyutlu yapılarının oluşumu ve translasyon sonrası modifikasyonlarının yanı sıra kalsiyum homeostazı, lipid ve steroid biyosentezi yer almaktadır(AL-Megrin et al., 2020; Liu et al., 2015). Çeşitli faktörler, ER'de katlanmamış veya yanlış katlanmış proteinlerin birikmesine neden olan endoplazmik retikulum stresi adı verilen hücresel bir süreci indükler (Semis et al., 2021c). ROT'ların da bu süreçte önemli bir rol üstlendiği bilinmektedir (Yardim et al., 2020). ER, stres durumu meydana geldiğinde, katlanmamış protein yanıtı (UPR) olarak bilinen bir dizi sinyal yolunun aktivasyonu ile bu sürece yanıt vermektedir (Jiang et al., 2009). İlk etapta UPR'nin meydana gelmesi ile hücrelerde normal fonksiyonlar geri getirilmeye çalışılır. Ancak UPR sürecindeki uzama hücrelerde işlev bozukluğu ile birlikte apoptoza neden olabilmektedir (Yardim et al., 2020). Memelilerde ER transmembran protein sensörleri olan enzim 1 gerektiren inositol (IRE1), çift sarmallı RNA ile aktive olan kinaz (PKR) benzeri ER kinaz (PERK) ve aktive edilmiş transkripsiyon faktörü 6 (ATF6) UPR'ye eşlik etmekte ve ER stresinin önemli göstergeleri olarak kabul edilmektedir (Deng et al., 2016). GRP78 ise ER tabanlı bir şaperon ve ER homeostazının merkezi düzenleyicisidir (Celik et al., 2020). DF'nin de ER stresini tetiklediğine dair kanıtlar bulunmaktadır (Foufelle and Fromenty, 2016). Sunulan çalışmada

DF'nin kalp dokusunda muhtemelen ROT'ların artışına bağlı olarak ER homeostazını bozduğu ve ATF-6, PERK, IRE1 ve GRP78 ekspresyonlarını tetikleyerek UPR yanıtını aktive ettiği belirlendi. Apoptotik belirteçler de göz önüne alındığında kalp dokusunda UPR yanıtının uzayarak apoptoza neden olduğu da söylenebilir. Öte yandan KRS tedavisinin kalp dokusunda ATF-6, PERK, IRE1 ve GRP78 ekspresyonlarını baskıladığı ve böylece ER stresini hafiflettiği görüldü. KRS'nin bu etkisi apoptozun baskılanması ile birlikte kalp dokusunu DF'nin hasar verici etkisinden koruduğunu düşündürmektedir.

Apoptoz, doku homeostazını sağlayan oksidatif hasar dahil olmak üzere çeşitli fizyolojik ve patolojik uyaranların yönlendirdiği normal bir hücre ölüm sürecidir (Kandemir et al., 2018). Yüksek derecelerde meydana gelen apoptoz, çeşitli patolojik durumların temel nedenlerinden biri olarak değerlendirilmektedir (Semis et al., 2021a). Ayrıca biriken kanıtlar oksidatif stres ve apoptoz arasında yakın bir ilişkinin olduğunu göstermektedir (Aja et al., 2020; Yesildag et al., 2021). Aktif merkezlerinde sistein kalıntıları bulunan ve aspartata özgü olan kaspazlar apoptotik sürecin merkezinde yer alan proteolitik enzimlerdir. Mitokondri tarafından üretilen ROT'lar sitokrom c salınımına ve bu da kaspaz-9 aktivasyonuna neden olur. Kaspaz-9 aktivasyonu ile apoptozun en önemli göstergelerinden biri olan kaspaz-3 etkin hale gelmektedir (Semis et al., 2021c; Yardim et al., 2020). Bcl-2 mitokondriyal dış membrana bağlanarak sitokrom c salınımını engellemekte ve böylece anti-apoptotik işlev göstermektedir (Ansari et al., 2017). Bax proteini ise büyüme faktörlerini tüketerek apoptozu desteklemektedir (Geng et al., 2015). Yakın tarihli bir çalışmada DF'nin kalp dokularında kaspaz-3 protein ekspresyonunun alan %'sinde önemli bir artışa ve bcl-2 protein ekspresyonunun alan %'sinde önemli bir düşüşe neden olduğu bildirilmiştir (Oda and Derbalah, 2018). Sunulan çalışmada RT-PCR ile analiz edilen Bax ve kaspaz-3 mRNA transkript seviyelerinin DF verilen grupta önemli derecede artış sergilediği belirlendi. Ayrıca DF'nin Bcl-2 ekspresyonunu baskılayarak muhtemel olarak mitokondriden sitokrom c ve kaspaz-3 gibi apoptotik faktörlerin serbest bırakılmasına ve böylece içsel bir apoptoza sebep olduğu düşünülmektedir. Öte yandan KRS tedavisinin Bax ve kaspaz-3 ekspresyonlarını baskıladığı Bcl-2 ekspresyonunu ise aktive ettiği görüldü. Böylece KRS'nin apoptozun baskılanması yoluyla DF kaynaklı kardiyotoksisiteyi hafifletebileceği belirlendi. Çeşitli toksik ajanlar tarafından farklı dokularda meydana getirilen toksisitelerde de KRS'nin anti-apoptotik etki göstererek dokuları koruduğu bildirilmiştir (Aksu et al., 2018; Çelik et al., 2020; Hanedan et al., 2018; Kandemir et al., 2020).

CK-MB, hücre enerji metabolizmasında hayati bir role sahip olan ve akut kardiyak hasar için kesin bir belirteç olarak kabul edilen kreatin kinazın alt tipidir. Bu biyobelirtecin serum düzeylerinin yükselmesi, dejeneratif veya nekrotizan kardiyak miyositlerin bozulmuş hücre membranından sızmasına bağlanabilir (Oda and Derbalah, 2018). Önceki bir çalışmada DF uygulamasının CK-MB seviyelerinde artışa neden olduğu bildirilmiştir (Arafa et al., 2020). Sunulan çalışmada da DF uygulaması ile kardiyak hasar meydana geldiği ve böylece serum CK-MB seviyelerinde artış olduğu düşünülmektedir. Öte yandan KRS'nin kalp dokusunu DF'nin yıkıcı etkisinden koruduğu CK-MB'nin seruma salınımının azalmasından anlaşılmaktadır.

SONUÇ

Çalışmadan elde edilen tüm veriler birlikte değerlendirildiğinde DF'nin oksidatif strese neden olarak zincirleme reaksiyonlarla ER stresine ve akabinde apoptoza neden olduğu ve buna bağlı olarak ratların kalp dokularında hasar meydana getirebileceği görüldü. Bununla birlikte KRS tedavisinin DF kaynaklı oksidatif stresi hafifleterek ER stresine ve apoptoza karşı koruma sağlayabileceği ve böylece kalp dokusunu DF'nin toksik etkilerine karşı koruyabileceği belirlendi.

Çıkar Çatışması: Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

Yazarların Katkı Oranı: [1]CG: %60, ÖK: %20, FMK: %20

Etik İzin: [1]Bu çalışma ATAUNİ HADYEK 10/248 numara ve 25.11.2021 tarih ile izin almıştır.
[2] Bu yazıda sunulan veri, bilgi ve belgeler akademik ve etik kurallar çerçevesinde elde edilmiştir.

Finansal Destek: Bu çalışmada herhangi bir finansal destek alınmamıştır.

KAYNAKLAR

Abdulmajeed N, Alnahdi H, Ayas N, Mohamed A. Amelioration of cardiotoxic impacts of diclofenac sodium by vitamin B complex. Eur Rev Med Pharmacol Sci. 2015; 19(4): 671-681.

Adeyemi WJ, Olayaki LA. Diclofenac–induced hepatotoxicity: Low dose of omega-3 fatty acids have more protective effects. Toxicol Rep. 2018; 5: 90-95.

Aja PM, Izekwe FI, Famurewa AC, Ekpono EU, Nwite FE, Igwenyi IO, Awoke JN, Ani OG, Aloke C, Obasi NA. Hesperidin protects against cadmium-induced pancreatitis by modulating insulin secretion, redox imbalance and iNOS/NF-κB signaling in rats. Life Sci. 2020; 259: 118268.

Aksu EH, Kandemir FM, Küçükler S, Mahamadu A. Improvement in colistin-induced reproductive damage, apoptosis, and autophagy in testes via reducing oxidative

stress by chrysin. J Biochem Mol Toxicol. 2018; 32(11): e22201.

AL-Megrin WA, Alomar S, Alkhuriji AF, Metwally DM, Mohamed SK, Kassab RB, Abdel Moneim AE, El-Khadragy MF. Luteolin protects against testicular injury induced by lead acetate by activating the Nrf2/HO-1 pathway. IUBMB life. 2020; 72(8): 1787-1798.

Alabi QK, Akomolafe RO. Kolaviron diminishes diclofenac-induced liver and kidney toxicity in wistar rats via suppressing inflammatory events, upregulating antioxidant defenses, and improving hematological indices. Dose-Response. 2020; 18(1): 1559325819899256.

Ansari MA, Raish M, Ahmad A, Alkharfy KM, Ahmad SF, Attia SM, Alsaad AM, Bakheet SA. Sinapic acid ameliorate cadmium-induced nephrotoxicity: In vivo possible involvement of oxidative stress, apoptosis, and inflammation via NF-κB downregulation. Environ Toxicol Pharmacol. 2017; 51: 100-107.

Arafa MH, Mohammad NS, Atteia HH. Rho-Kinase inhibitors ameliorate diclofenac-induced cardiotoxicity in chloroquine-treated adjuvant arthritic rats. Life sci. 2020; 254: 117605.

Aycan İÖ, Elpek Ö, Akkaya B, Kıraç E, Tuzcu H, Kaya S, Coşkunfırat N, Aslan M. Diclofenac induced gastrointestinal and renal toxicity is alleviated by thymoquinone treatment. Food Chem Toxicol. 2018; 118: 795-804.

Celik H, Kucukler S, Ozdemir S, Comakli S, Gur C, Kandemir FM, Yardim A. Lycopene protects against central and peripheral neuropathy by inhibiting oxaliplatin-induced ATF-6 pathway, apoptosis, inflammation and oxidative stress in brains and sciatic tissues of rats. Neurotoxicology. 2020; 80: 29-40.

Çelik H, Kucukler S, Çomaklı S, Caglayan C, Özdemir S, Yardım A, Karaman M, Kandemir FM. Neuroprotective effect of chrysin on isoniazid-induced neurotoxicity via suppression of oxidative stress, inflammation and apoptosis in rats. Neurotoxicology. 2020; 81: 197-208.

Deng H, Kuang P, Cui H, Chen L, Luo Q, Fang J, Zuo Z, Deng J, Wang X, Zhao L. Sodium fluoride (NaF) induces the splenic apoptosis via endoplasmic reticulum (ER) stress pathway in vivo and in vitro. Aging (Albany NY). 2016; 8(12): 3552.

Eldutar E, Kandemir FM, Kucukler S, Caglayan C. Restorative effects of Chrysin pretreatment on oxidant–antioxidant status, inflammatory cytokine production, and apoptotic and autophagic markers in acute paracetamol-induced hepatotoxicity in rats: an experimental and biochemical study. J Biochem Mol Toxicol. 2017; 31(11): e21960.

Foufelle F, Fromenty B. Role of endoplasmic reticulum stress in drug-induced toxicity. Pharmacol res perspect. 2016; 4(1): e00211.

Geng X, Shao H, Zhang Z, Ng JC, Peng C. Malathion-induced testicular toxicity is associated with spermatogenic apoptosis and alterations in testicular enzymes and hormone levels in male Wistar rats. Environ Toxicol Pharmacol. 2015; 39(2): 659-667.

Ghosh R, Goswami SK, Feitoza LFB, Hammock B, Gomes AV. Diclofenac induces proteasome and mitochondrial dysfunction in murine cardiomyocytes and hearts. Int J Cardiol. 2016; 223: 923-935.

Gur C, Kandemir FM, Darendelioglu E, Caglayan C, Kucukler S, Kandemir O, Ileriturk M. Morin protects against acrylamide-induced neurotoxicity in rats: an investigation into different signal pathways. Environ Toxicol Pharmacol. 2021a; 49808–49819.

Gur C, Kandemir O, Kandemir FM. Investigation of the effects of hesperidin administration on abamectin-induced testicular toxicity in rats through oxidative stress, endoplasmic reticulum stress, inflammation, apoptosis, autophagy, and JAK2/STAT3 pathways. Environ Toxicol. 2021b.

Gür C, Kandemir F, Genç A. Bortezomib ile Kalp Hasarı Oluşturulan Ratlarda Berberinin Oksidatif ve Nitrozatif Stres Üzerine Etkisi. Türk Doğa ve Fen Dergisi. 2020; 9(2): 118-126.

Hanedan B, Ozkaraca M, Kirbas A, Kandemir FM, Aktas MS, Kilic K, Comakli S, Kucukler S, Bilgili A. Investigation of the effects of hesperidin and chrysin on renal injury induced by colistin in rats. Biomed Pharmacother. 2018; 108: 1607-1616.

Ileriturk M, Benzer F, Aksu EH, Yildirim S, Kandemir FM, Dogan T, Dortbudak MB, Genc A. Chrysin protects against testicular toxicity caused by lead acetate in rats with its antioxidant, anti-inflammatory, and antiapoptotic properties. J Food Biochem. 2021; 45(2): e13593.

İleritürk M, Doğan T, Kandemir O. Investigation of the effect of berberine with arginase activity and oxidantantioxidant parameters on bortezomib-induced spleen injury in rats. Kocatepe Vet J. 2021; 14(1): 6-15.

Jiang CC, Yang F, Thorne RF, Zhu BK, Hersey P, Zhang XD. Human melanoma cells under endoplasmic reticulum stress acquire resistance to microtubule-targeting drugs through XBP-1-mediated activation of Akt. Neoplasia. 2009 (5); 11: 436-447.

Kandemir FM, Kucukler S, Caglayan C, Gur C, Batil AA, Gülçin İ. Therapeutic effects of silymarin and naringin on methotrexate-induced nephrotoxicity in rats: Biochemical evaluation of anti-inflammatory, antiapoptotic, and antiautophagic properties. J Food Biochem. 2017; 41(5): e12398.

Kandemir FM, Yıldırım S, Kucukler S, Caglayan C, Darendelioğlu E, Dortbudak MB. Protective effects of morin against acrylamide-induced hepatotoxicity and nephrotoxicity: A multi-biomarker approach. Food Chem Toxicol. 2020; 138: 111190.

Kandemir FM, Yildirim S, Kucukler S, Caglayan C, Mahamadu A, Dortbudak MB. Therapeutic efficacy of zingerone against vancomycin-induced oxidative stress, inflammation, apoptosis and aquaporin 1 permeability in rat kidney. Biomed Pharmacother. 2018; 105: 981-991.

Kucukler S, Benzer F, Yildirim S, Gur C, Kandemir FM, Bengu AS, Ayna A, Caglayan C, Dortbudak MB. Protective effects of chrysin against oxidative stress and inflammation induced by lead acetate in rat kidneys: a biochemical and histopathological approach. Biol Trace Elem Res. 2021; 199(4): 1501-1514.

Kuzu M, Kandemir FM, Yıldırım S, Çağlayan C, Küçükler S. Attenuation of sodium arsenite-induced cardiotoxicity and neurotoxicity with the antioxidant, anti-inflammatory, and antiapoptotic effects of hesperidin. Environ Toxicol Pharmacol. 2021; 28(9): 10818-10831.

Küçükler S, Kandemir FM, Yıldırım S. Protective effect of chrysin on indomethacin induced gastric ulcer in rats: role of multi-pathway regulation. Biotech Histochem. 2022; 1-14.

Liu W, Yang B, Wu L, Zou W, Pan X, Zou T, Liu F, Xia L, Wang X, Zhang D. Involvement of NRF2 in perfluorooctanoic acid-induced testicular damage in male mice. Biol Reprod. 2015; 93(2): 41, 41-47.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. methods. 2001; 25(4): 402-408.

Oda SS, Derbalah AE. Impact of diclofenac sodium on tilmicosin-induced acute cardiotoxicity in rats (tilmicosin and diclofenac cardiotoxicity). Cardiovasc Toxicol. 2018; 18(1): 63-75.

Owumi SE, Aliyu-Banjo NO, Odunola OA. Selenium attenuates diclofenac-induced testicular and epididymal toxicity in rats. Andrologia. 2020; 52(9): e13669.

Placer ZA, Cushman LL, Johnson BC. Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. Anal Biochem. 1966; 16(2): 359-364.

Prince SE. Diclofenac-induced renal toxicity in female Wistar albino rats is protected by the pre-treatment of aqueous leaves extract of Madhuca longifolia through suppression of inflammation, oxidative stress and cytokine formation. Biomed Pharmacother. 2018; 98: 45-51.

Radwan OK, Alzahrani YA, Kassab RB. Neuroprotective efficiency of Mangifera indica leaves extract on cadmium-induced cortical damage in rats. Metab Brain Dis. 2018; 33(4): 1121-1130.

Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem. 1968; 25: 192-205.

Semis HS, Gur C, Ileriturk M, Kandemir FM, Kaynar O. Evaluation of Therapeutic Effects of Quercetin Against Achilles Tendinopathy in Rats via Oxidative Stress, Inflammation, Apoptosis, Autophagy, and Metalloproteinases. Am J Sports Med. 2021a; 03635465211059821.

Semis HS, Gur C, Ileriturk M, Kaynar O, Kandemir FM. Investigation of the anti-inflammatory effects of caffeic acid phenethyl ester in a model of λ-Carrageenan–induced paw edema in rats. Hum Exp Toxicol. 2021b; 40(12-suppl): S721-S738.

Semis HS, Kandemir FM, Kaynar O, Dogan T, Arikan SM. The protective effects of hesperidin against paclitaxel-induced peripheral neuropathy in rats. Life Sci. 2021c; 287: 120104.

Taslimi P, Kandemir FM, Demir Y, İleritürk M, Temel Y, Caglayan C, Gulçin İ. The antidiabetic and anticholinergic effects of chrysin on cyclophosphamide-induced multiple organ toxicity in rats: Pharmacological evaluation of some metabolic enzyme activities. J Biochem Mol Toxicol. 2019; 33(6): e22313.

Temel Y, Kucukler S, Yıldırım S, Caglayan C, Kandemir FM. Protective effect of chrysin on cyclophosphamide-induced hepatotoxicity and nephrotoxicity via the inhibition of oxidative stress, inflammation, and apoptosis. Naunyn Schmiedebergs Arch Pharmacol. 2020; 393(3): 325-337.

Yardim A, Kandemir FM, Ozdemir S, Kucukler S, Comakli S, Gur C, Celik H. Quercetin provides protection against the peripheral nerve damage caused by vincristine in rats by suppressing caspase 3, NF-κB, ATF-6 pathways and activating Nrf2, Akt pathways. Neurotoxicology. 2020; 81: 137-146.

Yardım A, Kucukler S, Özdemir S, Çomaklı S, Caglayan C, Kandemir FM, Çelik H. Silymarin alleviates docetaxel-induced central and peripheral neurotoxicity by reducing oxidative stress, inflammation and apoptosis in rats. Gene. 2021; 769: 145239.

Yesildag K, Gur C, Ileriturk M, Kandemir FM. Evaluation of oxidative stress, inflammation, apoptosis, oxidative DNA damage and metalloproteinases in the lungs of rats treated with cadmium and carvacrol. Mol Biol Rep. 2021; 1-11.

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022):15(2):161-166

DOI: 10.30607/kvj. 1058115

RESEARCH ARTICLE

Investigation of *Theileria equi*, *Babesia caballi*, *Neospora* spp. and *Toxoplasma gondii* by Serological Methods in Horse Breed for Touristic Purpose in Nevşehir Province

Ufuk EROL[1*], Selçuk PEKKAYA[2], Cahit BABÜR[3], Yunus Emre BEYHAN[4], Fatih KUZUGÜDEN[5], İbrahim BALKAYA[6], Pınar DEMİR[7], Armağan Erdem ÜTÜK[8]

[1]Sivas Cumhuriyet University, Faculty of Veterinary Medicine, Department of Parasitology, 58140, Sivas, Turkey
[2]Republic of Türkiye Ministry of Agriculture And Forestry, Veterinary Control Central Research Institute, 06020, Ankara, Turkey
[3]Republic of Türkiye Ministry of Health Public Health General Directorate, National Parasitology Reference Laboratory, 06430, Ankara, Turkey
[4]Van Yüzüncü Yıl University, Faculty of Medicine, Department of Medical Parasitology, 65080, Van, Turkey
[5]Republic of Türkiye Ministry of Agriculture And Forestry, Nevşehir Directorate of Provincial Agriculture And Forestry, 50300, Nevsehir, Turkey
[6]Ataturk University, Faculty of Veterinary Medicine, Department of Parasitology, 25240, Erzurum, Turkey
[7]Kafkas University, Faculty of Veterinary Medicine, Department of Livestock Economics, 36100, Kars, Turkey
[8]Cukurova University, Faculty of Ceyhan Veterinary Medicine, Department of Parasitology, 01330 Adana-Turkey

ABSTRACT

Protozoan diseases cause deaths in horses and serious economic losses. Some of them have zoonotic potential and are important for public health. The aim of this study was to determine the seroprevalence of tissue (*Neospora* spp. and *Toxoplasma gondii*) and blood (*Theileria equi* and *Babesia caballi*) protozoa of horses, raised for touristic purposes, in the Nevşehir provice Central Anatolia Region of Turkey. For this aim, a total of 105 blood samples were collected from female horses from various breeds between the ages of 3-24 in Nevşehir province. Sera samples were tested against anti-*Neospora* spp., *T.equi* and *B.caballi* antibodies with c-ELISA method, while Sabin Feldman Dye Test was used for the detection of anti-*T.gondii* antibodies. At the end of the serologic examination, it was determined that two of 105 (1.90%) horses had anti-*Neospora* spp.; nine (8.57%) had anti-*T.gondii*; two (1.90%) had anti-*B.caballi* and 77 (73.33%) had anti-*T.equi* antibodies. Mixed infections were also detected in one horse (0.95%) caused by *T.equi*, *B.caballi* and *T.gondii*, one (0.95%) by *T.equi* and *B.caballi*, two (1.90%) by *Neospora* spp. and *T.equi* and in six horses (5.71%) by *T.gondii* and *T.equi*. There was no statistically significant difference between the age groups and *Neospora* spp., *T. gondii* and *B.caballi* seropositivity, while high *T.equi* seroprevalence was found to be significant in horses over 7 years old with the chi-square test. With this study, it was set forth that the exposure rate to *T.equi* in horses in Nevşehir province in the Central Anatolia region is quite high; also the prevalence increases with age, and protozoal mixed infections are likely to occur, which should not be ignored in treatment.

Keywords: Horse, Nevşehir, Blood and Tissue Protozoa, Serology, Turkey.

****

Nevşehir İlinde Turistik Amaçlı Yetiştirilen Atlarda *Theileria equii*, *Babesia caballi*, *Neospora* spp. ve *Toxoplasma gondii*'nin Serolojik Yöntemlerle Araştırılması

ÖZ

Protozoal hastalıklar atlarda ölümlere ve ciddi ekonomik kayıplara neden olur. Bazılarının zoonotik potansiyelleri ise halk sağlığı açısından önem arz eder. Bu çalışmanın amacı Nevşehir ilinde turistik amaçlarla yetiştirilen atlarda doku (*Neospora* spp. ve *Toxoplasma gondii*) ve kan (*Theileria equi* ve *Babesia caballi*) protozoonlarının yaygınlığını belirlemektir. Bu amaçla yaşları 3-24 arasındaki 105 dişi attan kan alınarak serumları çıkarılmıştır. Elde edilen serumlar *Neospora* spp., *T.equi* ve *B.caballi* antikorlarına karşı c-ELISA ve *T.gondii* antikorlarına karşı da Sabin-Feldman Dye testleri ile incelenmiştir. Çalışma sonucunda 105 atın ikisinde (%1,90) anti-*Neospora* spp., dokuzunda (%8.57) anti-*T.gondii*, ikisinde (%1,90) anti-*B.caballi* ve 77 (%73,33)'sinde de anti-*T.equi* antikorları tespit edilmiştir. Atlardan birinde (%0,95) *T.equi*, *B.caballi* ve *T.gondii*, birinde (%0,95) *T.equi* ve *B.caballi*, ikisinde (%1,90) *Neospora* spp. ve *T.equi* ve altısında (%5,71) ise *T.gondii* ve *T.equi*'nin neden olduğu miks enfeksiyonlar belirlenmiştir. Ki-kare testi ile ≤ 7 ve >7 yaş grupları ile *Neospora* spp., *T.gondii* ve *B.caballi* seroprevalansları arasında istatistiksel açıdan önemli bir fark bulunmazken, 7 yaş üzerindeki atlarda yüksek *T.equi* seroprevalansının önemli olduğu görülmüştür. Bu çalışma ile İç Anadolu bölgesinde Nevşehir ilinde atlarda *T.equi*'ye maruziyet oranının oldukça yüksek olduğu, prevalansın yaşla birlikte arttığı, ayrıca protozoon kaynaklı miks enfeksiyonların görülebileceği ve bunun tedavide göz ardı edilmemesi gereken bir durum olduğu ortaya konmuştur.

Anahtar kelimeler: At, Nevşehir, Kan ve Doku Protozoonları, Seroloji, Türkiye.

To cite this article:Erol U.,Pekkaya S., Babür C., Beyhan YE., Kuzugüden F., Balkaya İ., Demir P., Ütük AE. Investigation of Theileria equi, Babesia caballi, Neospora spp. and Toxoplasma gondii by Serological Methods in Horse Breed for Touristic Purpose in Nevşehir Province
Kocatepe Vet J. (2022):15(2):161-166
Submission: 15.01.2022 **Accepted**: 29.03.2022 **Published Online**: 13.04.2022
ORCID ID; *UE:0000-0002-6766-1335, SP:0000-0002-8291-9592, CB:0000-0002-6524-3260, YEB:0000-0002-1696-4803, FK:0000-0003-3606-1940, İB:0000-0002-1103-2812, PD:0000-0002-7010-0475, AEÜ:0000-0002-7986-3583*
*Corresponding author e-mail: ufukerol@cumhuriyet.edu.tr

GİRİŞ

Paraziter protozoonlar atlarda verim kayıplarına ve ölümlere neden olan önemli patojen etkenlerdendir. Bu protozoon etkenlerden en önemlileri arasında *Theileria equi*, *Babesia caballi Neospora* spp. ve *Toxoplasma gondii* bulunmaktadır (Dubey 2016, Taylor ve ark 2016, Dubey ve ark 2017).

Theileria equi ve *B.caballi* tektırnaklıların equine piroplasmosis etkenleridir (Wise ve ark, 2014). Etkenler atlara Ixodid keneler aracılığıyla biyolojik olarak veya enfekte atların kanıyla bulaşık iğne, cerrahi aletler ile enfekte hayvanlardan sağlıklı hayvanlara yapılan kan transfüzyonu ile mekanik olarak nakledilebilmektedir (Wise ve ark 2014, Taylor ve ark 2016). Equine piroplasmosis hemen hemen tüm dünyada görülmekle birlikte özellikle tropikal ve subtropikal bölgede endemiktir (Knowles 1996). Hastalık atlarda genellikle hemolitik anemiye neden olmakla birlikte bazen böbrek ve karaciğer bozukluklarına, abortlara ve çoklu organ yetmezliği gibi sistemik enfeksiyonlara ve nadiren de ölüme neden olabilmektedir (Wise ve ark 2014).

Equine neosporosis *Neospora* soyundaki türlerin atlarda meydana getirdiği hastalığa verilen addır. *Neospora* soyundaki *N.caninum* ve *N.hughesi* türleri atlarda klinik semptomlara neden olmaktadır. Bu türlerden *N.caninum*'un son konağı köpek, dingo, kurt ve çakal iken *N.hughesi*'nin son konağı bilinmemektedir (Marsh ve ark 1998, Dubey ve ark 2017). Etkenler ara konak olan atlara son konakların dışkılarıyla dış ortama çıkan ve sporlanan ookistlerin ağız yoluyla alınmasıyla bulaşmaktadır (Dubey ve ark 2017). Neosporosis atlarda sinirsel semptomlara, böbrek ve akciğer hasarına, abortlara ve nadiren de ölümlere neden olabilmektedir (Veronesi ve ark 2008, Dubey ve ark 2017).

Toxoplasma gondii tüm dünyada en önemli zoonoz protozoon etkenlerden birisidir. *Toxoplasma gondii*'nin son konağı evcil ve yabani kediler iken, ara konakları ise koyun, keçi, insan, at ve kuş gibi sıcakkanlı canlılardır (Dubey 2016). Etken hemen hemen tüm dünyada insanlarda oldukça yaygındır ve hatta Avrupa kıtasında her yıl yaklaşık 2 milyon insanın *T.gondii* ile enfekte olduğu tahmin edilmektedir (WHO 2021). *Toxoplasma gondii* insanlarda çoğunlukla asemptomatik olarak seyrederken immun sistemi zayıf insanlarda baş ağrısı, bitkinlik, kas ağrısı ve lenfadenopatiye, daha önce etkenle karşılaşmamış gebe annelerde ise düşüklere ve konjenital hasarlı çocukların doğmasına neden olmaktadır (Dubey 2016). Atlar etkenle; sporlanmış *T.gondii* ookistleri bulunan besin maddelerinin (ot ve su gibi) ağız yoluyla alınmasıyla enfekte olmaktadır. Atlarda toxoplasmosis genellikle subklinik enfeksiyonlara neden olurken, ateş, ataksi, retinal dejenerasyon, ensefalomyelitis ve atık gibi atipik klinik semptomlara da neden olabilmektedir (Miao ve ark 2013).

Türkiye'de atlarda kan ve doku protozoonlarının yaygınlığının serolojik yöntemlerle araştırılması amacıyla çok sayıda çalışma yapılmıştır (Akkan ve ark 2003, Akca ve ark 2004, Balkaya ve Erdoğmuş 2006a,b, Öncel ve ark, 2007, Acici ve ark 2008, Karatepe ve ark 2009, Karatepe ve ark 2010, Sarı ve ark 2010, Karatepe ve Karatepe 2012, Zhou ve ark 2016, Akkoyun ve Oğuz 2019). Ancak yapılan literatür taraması sonucunda İç Anadolu Bölgesinde bulunan ve turistik amaçlı at yetiştiriciliğinin yoğun olarak yapıldığı Nevşehir ilinde atlarda kan ve doku protozoonlarının yaygınlığı ile ilgili bir çalışmaya rastlanmamıştır. Bu çalışmanın amacı Nevşehir ilinde turistik amaçlı olarak yetiştirilen atlarda kan (*T. equi* ve *B.caballi*) ve doku (*Neospora* spp. ve T. gondii) protozoonlarının yaygınlığını serolojik yöntemlerle belirlemektir.

MATERYAL METOD

Çalışma materyali 2015-2016 yılları arasında Nevşehir ilinde turistik amaçlı yetiştirilen yaşları 3-24 arasında değişen, farklı ırklardan 105 dişi at ile oluşturulmuştur. Söz konusu atlardan kan örnekleri serum tüplerine alınarak soğuk zincir şartları sağlanarak Veteriner Kontrol Merkez Araştırma Enstitüsü'ne getirilmiştir. Kan örneklerinden serumlar oda sıcaklığında 4000 rpm'de 10 dk süreyle santrifüj yapılarak çıkarılmıştır. Çıkarılan serumlar 1,5 mL'lik mikrosantrifüj tüplerine alınarak serolojik yöntemlerle *T.equi*, *B. caballi*, *Neospora* spp. ve *T.gondii* yönünden incelenene kadar -20°C'lik derin dondurucularda saklanmıştır.

Çalışma kapsamında atlardan elde edilen kan serumlarında *T.equi*, *B.caballi* ve *Neospora* spp. antikorlarının yaygınlığının araştırılması amacıyla competitive ELISA (cELISA) kitleri (VMRD®, Pullman, ABD) kullanılmıştır. Testler üretici firmanın talimatlarına göre yapılmış ve sonuçlar 630 nm optik dansitede microplate okuyucu ile (ELx 800 UV, Universal Microplate Reader, Bio-Tec Instruments, Inc.) değerlendirilmiştir. Test sonuçlarının değerlendirilmesinde *T.equi* ve *B.caballi* yüzde inhibisyon değeri %40'dan fazla olan örnekler pozitif, %40'dan küçük olanlar ise negatif olarak değerlendirilmiştir. *Neospora* spp. yönünden sonuçların değerlendirilmesinde ise yüzde inhibisyon değeri %30'dan büyük olan örnekler pozitif, %30'dan küçük olanlar ise negatif olarak kaydedilmiştir.

Çalışma sırasında *T.equi*, *B.caballi* ve *Neospora* spp.'nin araştırılması amacıyla yapılan c-ELISA yönteminin doğru çalışıp çalışmadığının belirlenmesi amacıyla her testte üretici firma tarafından sağlanan pozitif ve negatif kontrol örnekler kullanılmıştır.

Serum örneklerinde anti-*T.gondii* antikorlarının varlığı Türkiye Halk Sağlığı Kurumu Parazitoloji Merkez Referans Laboratuvarında canlı takizoitler ve metilen mavisi kullanılarak Sabin-Feldman Boya Testi ile araştırılmıştır. Serum örnekleri, 56°C'de 30 dakika süreyle inaktive edildikten sonra serum fizyolojik ile 1/16, 1/64, 1/256 ve 1/1024 titrelerde sulandırılmıştır. Her bir dilüsyondan 25 µl alınarak *T.gondii* negative ve C2, C3, C4, Mg2 ve properdinden zengin activator serum ile eşit oranda karıştırılmıştır. Antijen olarak, 48 saat aralıklarla pasajlanmış 3-4 haftalık Swiss Albino cinsi sıçanlardan elde edilen *T.gondii* Rh suşu kullanılmıştır. Daha sonra 37°C'de 50 dakika inkübe edilen örnekler 25µl alkalin metilen mavisi (pH:11) ile muamele edilmiştir. Her bir örnekten 20µl alınarak ışık mikroskobunda 400 büyütmede incelenmiştir. Takizoitlerin %50'sinden fazlasının boya almadığı, 1:16 ve üzeri titreler pozitif olarak değerlendirilmiştir.

Bu çalışma için gerekli olan etik kurul izni Etlik Veteriner Kontrol Merkez Araştırma Enstitü Müdürlüğü Yerel Etik Kurulu'ndan alınmıştır (27.11.2015 tarih ve 2015/7 nolu karar).

Çalışma sonuçlarının istatistiksel değerlendirilmesinde Ki-kare (X^2) testi kullanılmıştır.

BULGULAR

Çalışma sonucunda 105 atın ikisinde (%1,90) anti-*Neospora* spp., dokuzunda (%8.57) anti-T.gondii, ikisinde (%1,90) anti-*B. caballi* ve 77 (%73,33)'sinde de anti-*T.equi* antikorları tespit edildi. Atlardan birinde (%0,95) *T.equi*, *B. caballi* ve *T.gondii*, birinde (%0,95) *T.equi* ve *B. caballi*, ikisinde (%1,90) *Neospora* spp. ve *T.equi* ve altısında (%5,71) ise *T.gondii* ve *T.equi*'nin neden olduğu mix enfeksiyonlar belirlendi (Tablo-1). Ki-kare (X^2) testi ile ≤ 7 ve >7 yaş grupları ile *Neospora* spp., *T.gondii* ve *B.caballi* seroprevalansları arasında istatistiksel açıdan önemli bir fark bulunmazken ($p>0.05$), 7 yaş üzerindeki atlarda yüksek *T.equi* seroprevalansının önemli olduğu ($p<0.05$) görüldü.

Tablo 1. Çalışmada atlarda tespit edilen protozoonlar ve yaş gruplarına göre yaygınlığı
Table 1. Protozoa detected in horses in the study and their prevalence according to age groups

Yaş	At Sayısı (n)	*Neospora* spp.		*T. gondii*		*T. equi*		*B. caballi*	
		n (+)	% (+)	n (+)	% (+)	n (+)	% (+)	n (+)	% (+)
≤ 7	43	1	2.32	4	9.30	26	60.46	-	0.00
>7	62	1	1.61	5	8.06	51	82.25	2	3.22
Toplam	**105**	**2**	**1.90**	**9**	**8.57**	**77**	**73.33**	**2**	**1.90**

TARTIŞMA

Atlar tüm dünyada olduğu gibi Türkiye'de de yıllarca tarım, ulaşım ve askeri alanda kullanılmak üzere yetiştirilmiştir. Günümüzde ise daha çok spor, yarış, hobi ve turistik amaçlı at yetiştiriciliği yapılmaktadır (Yilmaz ve Wilson 2013). Paraziter protozoonların insan ve diğer hayvan türlerinde olduğu gibi atlarda da verim kayıplarına ve hatta ölümlere neden olduğu bilinmektedir (Wise ve ark 2014, Dubey 2016, Taylor ve ark 2016, Dubey ve ark 2017). Bu çalışma ile Nevşehir ilinde turistik amaçlı olarak yetiştirilen atlar *T.equi*, *B.caballi*, *Neospora* spp. ve *T.gondii* yönünden serolojik yöntemlerle incelenmiştir.

Equine piroplasmosis atlarda görülen önemli kene kaynaklı protozoal hastalıklardandır. Hastalık at yetiştiriciliğinde verim kayıplarına ve ülkeler arasında at ticaretinin engellenebilmesi (özellikle hastalığın görülmediği ülkelere endemik bölgelerden yapılan at ticareti) nedeniyle ciddi ekonomik kayıplara neden olmaktadır (Wise ve ark 2014). Ayrıca *T.equi* ve *B.caballi* enfeksiyonlarından sonra atlar uzun süre etkenlerle enfekte kalabilmektedir ve enfekte atlar vektör keneler için enfeksiyon kaynağı olmaktadır (Wise ve ark 2014, Taylor ve ark 2016). Bu nedenle hem klinik enfekte hem de rezervuar atların belirlenmesi equine piroplasmosisten korunmada oldukça önemlidir (Wise ve ark 2014). Atlarda equine piroplasmosisin teşhisinde mikroskobik, moleküler ve serolojik teşhis yöntemleri kullanılmaktadır (OIE 2018). Mikroskobik teşhis yöntemleri akut enfeksiyonların teşhisinde başarılı bir şekilde kullanılırken, paraziteminin az olduğu olgularda veya kronik enfeksiyonların teşhisinde uygun bir yöntem değildir (OIE 2018). Moleküler teşhis yöntemleri ise hem akut hem de kronik enfeksiyonların teşhisinde başarılı bir şekilde kullanılabilmektedir. Ancak moleküler teşhis yöntemleri sırasında kullanılan alet-ekipmanlar ve kimyasallar pahalıdır (Wise ve ark., 2014). Serolojik teşhis yöntemleri ise kronik enfeksiyonların teşhisinde başarılı bir şekilde kullanılması ve kısa sürede çok sayıda hayvanın test edilmesine olanak sağlaması nedeniyle geniş çaplı epidemiyolojik çalışmalarda sıklıkla tercih edilmektedir (Wise ve ark 2014, OIE, 2018). Bu çalışmada atlarda *T.equi* ve *B.caballi*'nin serolojik teşhisinde c-ELISA yöntemi kullanılmıştır. Atlarda equine piroplasmosis dünyanın farklı bölgelerinde farklı oranlarda bildirilmiştir. Onyiche ve ark (2020) tarafından yapılan bir meta-analiz çalışmasında dünyanın farklı bölgelerinde atlarda *B.caballi*'nin %0-

90,9, *T.equi*'nin ise %0,5-82,9 arasındaki değişen oranlarda bulunduğu bildirilmiştir. Türkiye'de ise serolojik yöntemler kullanılarak yapılan çalışmalarda bölgelere göre değişmekle birlikte *B. caballi*'nin %0-38, *T.equi*'nin ise %0-64,45 oranlarında yaygın olduğu tespit edilmiştir (Akkan ve ark 2003, Balkaya ve Erdoğmuş 2006a,b, Acici ve ark 2008, Sevinc ve ark 2008, Karatepe ve ark 2009, Sarı ve ark 2010, Kurt ve Yaman 2012, Akkoyun ve Oğuz 2019). Bu çalışmada ise atlarda %1,90 (2/105) oranında *B.caballi*, %77,33 (77/105) oranında ise *T.equi* antikorları tespit edilmiştir. Bu çalışma ile edilen *B.caballi* yaygınlığı Türkiye'de yapılan birçok çalışmadaki değerlerden daha düşük iken, *T.equi* yaygınlığı ise Türkiye'de günümüze kadar yapılan çalışmalardaki yaygınlıklardan daha yüksek bulunmuştur (Akkan ve ark 2003, Balkaya ve Erdoğmuş 2006a,b, Acici ve ark 2008, Sevinc ve ark 2008, Karatepe ve ark 2009, Sarı ve ark 2010, Kurt ve Yaman 2012, Akkoyun ve Oğuz 2019). Çalışmamız ile diğer çalışmalardaki yaygınlık farklılığının nedenlerinin; çalışmalarda kullanılan testlerin farklı olmasına, örneklenen hayvan sayısının farklı olmasına ve ayrıca etkenlerin kene kaynaklı patojenler olması nedeniyle çalışma alanlarındaki uygun vektör türlerinin bulunup bulunmaması gibi faktörlere bağlı olabileceği düşünülmektedir. Ayrıca yapılan birçok çalışmada atlarda anti-*T.equi* antikorlarının anti-*B.caballi* antikorlarına oranla daha yüksek oranlarda tespit edildiği görülmüştür (Akkan ve ark 2003, Kurt ve Yaman 2012, Akkoyun ve Oğuz 2019, Onyiche ve ark 2020). Bu çalışmada da *T. equi* yaygınlığının *B.caballi*'ye oranla daha yüksek olduğu görülmüştür. Ayrıca çalışmada yedi yaşından büyük atlarda *T.equi*'nin yaygınlığının yedi yaşından küçük atlara oranla daha yüksek olduğu ve bu farklılığın istatistiksel açıdan önemli olduğu tespit edilmiştir. Bu durumun *T.equi*'nin kene kaynaklı bir patojen olması nedeniyle hayvanların yaşları arttıkça vektör kenelere maruz kalma ihtimalinin daha yüksek olmasından ve dolayısıyla enfeksiyona yakalanma ihtimalinin daha yüksek olmasından kaynaklandığı düşünülmektedir. Ayrıca Wise ve ark (2014)'nın çeşitli yazarlara atfen bildirdiğine göre *T.equi* enfeksiyonlarında tedavi uygulanmaz ise atlar ömür boyu parazitle persiste enfekte kaldığı, ancak *B.caballi* enfeksiyonlarda ise tedavi uygulanmazsa bile atların belli bir süre sonra paraziti tamamıyla elimine ettiğini bildirmişlerdir. Bu durumda atlarda *T.equi*'ye karşı ömür boyu bir antikor cevabı olurken *B.caballi*'ye kaşı ise daha sınırlı bir süre antikor cevabı olmaktadır. Bu nedenle bizim çalışmamızda ve diğer birçok çalışmada *T.equi* seroprevalansının *B.caballi*'ye oranla daha yaygın olduğu düşünülmektedir.

Equine neosporosis dünyanın farklı bölgelerinde bildirilmiş ve hastalığın yaygınlığının araştırılması amacıyla yapılan çalışmalarda; yaygınlığın Amerika kıtasında %0-85,7, Asya'da %2-77,8 ve Avrupa'da %0,3-55,2 oranında atlarda seropozitiflik saptanmıştır (Dubey ve ark 2017). Türkiye'de ise yapılan çalışmalarda %0-24 oranında seropozitiflik tespit edilmiştir (Sevgili ve ark 2003, Kilbaş ve ark 2008, Karatepe ve Karatepe 2012, Zhou ve ark 2016). Bizim çalışmamızda ise atların %1,90'nında *Neospora* spp. antikorları saptanmıştır. Atlarda neosporosisin teşhisinde NAT (*Neospora* direct agglutination test), IFAT (indirect fluorescent antibody test) ve ELISA (enzyme-linked immunosorbent assay) gibi farklı serolojik yöntemler kullanılabilmektedir (Dubey ve ark 2017). Yapılan çalışmalarda neosporosisin yaygınlığının farklı oranlarda çıkmasının nedeninin; numune alınan hayvan sayısına ve hayvanların yaşına, çalışmalarda kullanılan serolojik yöntemlerin farklı spesifite ve sensitiviteye sahip olmasıyla ve yöntemlerde belirlenen cut-off değerlerinin farklı olmasıyla ilgili olabileceği düşünülmektedir (Bártová ve ark 2010).

Toxoplasmosis Antartika kıtası dahil tüm dünyada görülen en önemli paraziter protozoondur (Dubey 2016). *Toxoplasma gondii* insan dahil birçok sıcak kanlı canlıda hastalık meydana getirdiği gibi atlarda da önemli klinik semptomlara neden olabilmektedir. Bu nedenle dünyada atlarda *T.gondii*'nin yaygınlığının araştırılması amacıyla birçok çalışma yapılmış ve yaygınlığın ülkelere göre değişmekle birlikte %0,40-72,2 arasında değişen oranlarda olduğu tespit edilmiştir (Younis ve ark 2015, Dubey 2016). Türkiye'de ise yaygınlığın %2,7-48,7 arasında değiştiği farklı araştırmalarla ortaya konulmuştur (Aktaş ve ark 1999, İnci ve ark 2002, Sevgili ve ark 2004, Akca ve ark 2004, Göz ve ark 2007, Güçlü ve ark 2007, Karatepe ve ark 2010, Gazyağci ve ark 2011, Zhou ve ark 2016). Bizim çalışmamızda ise atların %8,57 (9/105) anti-*T.gondii* antikorları tespit edilmiştir. Çalışmamızda tespit ettiğimiz yaygınlık Malatya (%6,4) (Aktaş ve ark 1999), Niğde (%7,2) (Karatepe ve ark 2010), Şanlıurfa (%7,5) (Sevgili ve ark 2004) ve İzmir'de (%3,7) (Zhou ve ark 2016) yapılan çalışmalarda daha yüksek diğer çalışmalardan ise düşük olduğu görülmüştür. Dünyada ve Türkiye'de yapılan çalışmalarda atlarda *T.gondii*'nin farklı oranlarda tespit edilmesi hayvanların beslenme şekline, çalışmalarda örneklenen hayvan sayılarına ve serolojik testlerin sensitivite ve spesifitelerine, hayvanların yaşlarına ve örneklenen hayvanların barınma alanlarının etrafında etkenin sonkonağının bulunup bulunmamasına göre farklı olabileceği düşünülmektedir.

SONUÇ

Bu çalışma ile bildiğimiz kadarıyla ilk kez Nevşehir ilinde turistik amaçlı yetiştirilen atlarda *T.equi*, *B.caballi*, *Neospora* spp. ve *T.gondii*'nin yaygınlığı serolojik yöntemlerle incelenmiş ve çalışma sonucunda atların bu protozoon türlerinden biriyle enfekte olabileceği gibi miks enfeksiyonların da görüldüğü tespit edilmiştir. Söz konusu paratizer protozoonların atlarda verim kayıplarına ve hatta ölümlere neden

olduğu bilinmektedir. Ayrıca yukarıda bahsedilen protozoonlar atlarda spesifik klinik semptomlara (anemi, ikterus ve abort gibi) neden olabildiği gibi bazen ise spesifik olmayan semptomlara (ateş, sinirsel semptomlar karaciğer ve böbrek yetmezliği gibi) da neden olabildiği bilinmektedir. Bu nedenle at yetiştiriciliğinin yapıldığı bölgelerde atlarda klinik semptomlar veya verim kayıplarının görülmesi durumunda *T.equi*, *B.caballi*, *Neospora* spp. ve *T.gondii* gibi protozoon türlerinin de veteriner hekimler tarafından göz önünde bulundurulması gerektiği düşünülmektedir.

Çıkar Çatışması: Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

Etik İzin: Bu çalışma Etlik Veteriner Kontrol Merkez Araştırma Enstitüsü Müdürlüğü Yerel Etik Kurulu 27.11.2015 tarih ve 2015/7 nolu kararı ile Etik İzni alınmıştır. Ayrıca yazarlar Araştırma ve Yayın Etiğine uyulduğunu beyan etmişlerdir.

Finansal Destek: --

Açıklama: --

KAYNAKLAR

Acici M, Umur S, Guvenc T, Arslan HH, Kurt M. Seroprevalence of equine babesiosis in the Black Sea region of Turkey. Parasitol Int. 2008; 57(2): 198-200.

Akca A, Babur C, Arslan MO, Gicik Y, Kara M, Kilic S. Prevalence of antibodies to *Toxoplasma gondii* in horses in the province of Kars, Turkey. Vet Med, 2004; 49(1), 9-13.

Akkan HA, Karaca M, Tutuncu M, Deger S, Keles I, Agaoglu Z. Serologic and microscopic studies on babesiosis in horses in the eastern border of Turkey. J Equine Vet Sci. 2003; 5(23): 181-183.

Akkoyun Z, Oguz B. Seroprevalance of *Theileria equi* and *Babesia caballi* in Horses of Mus Provice, Turkey. Alex J Vet Sci. 2019; 60(1): 22-29.

Aktaş M, Babür C, Köroğlu E, Dumanlı N. Sultansuyu Tarım işletmesi atlarında anti-*Toxoplasma gondii* antikorlarının Sabin-Feldman boya testi ile belirlenmesi. FÜ Sağlık Bil Derg. 1999; 13(2): 89-91.

Balkaya I, Erdogmus SZ. Investigation of prevalence of *Babesia equi* (Laveran, 1901) and *Babesia caballi* (Nuttall, 1910) in horses by serological methods in Elazig and Malatya province. Firat Uni J Health. 2006a; 20(1): 61-63.

Balkaya I, Erdogmus SZ. Prevalence of *Babesia equi* and *Babesia caballi* in horses by serological methods in the Mediterranean sea and the Black sea regions of Turkey. J Anim Vet Adv. 2006b; 5(2): 168-171.

Bártová E, Sedlák K, Syrová M, Literák I. *Neospora* spp. and *Toxoplasma gondii* antibodies in horses in the Czech Republic. Parasitol Res. 2010; 107(4): 783-785.

Dubey JP. Toxoplasmosis of animals and humans. 2nd Ed. CRC press. Taylor & Francis Group. New York, USA. 2016.

Dubey JP, Hemphill A, Calero-Bernal R, Schares G. Neosporosis in animals. 1st Ed. CRC press. Taylor & Francis Group. New York, USA. 2017.

Gazyağci S, Macun HC, Babür C. Investigation of seroprevalance of toxoplasmosis in mares and stallions in Ankara province, Turkey. Iranian J Vet Res. 2011; 12(4): 354-356.

Göz Y, Babür C, Aydin A, Kılıc S. Seroprevalence of toxoplasmosis, brucellosis and listeriosis in horses in Hakkari, eastern region of Turkey. Rev Med Vet. 2007; 158 (11): 534-539.

Güçlü Z, Karaer Z, Babür C, Kiliç S. Investigation of *Toxoplasma gondii* antibodies in sport horses bred in Ankara province. Türkiye Parazitol Derg. 2007; 31 (4): 264-267.

İnci A, Babür C, Aydın N, Çam Y. Kayseri yöresinde tektırnaklılarda (at, eşek ve katır) *Toxoplasma gondii* (Nicolle ve Manceaux, 1908) ve *Listeria monocytogenes*'in seroprevalansı üzerine araştırmalar. FÜ Sağ Bil Derg. 2002; 16 (2): 181 – 185.

Karatepe B, Babür C, Karatepe M, Kılıç S. Seroprevalence of toxoplasmosis in horses in Niğde Province of Turkey. Trop Anim Health and Prod. 2010; 42(3): 385-389.

Karatepe B, Karatepe M, Çakmak A, Karaer Z, Ergün G. Investigation of seroprevalence of *Theileria equi* and *Babesia caballi* in horses in Nigde province, Turkey. Trop Anim Health and Prod. 2009, 41(1): 109-113.

Karatepe M, Karatepe B. Niğde Yöresinde Atlarda *Neospora* spp. Seroprevalansının Araştırılması. Kafkas Üniv Vet Fak Derg. 2012; 18(Suppl-A): A39-A42.

Kilbaş Z, Adanir R, Avcioglu H. Seroprevalence of *Neospora caninum* in racehorses in Ankara, Turkey. Acta Parasitol. 2008; 53(3): 315-316.

Knowles Jr D. Equine babesiosis (piroplasmosis): a problem in the international movement of horses. Br Vet J. 1996; 152(2): 123-126.

Kurt C, Yaman M. Adana Yöresi Atlarında *Babesia equi* ve *Babesia caballi*'nin Yayılışının Mikroskobik ve Serolojik (cELİSA) Yöntemlerle Araştırılması. YYÜ Vet Fak Derg. 2012; 23(1): 1-4.

Marsh AE, Barr BC, Packham AE, Conrad PA. Description of a new *Neospora species* (Protozoa: apicomplexa: sarcocystidae). J Parasitol. 1998; 84(5): 983-991.

Miao Q, Wang X, She LN, Fan YT, Yuan FZ, Yang JF, Zhu XQ, Zou FC. Seroprevalence of *Toxoplasma gondii* in horses and donkeys in Yunnan Province, Southwestern China. Parasit Vectors. 2013; 6(168): 1-5.

World Organisation for Animal Health (OIE) Equine piroplasmosis,https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.05.08_EQUINE_PIROPLASMOSIS.pdf Erişim Tarihi: 28.09.2021. 2018.

Onyiche TE, Taioe MO, Molefe NI, Biu AA, Luka J, Omeh IJ, Yokoyama N, Thekisoe O. Equine piroplasmosis: An insight into global exposure of equids from 1990 to 2019 by systematic review and meta-analysis. Parasitology. 2020; 147(13): 1411-1424.

Öncel T, Vural G, Gicik Y, Arslan MO. Detection of *Babesia* (*Theileria*) equi (Laveran, 1901) in horses in the Kars province of Turkey. Turkiye Parazitol Derg. 2007; 31(3): 170-2.

Sarı B, Kırmızıgül AH, Deniz A, Taşçı GT. Kars ve Ardahan yöresinde kış mevsiminde atlarda *Babesia caballi* ve *Theileria equi*'nin seroprevalansı. Kafkas Üniv Vet Fak Derg. 2010; 16(4): 657-661.

Sevgili M, Babür C, Gökçen A, Nalbantoğlu S. Seroprevalance of *Toxoplasma gondii* (Nicolle and Manceaux, 1908) in thoroughbred Arabian mares as detected by Sabin-Feldman dye test in Şanlıurfa. FÜ Sağ Bil Derg. 2004; 18(1): 21-23.

Sevgili M, Şahin T, Çimtay İ, Çetin H, Keskin O, Gökçen A. Şanlıurfa yöresi safkan arap kısraklarında *Neospora caninum* antikorlarının belirlenmesi. YYÜ Vet Fak Derg. 2003; 14(2): 15-17.

Sevinc F, Maden M, Kumas C, Sevinc M, Ekici OD. A comparative study on the prevalence of *Theileria equi* and *Babesia caballi* infections in horse sub-populations in Turkey. Vet Parasitol. 2008; 156(3-4): 173-177.

Taylor MA, Coop RL, Wall RL. Veterinary parasitology, 4th edn. Blackwell Publishing, Oxford, England. 2016.

Veronesi F, Diaferia M, Mandara MT, Marenzoni ML, Cittadini F, Fioretti DP. *Neospora* spp. infection associated with equine abortion and/or stillbirth rate. Vet Res Commun. 2008; 32(1): 223-226.

Wise LN, Knowles DP, Rothschild CM. Piroplasmosis. In Equine infectious diseases, Ed; Sellon DC, Long MT, 2nd. Ed. Saunders Elsevier, Missouri, USA. 2014; pp. 467-475.

World Health Organization (WHO). Toxoplasmosis: greater awareness needed. Erişim Tarihi 27.09.2021.

Yilmaz O, Wilson RT. The domestic livestock resources of Turkey: occurrence and control of diseases of horses, donkeys and mules. J Equine Vet Sci. 2013; 33(12): 1021-1030.

Younis EE, Abou-Zeid NZ, Zakaria M, Mahmoud MR. Epidemiological studies on toxoplasmosis in small ruminants and equine in Dakahlia governorate, Egypt. Assiut Vet Med J. 2015; 61(145): 22-31.

Zhou M, Cao S, Sevinc F, Sevinc M, Ceylan O, Liu M, Wang G, Moumouni PFA, Jirapattharasate C, Suzuki H, Nishikawa Y, Xuan X. Enzyme-linked immunosorbent assays using recombinant TgSAG2 and NcSAG1 to detect *Toxoplasma gondii* and *Neospora caninum*-specific antibodies in domestic animals in Turkey. J Vet Med Sci. 2016; 78(12): 1877-1881.

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022):15(2):167-181
DOI: 10.30607/kvj. 1007942

RESEARCH ARTICLE

Feline Eosinophilic/Proliferative Keratitis: A Retrospective Study

Aynur DEMİR

Istanbul University- Cerrahpaşa, Veterinary Faculty, Surgery Department, 34315 Avcılar, Istanbul, Turkey

ABSTRACT

The medical records of 35 cats (44 eyes) with feline eosinophilic keratitis (FEK) diagnosed between 2016 and 2021 were reviewed. The mean age at the presentation of cases diagnosed with FEK was 4.12 years (3 months-13 years). Domestic shorthair was the dominant breed, accounting for 68.57% of cats (24/35). FEK was unilateral in 74.28% of the cases, and the most frequently affected position was the superotemporal quadrant of the cornea (75% of the eyes). The cytological examination of the cornea and conjunctiva showed a mixture of multiple eosinophils, plasma, and mast cells, confirming the diagnosis of FEK. Eosinophils were found in 86.36% of the corneal and conjunctival scrapings. We performed the polymerase chain reaction test for feline herpes virus type 1 in 12/35 cats. Viral DNA was detected in 20% of these cats. The cats were treated with a subconjunctival corticosteroid, a topical antibiotic-corticosteroid combination, artificial tear drops, and antiviral gel containing ganciclovir. At one to two weeks after the initial examination, the clinical signs markedly improved. The controlled and regular use of topical corticosteroids brought the lesions under control and resolved FEK without recurrence at least for six months.
Keywords: Eosinophilic, Feline, Keratitis, Proliferative, Steroids.

***

Kedilerde Eozinofilik/Proliferatif Keratitis: (2016-2021) Retrospektif Çalışma

ÖZ

2016 ve 2021 yılları arasında teşhis edilen 35 eozinofilik keratitisli kedi (FEK) vakasının (44 göz) tıbbi kayıtları gözden geçirildi. FEK tanısı konulan olguların ortalama sunum yaşı 4,12 yıldı (3ay-13 yıl). Domestik shorthair, kedilerin (24/35) %68.57'ni oluşturan baskın cinsti. Durum, vakaların %74,28'sinde tek taraflıydı ve en sık etkilenen pozisyon korneanın superotemporal kadranıydı (gözlerin %75'i). Kornea ve konjunktivanın sitolojik incelemesi, FEK tanısını doğrulayan çok sayıda eozinofil, plazma ve mast hücresi karışımı gösterdi. Eozinofiller, korneal ve konjonktival kazımaların %.86.36'sında bulundu. 12/35 kedi için kedi herpes virüsü tip 1 (FHV-1) için polimeraz zincir reaksiyonu (PCR) gerçekleştirdik. Bu kedilerin %20'sinde viral DNA tespit edildi. Kediler subkonjunktival kortikosteroid, topikal bir antibiyotik-kortikosteroid kombinasyonu, suni göz yaşı damlası ve gansiklovir içeren antiviral jel ile tedavi edildi. İlk muayene edildikten 1-2 hafta sonra klinik belirtiler dikkate değer bir şekilde iyileşti. Kontrollü ve düzenli topikal kortikosteroid kullanımı lezyonu kontrol altına aldı ve en az 6ay boyunca hastalığı nüks etmeden çözdü.
Anahtar kelimeler: Eozinofilik, Kedi, Keratitis, Proliferatif, Steroidler.

To cite this article: Demir A. Feline Eosinophilic/Proliferative Keratitis: A Retrospective Study. Kocatepe Vet J. (2022):15(2):167-181

Submission: 11.10.2021 **Accepted**: 14.03.2022 **Published Online**: 18.04.2022
ORCID ID; *AD: 0000-0002-5471-1655*
*Corresponding author e-mail: aynurdemir_1903@hotmail.com

INTRODUCTION

Feline eosinophilic keratitis (FEK), also known as proliferative keratitis, proliferative eosinophilic keratitis, or eosinophilic plaque, is a slowly progressive infiltrative keratopathy that starts with a progressive and superficial vascularization of the perilimbal cornea and is characterized by the infiltration of the corneal epithelium and stroma by mast cells, eosinophils, neutrophils, lymphocytes, and plasma cells (Ahn et al. 2010, Dean and Meunier 2013, Edwards et al. 2015, Lucyshyn et al. 2021). Although an association between FEK and feline herpes virus type 1 (FHV-1) has been suggested in cats, the precise pathogenesis of FEK remains unknown, and the cellular profile is typically consistent with type I (IgE-mediated) or type IV (Hodges 2005, Romaneck and Sebbag 2021). The literature also contains views suggesting a connection between this condition and the feline eosinophilic granuloma complex (Chisholm 1989).

Although the diagnosis of FEK is based on pathognomonic findings during the ophthalmic examination, the definitive diagnosis is confirmed by corneal cytology (Chisholm 1989, Spiess et al. 2009, Edwards et al. 2015). Treatment options vary and often involve a long-term process. Various drug combinations can be tried until the most effective treatment protocol is determined (Spiess et al. 2009. Treatment recommendations usually include corticosteroids and/or megestrol acetate. Initially, the use of topical or subconjunctival corticosteroids is recommended, and if this treatment fails, megestrol acetate may be required (Chisholm 1989).

This retrospective study aimed to investigate the breeds, age range, and clinical and cytological features of cats with FEK and determine the efficacy of corticosteroid and antiviral topical formulations in the treatment of the disease. The hypothesis was that corneal lesions would regress with the use of topical corticosteroids and recurrence would occur when this treatment was discontinued, but the systemic side effects of oral formulations, such as megestrol acetate would not be observed.

MATERIAL and METHODS

The records of cats referred to xxxxxx between January 2016 and April 2021 and were diagnosed with FEK were reviewed. The cats were included in the sample based on a confirmed diagnosis of FEK through the corneal cytology identification of inflammatory cells, such as eosinophils, mast, and plasma cells. The definitive diagnosis of FEK was made based on clinical examination findings and presence of eosinophils in corneal cytology.

Animals

The study material consisted of 35 cats of different breed, age, and sex characteristics, which were referred to our clinic between January 2016 and April 2021 and diagnosed with uni/bilateral FEK based on ophthalmoscopic findings, pathognomonic appearance of the ocular surface, and cytological examination. Only the cases that were registered in the system were included in the sample.

Methods

Data including breed, age, sex, ophthalmological findings, affected eye(s) and duration, location of the lesion, clinical status of the cases, diagnostic tests, medical treatment and time to resolution, and recurrence status were recorded. The distribution of FEK cases according to age group, breed, sex, duration, and eye is presented in table 1 and the distribution of lesion localization, cytology and clinical findings are reported in table 2. After obtaining the detailed anamnesis of the cases, physical and ophthalmological examinations were performed. The cases underwent a complete ophthalmic examination, including topical fluorescein staining, Schirmer's tear test, intraocular eye pressure measurement with tonometry (TonoVet, Kruuse, USA), indirect ophthalmoscopy after mydriasis (tropicamide ophthalmic solution), and fundus examination. The ophthalmoscopic examination revealed blepharospasm, ocular discharge, conjunctivitis, corneal opacity, and superficial vascularization, and cobblestone-like pink plaques affecting the cornea in varying locations (Fig 1). While the clinical diagnosis was tentatively based on the characteristic appearance of corneal lesions, e.g., proliferative, white-pink, edematous, irregular, and vascularized tissue and white-yellow corneal plaques (Fig 2), the definitive diagnosis was made upon the cytological examination of corneal/conjunctival scrapings, based on the presence of inflammatory cells, such as eosinophils, masts, plasma supporting the provisional diagnosis of FEK (Fig 3). Diagnostic tests performed included the cytological examination of corneal (Fig 4) and conjunctival specimens and real-time quantitative polymerase chain reaction (PCR) test for FHV-1. For the cytological evaluation, specimens were scraped from the cornea and conjunctival regions with pink and white plaques using a blunt-tipped corneal knife or cotton-tipped swabs with the cats under local anesthesia. The specimens spread on slides were air dried and stained with Diff-Quik. The cytological examination was performed using a light microscope at 40x magnifications. Lesion localizations in the cornea were classified as the relevant corneal quadrant (superotemporal (Fig 5), or inferonasal (Fig 6), central area, or whole cornea if the lesions covered most of

the corneal surface, as described by Dean and Meunier.

Treatment

Written informed consent to accept medical treatment was obtained from each guardian prior to initiating medical treatment. After local anesthesia, first topical dexamethasone (Decort, Deva, Turkey) was administered to all the eyes using a subconjunctival insulin injector of 2-4 mg per eye. In eyes where proliferative lesions were accompanied by corneal ulcers (10/35), a topical antibiotic ofloxacin (Exocin, Abdi İbrahim, Turkey, two drops for three to six times) and artificial tear drops (sodium hyaluronate (Dryex, Abdi İbrahim, Turkey, two drops for three to six times) and antiviral topical ointment ganciclovir (Virgan, Fharmila-thea, Turkey, two times) were applied.
For all the eyes without corneal ulcer or those with healed accompanying ulcers, moxifloxacin, and dexamethasone (Moxidexia®, Abdi İbrahim, Turkey, two drops for four to six times), sodium hyaluronate (Dryex, Abdi İbrahim, Turkey, two drops for four to six times) and 1.5 mg ganciclovir ointment (Virgan, Thea Pharma, Turkey, twice a day) were prescribed as the same treatment protocol.

In all the cases, positive results were obtained with medical treatment over varying durations, with the treatment lasting longer in some, but the use of immunosuppressive agents other than subconjunctival and topical steroids was not required in any of the cats. Despite the intensive and long-term use of topical steroids, including FHV-1 positive cases, none developed any complication (corneal infection, deep ulcer, or melting ulcer). Follow-up examinations were recommended at the first, second and four weeks after starting treatment. When the clinical complaints disappeared, topical steroids were continued for a minimum of three weeks to a maximum of two months to eliminate the possibility of recurrence. The frequency of treatment was gradually reduced depending on the response and severity of the initial disease. This reduction was typically undertaken after the complete resolution of lesions, with the daily treatment frequency being decreased to one dose per week (Fig 7 (a,b,c)). Photographs of the eyes were taken at each visit to assess the changes (Fig 8 (a,b). After the lesions healed, follow-up evaluations were performed at the second, sixth, 10th and 12th months, two and three years. The guardians of the cats that were not referred for a follow-up visits were contacted by phone, and information about the clinical status of the cases and photographs of the final appearance of the eyes were obtained.

RESULTS

Signalment

During the five-year study period, 35 cats (44 eyes) were diagnosed with FEK and treated. The mean age of the affected cats at the time of diagnosis was 4.12 years, and the age range ranged from three months to 13 years. There was male 23 (65.71%) and 12 (34.28%) female cats, of which five and four were non-sterile, respectively. Domestic cats, both shorthair and longhair, were the most affected breeds (82.85%, 29/35). In addition, three other breeds were also represented, namely Siamese (5.71%, 2/35) (Fig 9), British Shorthair (5.71%, 2/35), and Persian (5.71%, 2/35) (Fig 10).

Clinical findings

In most cats, only one eye was affected (74.28%, 26/35). The left eyes were more frequently affected than the right eyes (18/35 versus 8/35). Nine cats had bilateral lesions, of which eight had lesions in both eyes at presentation, while one cat developed eosinophilic lesions in the left eye at one year after presentation. Corneal lesions were most found in the superotemporal quadrant of the cornea (75%, 33/44), followed by the inferonasal quadrant (18.18%, 8/44) (Fig 11) and less frequently in the central surface (6.8%, 3/44). In two cases with lesions in the inferonasal quadrant, lesions were also seen in the nictitating membrane. It was determined that the proliferative lesions started from the limbal region in two cases (Fig 12) with the involvement of the superotemporal quadrant and three cases with that of the inferonasal quadrant and covered a very large area of the corneal surface starting from the limbal region and progressing toward the central cornea. Three cases had a history of stomatitis and mandibular sequester in addition to ocular lesions. The mean duration of clinical complaints was 2.47 months (range, 1 week-3 months). In three cats, clinical complaints had first started one or two years earlier and recurrence occurred a few weeks before the cases were referred to our department.

The preliminary diagnosis of the lesions was based on the clinical presentation and/or anamnesis of the cases. All the cases had corneal vascularization and infiltration. The eyes that were classified as minimally affected (11/44, 25%) had mild superficial corneal vascularization, edema, and/or fibrosis without plaque (Fig 13). In mildly, moderately, and severely affected eyes, there were plaques with or without corneal changes, covering approximately >25% (33/44, 75%), 26-75% (9/44, 20.45%), and 76-100% (2/44, 4.54%) of the cornea, respectively. Sandy-white corneal plaques were evident in 30 eyes (68.18%) of the 26 cats (Fig 14). A history of corneal ulceration before disease onset was recorded in 7/44

eyes (15.90%; 7/35 cats, 20%). At the time of diagnosis, 10/44 eyes (22.72%; 10/35 cats, 28.57%) had lesions and surrounding corneal ulcers. Of all the 35 cats diagnosed with FEK, 24 (68.57%) presented with additional conjunctivitis, and 17 eyes of 16 cases had non-healing corneal ulceration.

Cytological findings

Corneal and/or conjunctival cytology was performed in all the cases. Among the 44 eyes of 35 cats, eosinophils were detected in 38 (86.36%), mast cells in 25 (56.81%), plasma cells in 14 (31.81%), neutrophils in 28 (63.63%), and lymphocytes in six (13.63%).

Results of the PCR test for FHV-1

Twelve of the 35 cats were tested for FHV-1 using the PCR (tears) test. DNA was detected in 50% (6/34, 5 males and 1 female) of these cats, while 50% (6/34, 3 males and 3 females) did not have FHV-1. The remaining 23 cats were not tested for various reasons, such as the guardian's financial constraints and refusal of the test.

Treatment and Outcomes

All the 35 cats positively responded to treatment, evidenced by a reduction in the size of the lesions or disappearance of eosinophilic plaques, regression of corneal vascularization, and resolution of the previously described fluorescein-positive areas (Fig 15(a,b)). Healing times with topical medical treatment ranged from 12 days to 4 months, with a mean duration of 1.2 months. At the follow-up sessions undertaken at one-week intervals, significant regression of the corneal lesions was seen in 20.45% of the eyes at the first follow-up (10-14 days), 27.27 % at the second follow-up (15-21 days), 29.54% at the third follow-up (21-30 days), and 22.72% at the fourth follow-up (30-45 days and later). Three of the 35 cats made slow but gradual improvement, and their problem was eventually resolved within two to three months. The cases were followed up for a period of six months to three years after recovery. Corneal lesions reappeared in 11.76% of the cats within six months to one year. All the recurrences resulted from the discontinuation of treatment by the guardians. Response to topical steroid treatment took a long time in 6.81% of the cats. Blepharospasm and epiphora were observed in three cases, although their clinical complaints were not the same. Mild corneal scarring was observed in some of the cases (37.14%, 13/35) despite chronic topical steroid treatment.

The frequency of treatment required to maintain remission varied between the cats. Three of the 35 cats relapsed when treatment was discontinued. All these cats responded to treatment when the treatment was started again.

Table 1. Distribution of FEK cases according to the age group, sex, breed, duration, and eye

Item	**n**	**%**
Age group (yrs)		
≤1	9	25.71
1- ≤ 5	19	54.28
5-10	4	11.42
≥10	3	8.57
Sex		
Male	23	65.71
Female	12	34.28
Breed		
Domestic Shorthair	24	68.57
Domestic Longhair	5	14.28
Siamese	2	5.71
British Shorthair	2	5.71
Persian	2	5.71
Duration		
2-3 weeks	14	35
4-6 weeks	17	48.57
7+ weeks	4	11.42
Eye		
Left	18	51.42
Right	8	22.85
Both	9	25.71
TOTAL	35	100

Table 2. Distribution of lesion localisation, cytology, and clinical signs in FEK cases

Item	**n(eyes)**	**%**
Affected tissue/s		
Co	14	31.81
Conj	1	2.27
Co+Conj	27	61.36
Co+NM	2	4.54
Lesion location		
ST	33	75
IN	8	18.18
C	3	6.81
Cytology		
E	38	86.36
M	25	56.81
P	14	31.81
N	28	63.63
L	6	13.63
Clinical signs		
PL	32	72.72
H	31	70.45
V	37	84.09
ED	38	86.36
U	10	22.72

Co: Cornea, Con: Conjunctiva, IN: Inferonasal, ST: Superotemporal, C: Central, NM: Nictitans membrane, E: Eosinophil, M: Mast cells, P: Plasma cells, N: Neutrophil, L: Lymphocyte, PL: Plaque, H: Hyperemia, V: Vascularization, ED: Edema, UL: Ulcer

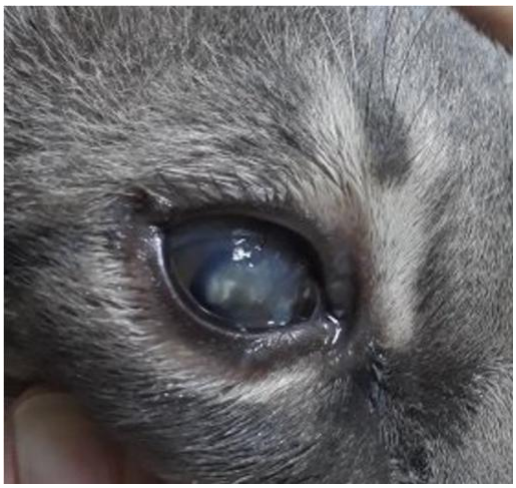


Figure 1. Cobblestone lesions covering the large surface of the cornea in a cat. (Case 13).

Figure 2. Corneal edema, irregular vascularized tissue, and white-yellow corneal plaques in a cat. (Case 9).

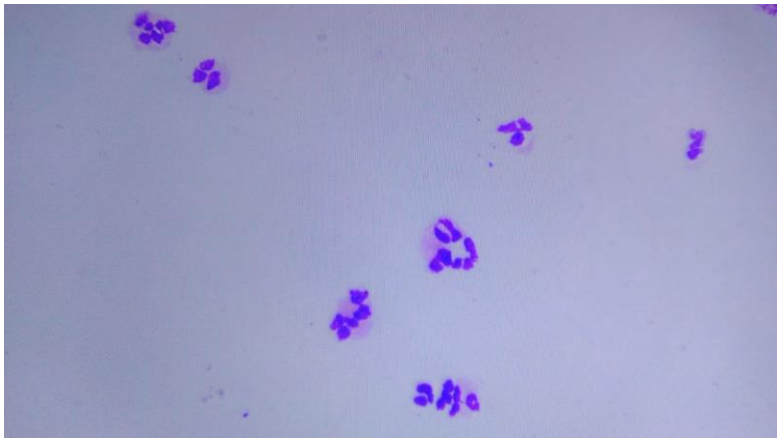


Figure 3. Corneal scraping of the left affected eye showing eosinophil and neutrophil. Diff-Quick staining, 40x magnification.

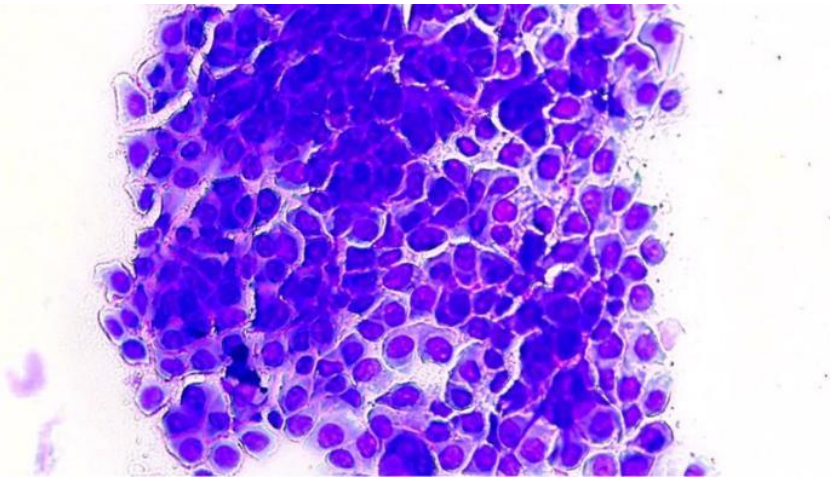


Figure 4. Superficial corneal epithelial cell deposits on cytological examination.

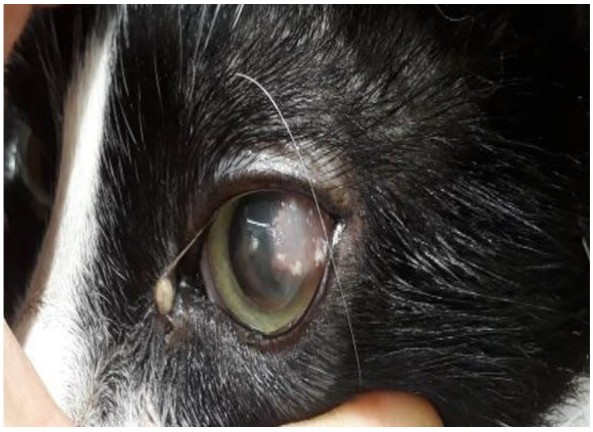


Figure 5. Gross appearance of feline eosinophilic keratitis as white plaques that partially cover superotemporal quadrant of the left eye in a 2-year cat.

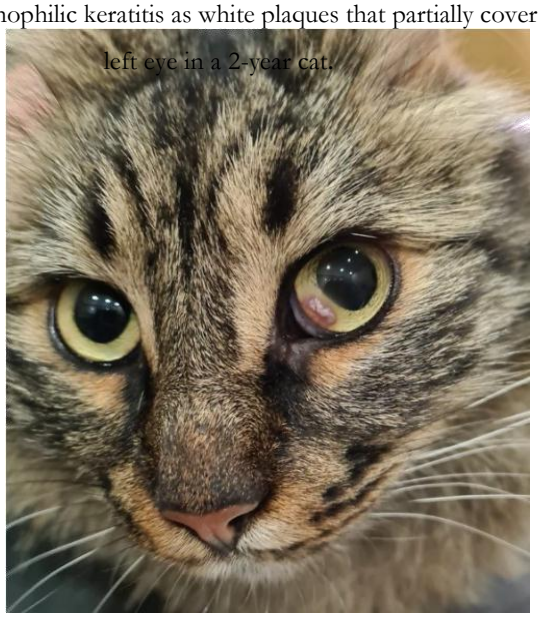


Figure 6. White plaque and neovascularization on the inferonasal quadrant of the cornea of the left eye in a 2-year-old Domestic longhair cat.

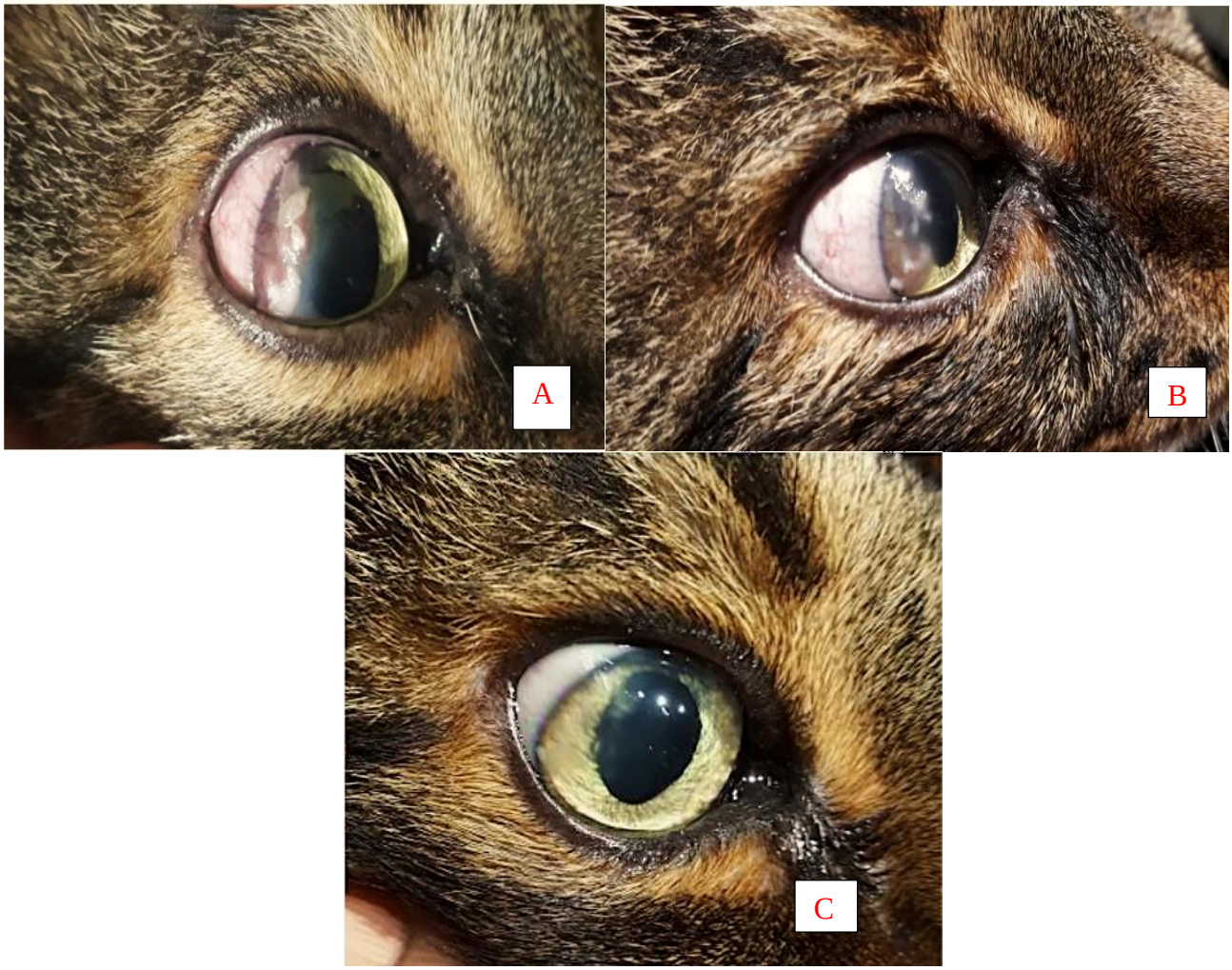


Figure 7. A. First appearance of eosinophilic keratitis in the right eye of a 13-year-old Domestic shorthair cat. B. 12 days with 1% topical dexamethasone, C. Appearance after 3 weeks of treatment.

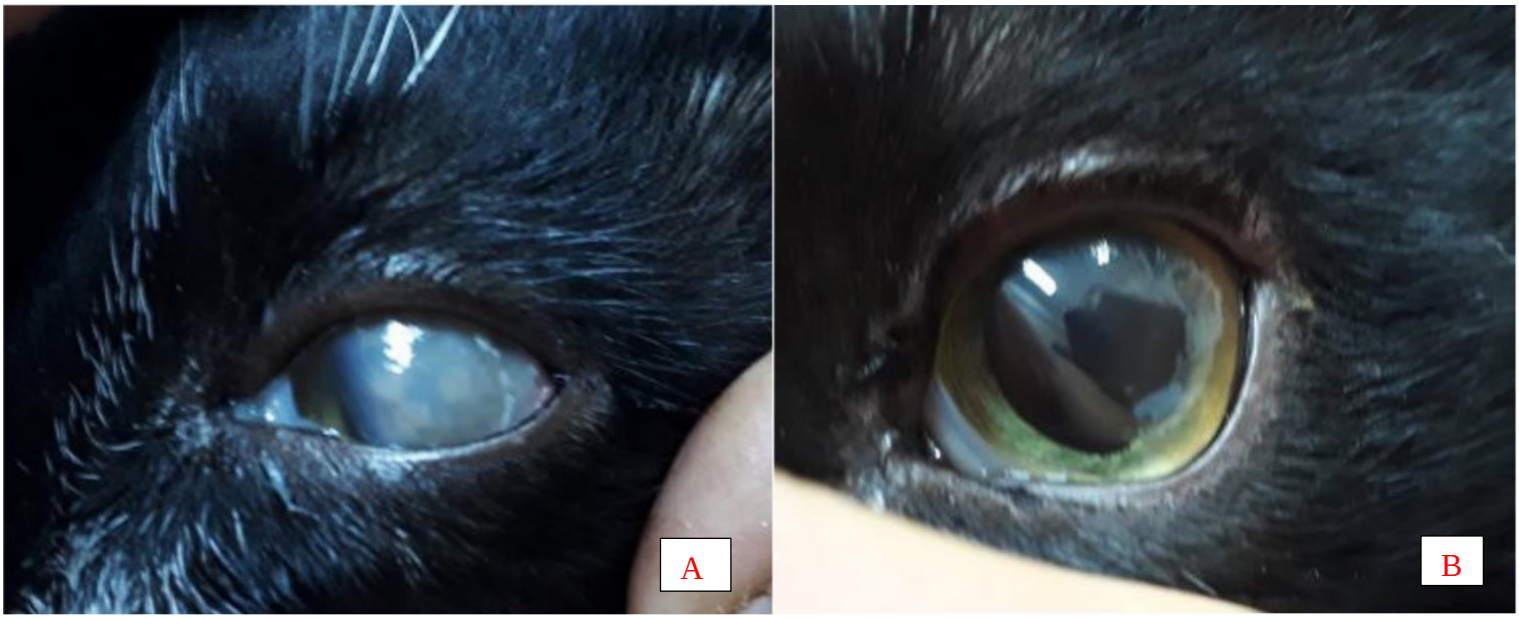


Figure 8. A. First appearance of the eosinophilic plaque covering the large part of the left cornea of case 6. B. Appearance of the left cornea after 4 weeks of treatment with 1% topical dexamethasone.

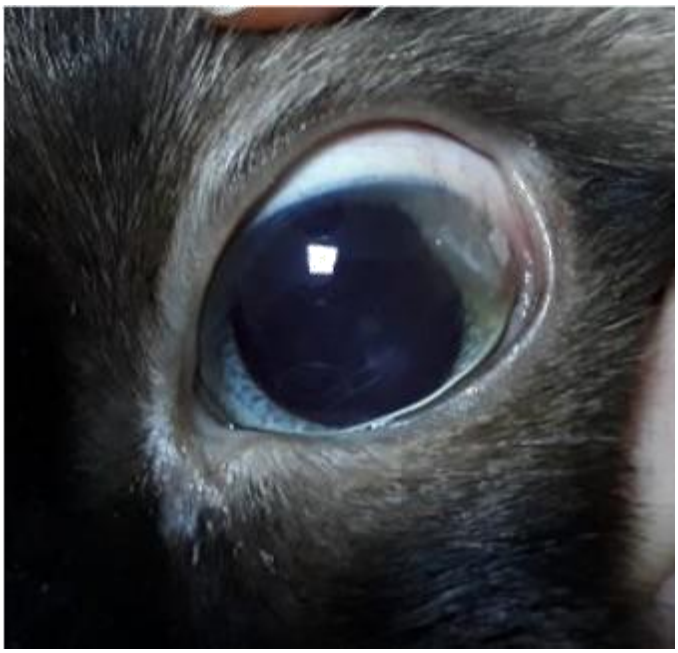


Figure 9. Corneal vascularization and mild plaque in the superotemporal quadrant in a 10-year-old Siamese cat.

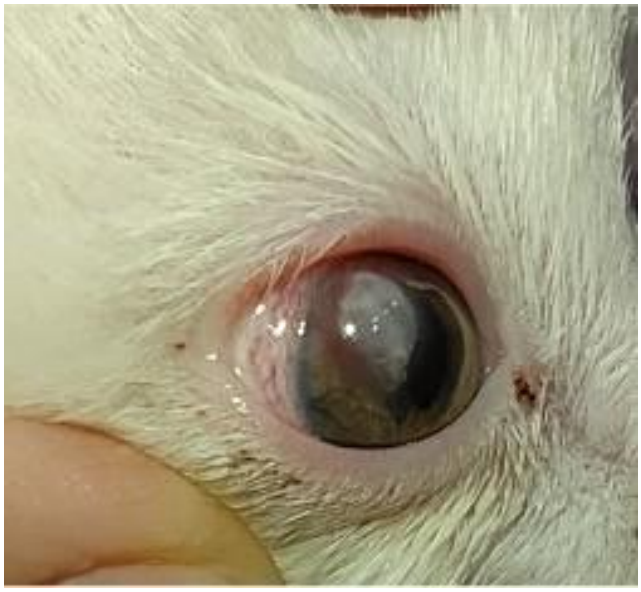


Figure 10. Corneal plaque and neovascularization in the right corneal superotemporal quadrant in a 3-month-old Persian cat.

Figure 11. Feline eosinophilic keratitis. Note the raised white corneal plaques and dense corneal edema.

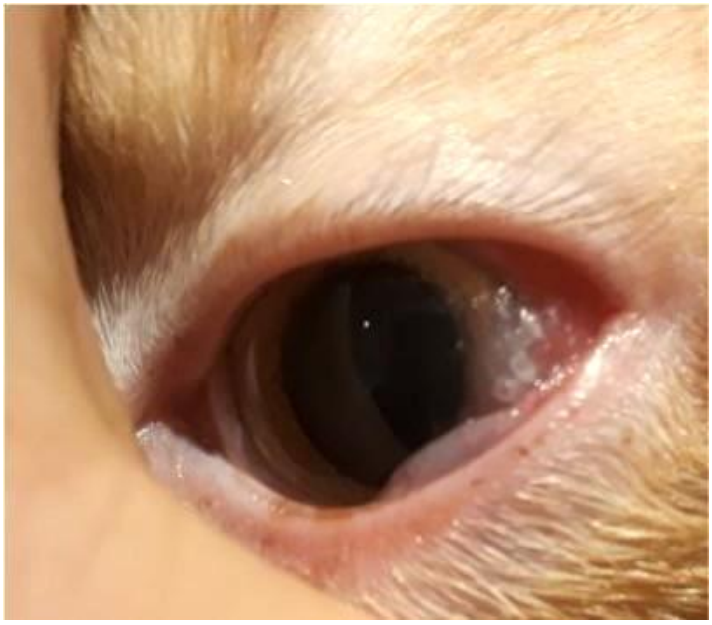


Figure 12. Corneal white plaques and conjunctival hyperemia originating in the limbal region in a one-year-old cat.

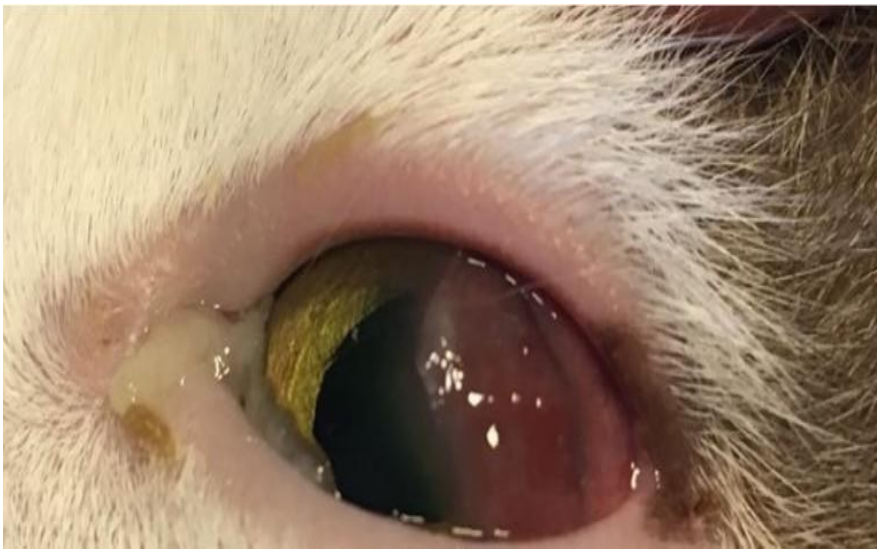


Figure 13. Superficial corneal vascularization, edema, and/or plaque-free fibrosis in a 1-year-old cat.

Figure 14. Proliferative (eosinophilic) keratitis. There is a thick white plaque on the lateral conjunctiva that extends over a dense neovascularized cornea.

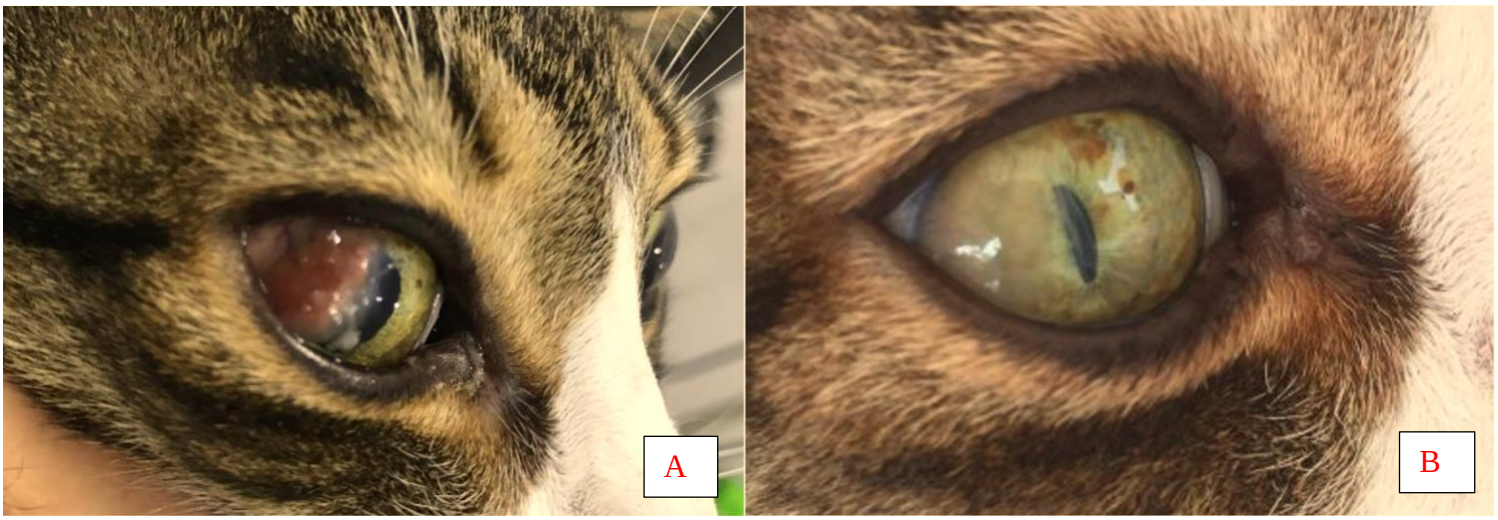


Figure 15. A. Dense proliferative keratitis of the right cornea in a 6-year-old cat. B. After 3 weeks of topical steroid therapy

DISCUSSION and CONCLUSION

Proliferative or eosinophilic keratitis has been described as a rare and progressive inflammation known to similarly affect the epithelium and stroma of the cornea and/or conjunctiva of cats and horses and characterized by a cellular infiltration of eosinophils, mast cells, neutrophils, lymphocytes, plasma cells, and sometimes histiocytes (Hodges 2005). It has been reported that lesions characteristically begin as single or multiple focal, raised pink plaques like granulation tissue in the peripheral area, progress toward superficial vascularization and stromal infiltration in the entire cornea, and have a granular or cobblestone appearance. In addition to the cornea, this condition can also invade the inner surface of the eyelids and the membrane of the bulbar conjunctiva, resulting in more widespread inflammation in the eye (Ahn et al. 2010).

In the current study, the corneal lesions were mostly located in the superotemporal quadrant of the cornea (75, 33/44), followed by the inferonasal quadrant (18.18%, 8/44) and less frequently observed in the central surface (6.81%, 3/44). Depending on the time from the emergence of the lesions to presentation, there were fluffy pink plaques like granulation tissue in the cats referred to our department at the initial stage, while in advanced stages, the cases presented with white or chalky plaques. These findings are consistent with the previous reports describing the formation of lesions and their position in the cornea. We also determined that the size of the lesions varied according to the chronicity, and the cats generally had more disturbing symptoms as the lesions progressed. All the cats had superficial vascularization and stromal infiltration of the cornea. Irregularly shaped, pink to white infiltrates with rough corneal plaques were present in 68.18% of the examined eyes. In two cases where the inferonasal quadrant was involved, lesions were also seen in the nictitating membrane. In five cases, the proliferative lesions started from the limbal region and progressed to the central cornea, covering a very large area of the corneal surface. Of all the cats with FEK, 22.72% also had corneal ulcers in and around their lesions at the time of the diagnosis of the disease. The rate of corneal ulcers accompanying FEK was 22.72% in our study, which was like the findings previously reported by Dean and Meunier (33.3%), Spiess (28.63%), and Morgan et al. (24%) and higher than the rate determined by Paulsen (13%).

FEK lesions tend to be more common in young and middle-aged, sterilized males, and domestic shorthair, longhair and purebred cats (Stiles and Coster 2016). Most of the data obtained from the current study, such as the mean age at diagnosis (4.19 years) (range 3 months to 13 years), breed and sex of the cats affected, and the eye affected by the lesion are consistent with previously published results. The affected cats varied in age at the first presentation, but FEK was usually seen in young to middle-aged adult cats. In previous studies and our study, domestic cats were identified as the most affected breeds (82.85%). However, in the current study, there were also three pure cat breeds representing Persian (5.71%), Siamese (5.71%) and British Shorthair (5.71%) cats. FEK lesions are usually unilateral, but if left untreated or treated inappropriately, the disease can affect both eyes (Ahn et al. 2010). Generally, first, one eye is affected, and a few months later, the second eye begins to show symptoms. Bilateral lesions usually occur when the treatment of the first lesion is ineffective (Ahn et al. 2010). According to a retrospective study, approximately 80% of the FEK cases were unilateral (Ahn et al. 2010). In a study indicating no age, breed, or sex predisposition to the disease, it was reported that the lesions occurred unilaterally in 66% of the cases (Chisholm 1989). In our study, most of the cases, 74.28% (n = 26), presented with a unilateral lesion, while the remaining nine cases had bilateral lesions, and the left eyes were more frequently affected than the right eye. According to Dean and Meunier, the right and left eyes were equally affected. In two cases, one eye was affected first, while the second eye showed symptoms months later. Bilateral involvement was defined as disease progression. While the sex ratio of the male and female cats affected by these lesions was close to 1 according to the studies of Nasisse et al. and Dean and Meunier, it was observed that males (23/35) were predominantly affected by these lesions at a rate of 65.71%. Similarly, previous studies by Spiess, Morgan et al. and Paulsen et al. reported that the lesions affected male cats more.

The probable diagnosis of FEK is often based on the characteristic appearance of lesions on an ophthalmic examination, and this disease is suspected when there is a history of failure to respond to antibiotic therapy (Chisholm 1989, Hodges 2005). Under normal conditions, eosinophils and mast cells are not seen in healthy cat corneas. The clinical appearance and corneal infiltration of one of these two inflammatory cells in the corneal cytology is pathognomonic for proliferative FEK. Although this disease may have a classical, almost pathognomonic appearance in cats, the definitive diagnosis is made based on the cytological examination of corneal scrapings or light microscopy of corneal biopsies (Hodges 2005, Ahn et al. 2010). Diagnostic samples are obtained using the Microbrush® applicator, cotton swabs, Kimura spatula, or the blunt (handle) tip of a scalpel blade (Spiess et al. 2009). In the current study, a history of unresponsiveness to long-term antibiotic therapy and ophthalmological examination findings of some cases created suspicion of the disease, but the definitive diagnosis was confirmed based on cytological findings, as described in previous publications (Spiess et al. 2009, Stiles and Coster 2016, Sancak et al. 2011).

A blunt-tipped corneal knife or cotton swabs were used in the cytology of the affected areas. The cytological appearance was like what was previously described (Spiess et al. 2009, Dean and Meunier 2013, Stiles and Coster 2016). The results showed the presence of mainly eosinophils (86.36%), neutrophils (63.63%) and mast cells (72.85%), while lymphocytes and plasma cells were seen less frequently.

The etiology of eosinophilic keratitis has not yet been fully determined, but the literature contains various assumptions, including parasite invasion, allergic components, and immune reactions (Stiles and Coster 2016). Some studies suggest that eosinophilic keratitis emerges as an immune response to an antigenic stimulus. Considering the cellular profile, it is suggested that the eosinophilic response can be explained by a type I or type IV hypersensitivity reaction. In a previous study, the disease was considered to develop idiopathically (Hodges 2005). However, none of these ideas have been confirmed to date. In another study, eosinophilic keratitis was associated with FHV-1 diagnosed based on a PCR analysis (Stiles and Coster 2016). In the current study, hypotheses based on systemic clinical findings and anamnesis were made in some of the cases in which the disease etiology could not be precisely determined due to the necessary tests not being performed for reasons such as the financial constraints of the guardians or due to the results of the examination not being significant. We determined that 20% of the cases were positive for FHV-1. However, as reported in previous studies (Chisholm 1989, Hodges 2005), we consider that FHV-1 positivity in the samples taken from the eyes of cats does not necessarily indicate that the two diseases have a cause-and-effect relationship.

The most common form of treatment for FEK has been identified as medical therapy with anti-inflammatory drugs. Due to its chronic character, the disease can only be controlled but not cured. Long-term or even life-long treatment at a low and effective dose is required to control the disease. Topical steroids are the first choice for treatment due to their high efficacy and minimal side effects, and they are crucial in the success of treatment (Raczynska et al. 2003). However, their use in FEK is not completely risk-free. In the presence of FHV-1, which plays an important pathogenic role in the etiology of the disease, long-term immunosuppressive therapy can theoretically facilitate the recurrence of an FHV-1 infection (Chisholm 1989, Hodges 2005). Nevertheless, the topical application of antiviral drugs provides an advantage for treating suspected cases of herpetic keratitis. Other recommended treatments for the disease include systemic corticosteroids and megestrol acetate (Ovaban; Schering-Plough Animal Health, Pointe Claire, Quebec) and recently applied topical formulations of cyclosporine, tacrolimus (Aydın and Aktaş 2021) and megestrol acetate (Stiles and Coster 2016). However, oral corticosteroids should not be used as first-line drugs due to the high dose required for immunosuppression and possible side effects. Megestrol acetate has been suggested to be beneficial in cats with a history of recurrent FEK, but caution should be exercised since it causes adrenocortical suppression, polyphagia, behavioral changes, diabetes mellitus, mammary hyperplasia, and neoplasia, and it is not licensed for use in cats and has a half-life of eight days. Depending on the clinical manifestation, other methods can be used in addition to local and systemic drug use (Chisholm 1989, Hodges 2005). In large-scale granulomas, a superficial keratectomy can usually accelerate the healing process (Hodges 2005). In our study, the size of the lesions, their clinical appearance, and the symptoms of the cases widely differed, the etiology of the disease could not be precisely determined, and the viral etiology was dubious; therefore, topical corticosteroids were simultaneously applied with antivirals. After treatment, no ocular clinical signs related to an FHV-1 infection were observed in any of the cases with FHV-1 positivity at the time of presentation. In most of our cases, the topical steroid application resulted in the disappearance of white plaques and neovascularization by the second to fourth day of treatment, and the disease was taken under control within a few weeks. However, a few cats required intermittent drugs to keep the disease under control. Morgan reported that the recurrence rate of FEK was over 64%. The author noted that recurrence developed at six months after treatment, and clinical symptoms disappeared after 20 days of the topical application of 1% prednisolone. In another study conducted with 65 cats, Morgan et al. reported a recurrence rate of 64%, and recurrence was observed in all treatment modalities (Hodges 2005). In our study, the recurrence rate in controlling FEK with topical steroids was determined as 11.76%. Recurrence developed after 6 months, and the clinical complaints disappeared after two to three weeks of topical dexamethasone. In conclusion, as in previous studies, it was determined that feline eosinophilic keratitis was mostly seen in unilateral domestic short/longhair cats. The clinical appearance of the lesions was pathognomonic; however, it was easily performed by cytological examination at 40x magnification to confirm the diagnosis. Also, topical steroid and antiviral ophthalmic solutions were determined to be effective treatments in the control of FEK in all cases presenting to our referral center.

Çıkar Çatışması: Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

Etik İzin: Bu çalışma kliniğimize rutin göz hastalıkları şikâyeti ile sevk edilen ve medikal tedavi uygulanan klinik çalışmadır. Her hastaya reçete edilen

medikal tedavi için hasta sahiplerinden tedaviyi kabul ettiklerini gösteren imzalı onam belgesi alınmıştır.

Finansal destek: Finansal destek alınmamıştır.

Teşekkür: Bu çalışmada sitolojik inceleme sırasında desteklerini veren Veteriner Hekim Erhan GÜLÇİK'e teşekkürlerimi sunarım.

REFERENCES

Ahn JT, Jeong MB, Kim SE, Park YW, Kim TH, Ahn JS, Seo KM. A Case of Feline Proliferative Eosinophilic Keratitis Treated by Topical Cyclosporine and Corticosteroids. Journal of Veterinary Clinics. 2010; 27(6): 751-754.

Aydın Ö, Aktaş MS. Kedi ve Köpeklerde Kullanılan Bazı İmmünsupresif İlaçlar ve Kullanım Amaçları. Bozok Veterinary Sciences. 2021; 2(2): 101-107.

Chisholm WH. Feline eosinophilic keratitis. The Canadian Veterinary Journa. 1989; 30(5): 438.

Dean E, Meunier V. Feline eosinophilic keratoconjunctivitis: a retrospective study of 45 cases (56 eyes).

Journal of feline medicine and surgery. 2013; 15(8): 661-666.

Edwards S, Clode AB, Gilger BC. Equine eosinophilic keratitis in horses: 28 cases (2003–2013). Clinical case

reports 2015; 3(12): 1000.

Hodges A. Eosinophilic keratitis and keratoconjunctivitis in a 7-year-old domestic shorthaired cat. The Canadian Veterinary Journal. 2005; 46(11): 1034.

Lucyshyn DR, Good KL, Knickelbein KE, Chang MW, Strøm AR, Hollingsworth SR, Maggs DJ. Subcutaneous administration of triamcinolone as part of the management of feline eosinophilic keratoconjunctivitis. Journal of Feline Medicine and Surgery. 2021; 23(6): 575-583.

Morgan RV. Feline Eosinophilic Keratitis. *http://www.saintfrancis.org* › wp-content › uploads

Morgan RV, Abrams KL, Kern TJ. Feline eosinophilic keratitis: A retrospective study of 54 cases. Vet Comp

Ophthalmol. 1996; 6: 131-134.

Nasisse MP, Glover TL, Moore CP. Detection of feline herpesvirus 1 DNA in corneas of cats with eosinophilic keratitis or corneal sequestration. Am J Vet Res. 1998; 59: 856–858

Paulsen ME, Lavach JD, Severin GA, Eichenbaum JD. Feline eosinophilic keratitis: A review of 15 clinical

cases. J Am Anim Hosp Assoc. 1987; 23: 63-69

Raczyńska K, Iwaszkiewicz-Bilikiewicz B, Stozkowska W, Sadlak-Nowicka J. Clinical evaluation of provitamin B5 drops and gel for postoperative treatment of corneal and conjuctival injuries. Klinika oczna. 2003; 105(3-4): 175-178.

Romaneck AK, Sebbag L. Case Report: Clinical Remission in a Cat with Severe Bilateral Eosinophilic Keratitis Receiving Combined Immunosuppressive Therapy (Triamcinolone Acetonide and Tacrolimus). Frontiers in Veterinary Science. 2021; 8: 401

Sancak İG, Eser Özgencil F, Arda Sancak A. Kedide eozinofilik keratitis. Veteriner Hekimler Derneği Dergisi. 2011; 82(2): 3-6.

Spiess AK, Sapienza JS, Mayordomo A. Treatment of proliferative feline eosinophilic keratitis with topical 1.5% cyclosporine: 35 cases. Veterinary ophthalmology. 2009; 12(2): 132-137.

Stiles J, Coster M. Use of an ophthalmic formulation of megestrol acetate for the treatment of eosinophilic keratitis in cats. Veterinary Ophthalmology. 2016; 19: 86–90. doi:10.1111/vop.12371

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022):15(2):182-188
DOI: 10.30607/kvj. 1061377

RESEARCH ARTICLE

Retrospective Evaluation of Cardiopulmonary Diseases in Cats and Dogs: 570 Cases

Ekrem Çağatay ÇOLAKOĞLU[1]*, Kadir SEVİM[1], Aslan KALINBACAK[1], Ufuk KAYA[2]

[1]Ankara University Faculty of Veterinary Medicine, Animal Hospital, Department of Internal Medicine, Cardiology Unit, Ankara, Turkey
[2]Hatay Mustafa Kemal University Faculty of Veterinary Medicine, Department of Biostatistics, Hatay, Turkey

ABSTRACT

Retrospective evaluation of the cardiopulmonary data is the main point in prevention and treatment of the diseases. In this study, it was aimed to retrospectively evaluate the data of 570 client-owned patients (321 dogs and 249 cats) referred to cardiology unit. The clinical findings, electrocardiographic data, echocardiography and blood analyses were evaluated. Acquired heart disease in 214 patients (37.54%), congenital heart disease in 38 patients (6.6%), and diseases affecting the lower and/or upper respiratory tract in 98 patients (17.19%) were found. Dilated cardiomyopathy was the most common acquired heart disease, and patent ductus arteriosus was the most common congenital heart disease in dogs. In cats, hypertrophic cardiomyopathy was the most common acquired heart disease, while tricuspid valve dysplasia was the most common congenital heart disease. It was concluded that clinical findings, physical examination and diagnostic applications should be evaluated together in the diagnosis of cardiopulmonary diseases.

Key words: Cardiopulmonary, cat, dilated cardiomyopathy, dog, heart disease, mitral

***

Kedi ve Köpeklerde Kalp Hastalıklarının Retrospektif Değerlendirilmesi: 570 Hasta

ÖZ

Kardiyopulmoner verilerin retrospektif değerlendirilmesi hastalıkların önlenmesinde ve tedavisinde temel noktadır. Bu çalışmada kardiyoloji birimine getirilen 321'i köpek ve 249'u kedi olmak üzere toplam 570 sahipli hastanın verilerinin retrospektif değerlendirilmesi amaçlandı. Hastaların klinik bulguları, elektrokardiyografik verileri, ekokardiyografileri ve kan analizleri değerlendirildi. 214 hastada (%37,54) edinsel kalp hastalığı, 38 hastada (%6.6) konjenital kalp hastalığı, 98 hastada (%17,19) alt ve/veya üst solunum yollarını etkileyen hastalıklara rastlandı. Köpeklerde edinsel kalp hastalıklarından en çok dilate kardiyomyopati, konjenital kalp hastalıklarından ise patent ductus arteriosus tespit edildi. Kedilerde ise hipertrofik kardiyomyopati en çok karşılaşılan edinsel kalp hastalığı olurken, triküspit kapak displazisi ise en çok görülen konjenital kalp hastalığıydı. Sonuç olarak kardiyopulmoner hastalıkların teşhisinde klinik bulgular, fiziksel muayene bulguları ve diyagnostik metodların beraber değerlendirilmesi gerektiği kanısına varıldı.

Anahtar kelimeler: Dilate kardiyomyopati, kalp hastalıkları, kardiyopulmoner, kedi, köpek, mitral.

To cite this article: Çolakoğlu E.Ç. Sevim K. Kalınbacak A. Kaya U. Retrospective Evaluation of Cardiopulmonary Diseases in Cats and Dogs: 570 Cases. Kocatepe Vet J. (2022):15(2):182-188

Submission: 21.01.2022 **Accepted**: 22.03.2022 **Published Online**: 18.04.2022
ORCID ID; *EÇÇ: 0000-0003-2789-035X, KS: 0000-0002-1959-1010, AK: 0000-0002-8057-2871, UK: 0000-0002-4805-0993*
*Corresponding author e-mail: colakoglu@ankara.edu.tr

INTRODUCTION

Quality time between pets and owners has increased associated with the restrictions in people's social lives due to COVID-19 pandemic in all over the world (Ho et al. 2021). Increased interest and concern of owners with their pets has resulted in increased contact between veterinarians and owners as well (Jalongo 2021).

According to the data collected from different veterinary clinics in 2014, Özen et al. reported that the number of client-owned cats and dogs in Ankara region is between 15-20 thousand and 25-30 thousand, respectively (Doğukan et al. 2014). A better understanding of the absolute and relative prevalences of common diseases in a certain population can be used to identify differential diagnoses and prioritize breed, research, and health control efforts. In addition, results from prevalence studies can facilitate the identification of strategic plans necessary for animal welfare. For this purpose, the prevalence of the diseases in 3,884 dogs were evaluated and published with data obtained from different centers (O'Neill et al. 2014b). Cardiopulmonary system diseases were among the most frequently described disorders. Similarly, in a study conducted on 3584 cats, it was reported that the prevalence of cardiac diseases was particularly high (O'Neill et al. 2014a).

Cardiopulmonary diseases are common in dogs and cats. Late diagnosis in early stages of cardiac disorders leads late treatment applications with poor prognosis. Defining the etiology and incidence of cardiopulmonary disorders is the main point in preventing the disease and treatment applications. Although the presence of some retrospective studies including cardiopulmonary disorders in Turkey (Cihan and Yılmaz 2011, Kibar et al. 2008), there is few cases in these studies.

The purpose of the current study was therefore to reflect the retrospective evaluation of cardiopulmonary disorders in dogs and cats.

MATERIAL and METHOD

Animals

The study population consisted of 570 non-pregnant client-owned dogs and cats referred with cardiopulmonary signs or routine cardiopulmonary examination during the time period between 26.09.2020-26.09.2021. All cases were evaluated for signalment, anamnesis, clinical signs and cardiopulmonary examination findings. Cardiopulmonary diagnosis were defined by the conbinations of clinical applications including blood analyses (WBC, LYM, NEUT, EOS, LYM, RBC, HCT, MCV, MCH, MCHC, RDW, PLT, Urea, Creatinin, Glucose, Total Bilirubin, ALP, ALT, GGT, CK, Na, K, P) and imaging procedures (Electrocardiography, echocardiography). 2D B and M mode, color-flow and spectral doppler Echocardiography (Philips Affinity 50® Echocardiography System) obtained from the right and left parasternal long and short axis positions and apical views were performed to identify the structural heart disease in all cases. Clinic ECG were recorded by a 12-lead ECG machine (Edan SE 1201®, 50 mm/sec,10 mm/mV) for 2 minutes in some cases lying right lateral recumbency without any sedation. Laterolateral and ventrodorsal thoracic radiographic images were also obtained in some cases. Diagnosis of heartworm disease were performed using rapid test kits (Uranotest Quattro®) and peripheral smears. All cases had antiparasitic therapy and routine vaccination. All cases were treated with appropriate medications.

Statistical Analysis

Descriptive statistics were performed using IBM SPSS Statistics software Version 23.0. Categorical variables included in the study were calculated as "Frequency (n) - Percent (%)". Quantitative variables were shown as "Mean ± Standard deviation". Results were presented as a table in all data.

RESULTS

The data of 570 client-owned dogs and cats referred with cardiopulmonary clinical signs or routine cardiopulmonary examination were retrospectively evaluated. While 214 patients (37.54%) had acquired heart disease, congenital heart disease was diagnosed in 38 patients (6.6%) (Table 1). The most common acquired heart disease in dogs was dilated cardiomyopathy (DCM) (45.19 %), followed by myxomatous mitral valve disease (MMVD) with 34.07 %. In cats, hypertrophic cardiomyopathy (HCM) (68.35%) was the most common acquired heart disease, followed by restrictive cardiomyopathy (RCM) with 10.13% (Table 1). In other referred patients; chronic bronchitis (42/570, 7.36%), tracheitis/laryngitis (25/570, 4.38%), lung metastasis following mammary tumor operation (8/570, 1.40%), acute bronchitis (6/570, 1.05%), bronchopneumonia (5/570, 0.87%), tracheal collapse (6/570, 1.05%), tracheobronchitis (6/570, 1.05%), diaphragmatic hernia (3/ 570, 0.5%), idiopathic pleural effusion (3/570, 0.5%), brachiocephalic syndrome (2/570, 0.35%), and peritoneopericardial diaphragmatic hernia (PPDH) (2/570, 0.35%) determined. 111 (19.47%) patients were referred for preoperative cardiopulmonary examination, while 99 (17.36%) were referred for routine cardiopulmonary controls.

Breed distributions in patients with various heart disease were shown in Table 2. Distribution of clinical signs in patients with DCM included exercise intolerance and dyspnea (15/61, 24.59%), ascites (10/61, 16.39%), exercise intolerance and coughing

(8/61, 13.11%), coughing (7/61, 11%), 47), dyspnea (6/61, 6.5%), dyspnea and ascites (2/61, 3.27%) and exercise intolerance (15/61, 24.59%). Secondary hypothyroidism in 10 dogs with DCM were also defined. Clinical signs in MMVD dogs were coughing (23/47, 48.93%), exercise intolerance (16/47, 34%), coughing and exercise intolerance (7/47, 14.89%), and dyspnea (1/47, 2.12%). Mitral regurgitation with concomitant tricuspid disease was defined in 8 MMVD dogs. The most common clinical signs in cats with HCM was dyspnea (43/54, 80%). There was no clinical signs in 11 cats (20.37%) diagnosed with HCM. In RCM cats, the most common clinical sign was also dyspnea.

Electrocardiographic findings in dogs with DCM (n=38) included atrial fibrillation (n=15, 39.47%), left ventricular enlargement pattern (n=9, 23.7%), ventricular tachycardia (n=8, 21%), biventricular enlargement pattern (n=3, 7.9%), left bundle branch block (n=2, 5.26%) and P mitrale (n=1, 2.63%). Echocardiographic examinations revealed left ventricular dilatation (n=32, 51%), left ventricular and atrial dilatation (n=18, 29%), biatrial dilatation (n=6, 9.7%), and mitral regurgitation (n=6, 9.7%). The electrocardiographic findings (n=27) of dogs with MMVD were as follows; P mitrale (n=12, 44%), sinus arrhythmia (n=8, 26.6%), ST depression (n=3, 11%), P pulmonale (n=3, 11%), and biphasic T wave (n=1, 3%). In echocardiographic examinations in MMVD dogs, mitral regurgitation (n=47, 100%), left atrial enlargement (n=19, 40.47%), tricuspid regurgitation (n=8, 17%) and mitral prolapse (n=5,10.6%) was determined as well. In cats with HCM (n=20), electrocardiography revealed ventricular premature complex (n=8 , 40%), atrial fibrillation (n=5, 25%), ventricular tachycardia (n=3, 15%), atrial premature complex (n=2, 10%) and sinus tachycardia (n=2, 10%). Echocardiographic examination of cats with hypertrophic cardiomyopathy revealed thickening of the intraventricular septum and/or left ventricular free wall in all cats. Obstructive aortic stenosis was detected in five cats (9%). Other findings were left atrial enlargement (n=24, 44%), systolic anterior motion (SAM) (n=12, 22.2%) and smoke in the left atrium (n=7, 13%), respectively.

Table 1. Characterisation of Cardiac Diseases

				Age		**Sex**	
AQUIRED HEART DISEASE	**Dog n (%)**	**Cat n (%)**	**Total n (%)**	**Dog**	**Cat**	♂	♀
DCM (n:10/65 hypothyroid dogs)	61 (45.19)	4 (5.06)	65 (30.37)	8.64±3.67	7.75±4.19	33	28
HCM (HOCM included)	-	54 (68.35)	54 (25.23)	-	5.41±3.91	41	13
MMVD	46 (34.07)	-	46 (21.50)	11.72±3.14	-	31	16
RCM	-	8 (10.13)	8 (3.74)	-	8±4.6	7	1
Endocarditis		5 (6.33)	5 (2.34)	-	3±2.01	2	3
Cardiac Mass	3 (2.22)	-	3 (1.40)	8±2.11	-	2	1
Dirofilariasis	2 (1.48)	-	2 (0.93)	4±3.12	-	2	0
Cardiorenal Syndrome	13 (9.63)	-	13 (6.07)	7±2.12	-	9	4
Mitral Regurgitation	4 (2.96)	3 (3.80)	7 (3.27)	8±3.21	5±2.1	5	2
FATE	-	5 (6.33)	5 (2.34)	-	3±1.2	3	2
Mitral Prolapse	3 (2.22)	-	3 (1.40)	6±3.2	-	1	2
Tricuspid Prolapse	3 (2.22)	-	3 (1.40)	3±1.1	-	1	2
CONGENITAL HEART DISEASE							
PDA	7 (29.17)	-	7 (18.42)	5±2.5	-	7	-
ASD	2 (8.33)	4 (28.57)	6 (15.79)	4.5±1.0	1±1.8	2	4
TVD veya TR	2 (8.33)	4 (28.57)	6 (15.79)	11±2.0	0.8±2.2	4	2
VSD (Muscular, Supracristal VSD included)	1 (4.17)	5 (35.71)	6 (15.79)	2	1.4±2.0	2	4
AS	5 (20.83)	-	5 (13.16)	11±1.9	-	2	3
PS	5 (20.83)	-	5 (13.16)	2.4±1.2	-	3	2
Aortopulmonary Window	1 (4.17)	-	1 (2.63)	1	-	-	1
SSS	1 (4.17)	-	1 (2.63)	5	-	-	1
Kartegener Syndrome	-	1 (7.14)	1 (2.63)	-	4	-	1
TOTAL	**159**	**93**	**252**				

DCM: Dilated Cardiomyopathy, HCM: Hypertrophic Cardiomyopathy, HOCM: Hypertrophic Obstructive Cardiomyopathy, MMVD: Myxomatous Mitral Valve Disease, RCM: Restrictive Cardiomyopathy, FATE: Feline Arterial Thromboembolism, PDA: Patent Ductus Arteriosus, ASD: Atrial Septal Defect, VSD: Ventricular Septal Defect, TVD: Tricuspid Valve Dysplasia, TR: Primary Tricuspid Regurgitation, AS: Aortic Stenosis, PS: Pulmonary Stenosis, SSS: Sick Sinus Syndrome.

Table 2. Breed Distributions

Dog Breeds	DCM, n (%)	MMVD, n (%)	HCM, n (%)	RCM, n (%)
Golden Retriever	22 (36.1)	-	-	-
Mix Breed Dogs	10 (16.4)	-	-	-
Cocker Spaniel	8 (13.1)	1 (2.1)	-	-
Malaklı	1 (1.6)	-	-	-
Pomeranian	1 (1.6)	-	-	-
Setter	2 (3.3)	-	-	-
Terrier types	3 (4.9)	18 (38.3)	-	-
Kurzhaar	2 (3.3)	-	-	-
Kangal	5 (8.2)	-	-	-
Bulldog	1 (1.6)	-	-	-
Belgian Malinois	1 (1.6)	-	-	-
Rottweiler	4 (6.6)	-	-	-
Beagle	1 (1.6)	-	-	-
Cavalier King Charles	-	7 (14.9)	-	-
Kai	-	3 (6.4)	-	-
Pekingese	-	13 (27.7)	-	-
Chihuahua	-	3 (6.4)	-	-
Pincher	-	1 (2.1)	-	-
Jack Russell	-	1 (2.1)	-	-
Cat Breeds				
British Short Hair	-	-	12 (22.2)	1 (12.5)
Mix Breed Cats	-	-	13 (24.1)	4 (50)
Scottish Fold	-	-	18 (33.3)	-
Persian	-	-	9 (16.7)	-
Chinchilla	-	-	1 (1.9)	-
Siamese	-	-	1 (1.9)	-
Van Cat	-	-	-	1 (12.5)
Ankara Cat	-	-	-	2 (25)

DCM: Dilated Cardiomyopathy (n:61 dogs), HCM Hypertrophic Cardiomyopathy (n:54), MMVD: Myxomatous Mitral Valve Disease (n:46), RCM: Restrictive Cardiomyopathy (n:8).

DISCUSSION

Although some difficulties are possible in determining the prevalence of congenital heart disease in dogs and cats, some malformations can cause directly perinatal mortality without any cardiac murmurs. Breed predispositions may also affect the type of possible congenital heart disease (Oliveira et al. 2011). In the study presented here, congenital heart disease was detected in 24 dogs and 14 cats. In accordance with the current literatures (Côté et al. 2011a, Oyama and Strickland 2015, Garncarz et al. 2017), the most common congenital heart diseases in dogs and cats were patent ductus arteriosus (PDA, n=7) and tricuspid valve dysplasia (n=6), respectively. Dilated cardiomyopathy (DCM) characterized by systolic dysfunction and poor prognosis usually affects large breed dogs (Martin et al. 2010). Researches have reported the predispositions in large breed dogs including Doberman Pincher, Irish Wolfhound, Great Danes, Boxer, American Cocker, Bulldog, Golden Retriever and Saint Bernard (Dukes-McEwan et al. 2003, Vollmar et al. 2013, Bélanger et al. 2005, Fascetti et al. 2003, Backus et al. 2006, Backus et al. 2003). In the current study, similar findings were also observed. 22 dogs diagnosed with DCM phenotype were Golden Retrievers. It is also known the reasons of genetic, nutrition, inflammation, infiltration, ischemia, drug/toxin-induced cardiomyopathy, immunological disorders, metabolic diseases, biochemical changes and chronic tachycardia are the possible etiological factors in DCM (McCauley et al. 2020, Freid et al. 2021, Beier et al. 2015). Therefore, other predisposing factors should be considered in patients with DCM. In this study, many of them have been ruled out by the diagnostic procedures and concomitant hypothyroidism was detected in 10 dogs with dilated cardiomyopathy. In the present study, most of the dogs with DCM were male and over the middle-aged as consistent with the reports previously reported (Freid et al. 2021, Çolakoğlu et al. 2017, Martin et al. 2009, Dukes-McEwan 2016).

Similar to the results of previous studies, atrial fibrillation was most common arrhythmia in

electrocardiographic records of dogs with DCM (Martin et al. 2009). Echocardiographic examination findings in this study have also been found to be compatible with the literature (Bonagura and Visser 2021, Tidholm et al. 2001).

Myxomatous mitral valve disease (MMVD) is a chronic degenerative heart disease usually occurring in middle-aged small breed dogs. Degenerative changes preventing valve function over time cause mitral insufficiency. Concomitant degenerative changes on tricuspid valves have also been defined with mitral valve changes. Congestive heart failure, pulmonary hypertension, pulmonary edema and arrhythmias may occur in the later stages of the disease as well (Borgarelli and Buchanan 2012). In the study here, all MMVD dogs were small breeds (<15 kg) as consistent with the previous studies (Mattin et al. 2015, Kim et al. 2017). 6 years or older dogs with MMVD in the study presented here have supported the fact that mitral valve disease was more common in older ages of dogs (Çolakoğlu et al. 2017, Kim et al. 2017, Mattin et al. 2015, Borgarelli and Buchanan 2012). Similar to the studies reporting that degenerative mitral valve disease was more common in male animals (Keene et al. 2019, Mattin et al. 2015, Petrič 2015), male dogs were majority among dogs with MMVD. Although myxomatous mitral valve disease mainly affects the mitral valve, it is known that the tricuspid valve is also affected in 30% of cases (Keene et al. 2019). In the study, degenerative changes and related tricuspid regurgitation were observed in the tricuspid valve in 8 dogs. Kim et al. have reported the sinus arrhythmia in 40 dogs (40/168, 23.8%) as a result of their retrospective study of MMVD (Kim et al. 2017). These results appear to be similar to our study. Increased LA volume and stroke volume in dogs with MMVD occurs due to regurgitant flow from the left ventricle. This is a compensatory mechanism necessary to prevent pulmonary congestion and maintain ventricular filling (Höllmer et al. 2017). In the study here, left atrial enlargement was also remarkable in 19 dogs (n=19, 40.47%).

Hypertrophic cardiomyopathy (HCM) is the most common acquired heart disease in cats. The disease is characterized by a non-dilated and thickened left ventricle free wall. For the exact diagnosis of the disease as primary cardiomyopathy, secondary disorders including hypertension and hyperthyroidism associated with left ventricular thickening should be ruled out (Trehiou-Sechi et al. 2012, Payne et al. 2013). While the disease can cause congestive heart failure, thromboembolism or sudden death, some cats may remain asymptomatic for years and may not cause any systemic disorders (Payne et al. 2013). In accordance with the literatures (Wilkie et al. 2015, Payne et al. 2015), HCM fenotype was most frequently identified in cats with heart disease referred to cardiology unit. Although there are studies showing a higher risk of developing HCM in pure breeds (Luis Fuentes et al. 2020), further studies are required. In this study, the majority of cats with HCM phenotype were pure breeds (n=40). We think this situation is associated with the distribution of cats in Ankara region. In consistent with the reports previously described (Trehiou-Sechi et al. 2012, Spalla et al. 2016, Payne et al. 2013, Rush et al. 2002), cats with HCM are predominantly male (n=41) in this study as well. The higher incidence of ventricular premature complexes and atrial fibrillation in our study is consistent with the reports (Trehiou-Sechi et al. 2012, Payne et al. 2010). Echocardiography is the best method for diagnosing HCM. Thickening of the left ventricular free wall, interventicular septum or papillary muscles are the main echocardiographic changes in this disease (Ware and Ward 2019). The echocardiographic findings in the study were similar to the data previously published (Trehiou-Sechi et al. 2012, Spalla et al. 2016, Ferasin et al. 2003).

Restrictive cardiomyopathy (RCM) is a myocardial disease characterized by severe diastolic dysfunction, atrial enlargement, normal left ventricular wall thickness, and normal/slightly decreased systolic function. Echocardiography is also the best method for the diagnosis of RCM including myocardial and endocardial forms (Locatelli et al. 2018). In retrospective studies, RCM has been described as the second most common cardiomyopathy in cats (Locatelli et al. 2018, Spalla et al. 2016). RCM cats in this study were also the second most common cardiomyopathy as consistent with the reports previously described. In a necropsy study of 304 cats, Kimura et al. (Kimura et al. 2016) most observed the endocardial form of RCM and reported that RCM is much more common in cats contrary to popular belief. They have attributed the reasons why the incidence of the disease is known to be low to the localization of endocardial fibrotic tissues, the quality of the devices for imaging, and the experience of the operator. The predominance of male sex (8/9) in cats with RCM in our study was consistent with previous studies (Kimura et al. 2016, Locatelli et al. 2018). Similar to the studies reporting the wide age range of the RCM (Locatelli et al. 2018, Kimura et al. 2016, Chetboul et al. 2019), in the study herein we also observed the wide age range in cats (3-15 years).

Although the prevalence of dilated cardiomyopathy in cats is not clearly known, it is known that it is less common compared to hypertrophic cardiomyopathy. Despite the addition of taurine to commercial cat foods, it is still possible to observe the dilated cardiomyopathy in cats (Côté et al. 2011b). The main cause of idiopathic dilated cardiomyopathy in cats with normal taurine levels is not fully understood (Sevim and Çolakoğlu 2021, Ferasin et al. 2003). In our study, dilated cardiomyopathy was diagnosed in 4 cats known to be fed commercial foods. Levels in 2 cats measured taurine concentrations were observed

within reference ranges. As a result, only two cats could be classified as idiopathic dilated cardiomyopathy. In remaining two cats, the taurine level could not be determined due to economic concerns.

CONCLUSION

It was concluded that clinical symptoms and physical examination findings with diagnostic applications should be considered together in the evaluation process of cardiopulmonary diseases. It was also considered that DCM and MMVD were the most common heart disease in dogs.

Ethics Committee Information: This study does not present any ethical concerns.

Conflict of interest: The authors declare that there is no conflict of interest.

Financial support: Study (Project name: Elimination of the Deficiencies of the Veterinary Cardiology Unit) was funded by Ankara University BAP Coordination Unit with the Project number 20A0239001.

Authors Contribution Rate: The authors declared that they contributed equally to the article.

REFERENCES

Backus RC, Cohen G, Pion PD, Good KL, Rogers QR, Fascetti AJ. Taurine deficiency in Newfoundlands fed commercially available complete and balanced diets. J. Am. Vet. Med. Assoc. United States; 2003;223(8): 1130–6.

Backus RC, Ko KS, Fascetti AJ, Kittleson MD, Macdonald KA, Maggs DJ, Berg JR, Rogers QR. Low plasma taurine concentration in Newfoundland dogs is associated with low plasma methionine and cyst(e)ine concentrations and low taurine synthesis. J. Nutr. United States; 2006;136(10): 2525–33.

Beier P, Reese S, Holler PJ, Simak J, Tater G, Wess G. The role of hypothyroidism in the etiology and progression of dilated cardiomyopathy in Doberman Pinschers. J. Vet. Intern. Med. Wiley Online Library; 2015;29(1): 141–9.

Bélanger MC, Ouellet M, Queney G, Moreau M. Taurine-deficient dilated cardiomyopathy in a family of golden retrievers. J. Am. Anim. Hosp. Assoc. United States; 2005;41(5): 284–91.

Bonagura JD, Visser LC. Echocardiographic assessment of dilated cardiomyopathy in dogs. J. Vet. Cardiol. Elsevier; 2021.

Borgarelli M, Buchanan JW. Historical review, epidemiology and natural history of degenerative mitral valve disease. J. Vet. Cardiol. Elsevier; 2012;14(1): 93–101.

Chetboul V, Passavin P, Trehiou-Sechi E, Gouni V, Poissonnier C, Pouchelon J, Desquilbet L. Clinical, epidemiological and echocardiographic features and prognostic factors in cats with restrictive cardiomyopathy: A retrospective study of 92 cases (2001-2015). J. Vet. Intern. Med. Wiley Online Library; 2019;33(3): 1222–31.

Cihan H, Yılmaz Z. Bursa'daki köpeklerde kalp hastalıklarının prevalansı. Uludağ Üniversitesi; 2011.

Çolakoğlu E, Kurtdede A, Haydardedeoğlu A, Alihosseini H, Özen D, Aydemir E. Evaluation of the cardiopulmonary consultation requests in 88 dogs with heart disease. Vet. Fakültesi Derg. 2017;64: 191–8.

Côté E, MacDonald KA, Meurs KM, Sleeper MM. Feline Cardiology. Feline Cardiol. 2013.

Doğukan O, GÜRCAN S, Ufuk K. Ankara ilinde yer alan sahipli kedi ve kopek populasyonunun belirlenmesi. Vet. Hekimler Derneği Derg. 2014;85(1): 9–16.

Dukes-McEwan J, Borgarelli M, Tidholm A, Vollmar AC, Häggström J. Proposed Guidelines for the Diagnosis of Canine Idiopathic Dilated Cardiomyopathy. J. Vet. Cardiol. 2003;5(2): 7–19.

Fascetti AJ, Reed JR, Rogers QR, Backus RC. Taurine deficiency in dogs with dilated cardiomyopathy: 12 cases (1997-2001). J. Am. Vet. Med. Assoc. United States; 2003;223(8): 1137–41.

Ferasin L, Sturgess CP, Cannon MJ, Caney SMA, Gruffydd-Jones TJ, Wotton PR. Feline idiopathic cardiomyopathy: a retrospective study of 106 cats (1994-2001). J. Feline Med. Surg. England; 2003;5(3): 151–9.

Freid KJ, Freeman LM, Rush JE, Cunningham SM, Davis MS, Karlin ET, Yang VK. Retrospective study of dilated cardiomyopathy in dogs. J. Vet. Intern. Med. Wiley Online Library; 2021;35(1): 58–67.

Fuentes VL, Johnson LR, Dennis S, Avenue S, Johnson LR. BSAVA Manual of Canine and Feline Cardiorespiratory Medicine Second edition. 2016; 330.

Garncarz M, Parzeniecka-Jaworska M, Szalus O. Congenital heart defects in dogs: A retrospective study of 301 dogs. Med. Weter. 2017;73: 651–6.

Ho J, Hussain S, Sparagano O. Did the COVID-19 Pandemic Spark a Public Interest in Pet Adoption? Front. Vet. Sci. 2021;8: 444.

Höllmer M, Willesen JL, Tolver A, Koch J. Left atrial volume and function in dogs with naturally occurring myxomatous mitral valve disease. J. Vet. Cardiol. Elsevier; 2017;19(1): 24–34.

Jalongo MR. Pet Keeping in the Time of COVID-19: The Canine and Feline Companions of Young Children. Early Child. Educ. J. Springer; 2021; 1–11.

Keene BW, Atkins CE, Bonagura JD, Fox PR, Häggström J, Fuentes VL, Oyama MA, Rush JE, Stepien R, Uechi M. ACVIM consensus guidelines for the diagnosis and treatment of myxomatous mitral valve disease in dogs. J. Vet. Intern. Med. 2019;33(3): 1127–40.

Kibar M, Oğrak Y, Apaydın N, Çam Y. Kangal Irkı Köpeklerde Doğmasal Kalp Hastalıklarında Doppler Ekokardiografik Muayene Bulguları. Erciyes Üniversitesi Vet. Fakültesi Derg. 2008;5(2): 73–9.

Kim H-T, Han S-M, Song W-J, Kim B, Choi M, Yoon J, Youn H-Y. Retrospective study of degenerative mitral valve disease in small-breed dogs: survival and prognostic variables. J. Vet. Sci. The Korean Society of Veterinary Science; 2017;18(3): 369–76.

Kimura Y, Fukushima R, Hirakawa A, Kobayashi M, Machida N. Epidemiological and clinical features of the endomyocardial form of restrictive cardiomyopathy in cats: a review of 41 cases. J. Vet. Med. Sci. Japanese Society of Veterinary Science; 2016; 15–373.

Locatelli C, Pradelli D, Campo G, Spalla I, Savarese A, Brambilla PG, Bussadori C. Survival and prognostic factors in cats with restrictive cardiomyopathy: a review of 90 cases. J. Feline Med. Surg. Sage Publications Sage UK: London, England; 2018;20(12): 1138–43.

Luis Fuentes V, Abbott J, Chetboul V, Côté E, Fox PR, Häggström J, Kittleson MD, Schober K, Stern JA. ACVIM consensus statement guidelines for the classification, diagnosis, and management of cardiomyopathies in cats. J. Vet. Intern. Med. Wiley Online Library; 2020;34(3): 1062–77.

Martin MWS, Stafford Johnson MJ, Celona B. Canine dilated cardiomyopathy: a retrospective study of signalment, presentation and clinical findings in 369 cases. J. Small Anim. Pract. Wiley Online Library; 2009;50(1): 23–9.

Martin MWS, Stafford Johnson MJ, Strehlau G, King JN. Canine dilated cardiomyopathy: a retrospective study of prognostic findings in 367 clinical cases. J. Small Anim. Pract. Wiley Online Library; 2010;51(8): 428–36.

Mattin MJ, Boswood A, Church DB, López-Alvarez J, McGreevy PD, O'Neill DG, Thomson PC, Brodbelt DC. Prevalence of and risk factors for degenerative mitral valve disease in dogs attending primary-care veterinary practices in England. J. Vet. Intern. Med. Wiley Online Library; 2015;29(3): 847–54.

McCauley SR, Clark SD, Quest BW, Streeter RM, Oxford EM. Review of canine dilated cardiomyopathy in the wake of diet-associated concerns. J. Anim. Sci. Oxford University Press US; 2020;98(6): skaa155.

O'Neill DG, Church DB, McGreevy PD, Thomson PC, Brodbelt DC. Prevalence of disorders recorded in cats attending primary-care veterinary practices in England. Vet. J. [Internet]. 2014;202(2): 286–91.

O'Neill DG, Church DB, McGreevy PD, Thomson PC, Brodbelt DC. Prevalence of Disorders Recorded in Dogs Attending Primary-Care Veterinary Practices in England. PLoS One. Public Library of Science; 2014;9(3): e90501.

Oliveira P, Domenech O, Silva J, Vannini S, Bussadori R, Bussadori C. Retrospective review of congenital heart disease in 976 dogs. J. Vet. Intern. Med. United States; 2011;25(3): 477–83.

Payne J, Luis Fuentes V, Boswood A, Connolly D, Koffas H, Brodbelt D. Population characteristics and survival in 127 referred cats with hypertrophic cardiomyopathy (1997 to 2005). J. Small Anim. Pract. Wiley Online Library; 2010;51(10): 540–7.

Payne JR, Borgeat K, Connolly DJ, Boswood A, Dennis S, Wagner T, Menaut P, Maerz I, Evans D, Simons VE. Prognostic indicators in cats with hypertrophic cardiomyopathy. J. Vet. Intern. Med. Wiley Online Library; 2013;27(6): 1427–36.

Payne JR, Brodbelt DC, Fuentes VL. Cardiomyopathy prevalence in 780 apparently healthy cats in rehoming centres (the CatScan study). J. Vet. Cardiol. Elsevier; 2015;17: S244–57.

Petrič AD. Myxomatous mitral valve disease in dogs− An update and perspectives. Maced. Vet. Rev. 2015;38: 13–20.

Rush JE, Freeman LM, Fenollosa NK, Brown DJ. Population and survival characteristics of cats with hypertrophic cardiomyopathy: 260 cases (1990–1999). J. Am. Vet. Med. Assoc. Am Vet Med Assoc; 2002;220(2): 202–7.

Sevim K, Çolakoğlu EÇ. Non-taurine responsive dilated cardiomyopathy in 2 cats. Ankara Üniversitesi Vet. Fakültesi Derg. 2021.

Smith FWK, Tilley LP, Oyama M, Sleeper MM. Manual of canine and feline cardiology-E-Book. Elsevier Health Sciences; 2015.

Spalla I, Locatelli C, Riscazzi G, Santagostino S, Cremaschi E, Brambilla P. Survival in cats with primary and secondary cardiomyopathies. J. Feline Med. Surg. Sage Publications Sage UK: London, England; 2016;18(6): 501–9.

Tidholm A, Häggström J, Borgarelli M, Tarducci A. Canine idiopathic dilated cardiomyopathy. Part I: aetiology, clinical characteristics, epidemiology and pathology. Vet. J. Elsevier; 2001;162(2): 92–107.

Trehiou-Sechi E, Tissier R, Gouni V, Misbach C, Petit AMP, Balouka D, Carlos Sampedrano C, Castaignet M, Pouchelon J, Chetboul V. Comparative echocardiographic and clinical features of hypertrophic cardiomyopathy in 5 breeds of cats: a retrospective analysis of 344 cases (2001–2011). J. Vet. Intern. Med. Wiley Online Library; 2012;26(3): 532–41.

Vollmar AC, Fox PR, Servet E, Biourge V. Determination of the prevalence of whole blood taurine in Irish wolfhound dogs with and without echocardiographic evidence of dilated cardiomyopathy. J. Vet. Cardiol. Off. J. Eur. Soc. Vet. Cardiol. Netherlands; 2013;15(3): 189–96.

Wilkie LJ, Smith K, Fuentes VL. Cardiac pathology findings in 252 cats presented for necropsy; a comparison of cats with unexpected death versus other deaths. J. Vet. Cardiol. Elsevier; 2015;17: S329–40.

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022):15(2):189-193

DOI: 10.30607/kvj.1073174

RESEARCH ARTICLE

Comparative Evaluation of PSA and USG Results in the Diagnosis of Prostate Hyperplasia in Geriatric Dogs

Ömer Cankan ALPAY[1], Turan CİVELEK[2*]

[1] Pasteur Veterinary Polyclinic, 41050, İzmit, Turkey

[2] Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Internal Medicine, 03200, Afyonkarahisar, Turkey*

ABSTRACT

In geriatric dogs, there is an increase in the incidence of clinical manifestations of different diseases in relation to age. One of the most important of these diseases is prostatic hyperplasia. It is known that 3-10% of male dogs brought to veterinarians for examination have prostatic hyperplasia. Prostate diseases are generally not differentiated by evaluation of clinical signs. Ultrasonography, enzymatic measurements and biopsy are required for definitive diagnosis. Determining which diagnostic tools to use in possible situations is of great importance in establishing the correct diagnosis and correct treatment protocol. In this study, it was aimed to evaluate the results of ultrasonography and specific enzyme measurement in the diagnosis of prostatic hyperplasia in geriatric dogs. In the ultrasonographic examination performed in 24 geriatric dogs, the mean value was determined as prostate length 4.88±0.15 cm, prostate width 3.98±0.11 cm, prostate height 3.46±0.13 cm and prostate volume 35.18±2.01 cm^3. In dogs diagnosed only with prostatic hyperplasia; as the mean value, prostate length was 4.93±0.24 cm, prostate width was 3.74±0.11 cm, prostate height was 3.63±0.11 cm, and prostate volume was 34.97±2.44 cm^3. PSA level was measured as <0.006 ng/mL in all dogs in the study.

Keywords: Hyperplasia, Dog, Prostate, PSA, Ultrasonography.

***

Geriatrik Köpeklerde Prostat Hiperplazisinin Tanısında PSA ve USG Sonuçlarının Karşılaştırmalı Değerlendirilmesi

ÖZ

Geriatrik dönem köpeklerde, yaşla ilişkili olarak, farklı hastalıkların klinik görülme insidansında bir artış şekillenir. Bu hastalıkların en önemlilerinden biri de prostat hiperplazisidir. Veteriner Hekimlere muayene için getirilen erkek köpeklerin % 3-10'unda prostat hiperplazisine rastlandığı bilinmektedir. Prostat hastalıkları, genellikle, klinik belirtilerin değerlendirilmesiyle ayırt edilememektedir. Kesin tanı için ultrasonografi, enzimatik ölçümler ve biyopsi gerekir. Olası durumlarda hangi teşhis araçlarının kullanılacağını belirlemek doğru tanı ve doğru tedavi protokolünün ortaya konmasında büyük önem arz eder. Bu çalışmada geriatrik dönem köpeklerde prostat hiperplazisinin tanısında ultrasonografi ve spesifik enzim ölçüm sonuçlarının değerlendirilmesi amaçlandı. Geriatrik 24 köpekte yapılan ultrasonografik muayenede, ortalama değer olarak, prostat boyu 4.88±0.15 cm, prostat eni 3.98±0.11 cm, prostat yüksekliği 3.46±0.13 cm ve prostat hacmi ise 35.18±2.01 cm^3 olarak belirlendi. Sadece prostat hiperplazisi tanısı konan köpeklerde ise; ortalama değer olarak, prostat boyu 4.93±0.24 cm, prostat eni 3.74±0.11 cm, prostat yüksekliği 3.63±0.11 cm ve prostat hacmi ise 34.97±2.44 cm^3 olarak tespit edildi. Çalışmadaki köpeklerin tamamında PSA düzeyi <0.006 ng/mL olarak ölçüldü.

Anahtar Kelimeler: Hiperplazi, Köpek, Prostat, PSA, Ultrasonografi.

To cite this article: Alpay Ö.C. Civelek T. Comparative Evaluation of PSA and USG Results in the Diagnosis of Prostate Hyperplasia in Geriatric Dogs. Kocatepe Vet J. (2022):15(2):189-193

Submission: 14.02.2022 **Accepted**: 27.04.2022 **Published Online**: 28.04.2022

ORCID ID; *ÖCA: 0000-0002-2536-1318, TC: 0000-0002-4819-3484*

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

GİRİŞ

Köpek prostatı proksimal üretrayı çevreleyen çift loblu bir yapıdadır. Dorsal ve ventral sulkusa sahip olan bu bez aynı zamanda fibromüsküler bir kapsülle çevrilidir (Evans ve Christensen, 1993). Prostatın sağ ve sol loblarını ayıran belirgin bir medyan septumu bulunmaktadır (Kutzler ve Yeager, 2005). Her bir lob ayrıca trabeküllerle lobüllere bölünür. Tubuloalveolar bezler lobüller içinde yer alır ve prostat salgıları bezleri üretraya boşalan küçük kanallar yoluyla terk eder (Smith, 2008).

Köpeklerde görülen yaygın prostat hastalıkları arasında prostat hiperplazisi ((bening prostat büyümesi (BPH)), prostatit, prostat kistleri ve prostat adenokarsinomu yer almaktadır. Köpeklerde prostat hastalıkları vakalarının %50'den fazlası bening prostat hiperplazisi (BPH) ile ilişkilidir (Krawiec ve ark, 1992). BPH genellikle yaşa bağlı ortaya çıkan bir hastalık olarak dikkat çekmektedir (Lévy ve ark, 2014). BPH beş yaşın üzerindeki erkek köpeklerde %80 düzeyinde görülür. Özellikle dokuz yaşın üzerindeki köpeklerin ise %95'inin etkilendiği bildirilmiştir (Korodi ve ark, 2008). Bu hastalıkta klinik olarak; kanlı prostat sıvısı, kabızlık, disüri ve tenesmus izlenir. Kısırlaştırılmamış köpekler vakaların büyük çoğunluğunu teşkil etmektedir. Bununla birlikte kısırlaştırılmış köpeklerde de prostat tümörleri ve enfeksiyonları gözlenebilir (Wilson, 2011).

Köpeklerde prostat bezinin başlıca salgı ürünü olan köpek prostata özgü esteraz (CPSE), insan prostata özgü antijenine (PSA) benzer ve PSA gibi serin proteaz yapıdadır. CPSE ve PSA hormonal düzenleme altındadır ve serum testosteron aktivitelerindeki düşüşler serum ve seminal plazma konsantrasyonlarında azalmaya neden olur (Dube ve ark, 1986). PSA'nın prostat kanseri (PCa) ve BPH gibi insan prostat bozukluklarında klinik uygulamada önemli bir biyokimyasal belirteç olduğu bildirilmiştir. PSA prostatın epitel hücrelerinde üretilen glikoprotein yapıda bir enzimdir. İmmünohistokimya kullanılarak prostatik dokuda gösterilebilir ve serum ve seminal sıvıda tespit edilebilir. Aşırı PSA üretimi ve/veya epitelin bozulması ve kan-glandüler bariyerindeki değişiklik PSA'nın epitel çevresindeki dokuya ve sonuç olarak kan dolaşımına difüzyonuna yol açarak serum PSA seviyelerinin yükselmesine neden olur (Roehrborn, 1999).

Noninvaziv bir yöntem olan ultrasonografi insanlarda prostatın değerlendirilmesi amacıyla ilk kez 1967 yılında kullanılmıştır (Edson Pontes ve ark, 1984). O zamandan günümüze, ultrasonografi prostat anormalliklerini araştırmak ve özellikle parankim boyutunu ve ekotekstürünü incelemek için kullanılmaktadır (Günzel ve ark, 2001). Prostatın ultrasonografik görüntülemesi; prostatın boyutu, şekli, iç yapısı, ekojenitesi ve ekotekstüründeki değişiklikler ve yanı sıra sınır yapısı ve kapsülü hakkında da bilgi sahibi olmamızı sağlar (Zohel ve Castellano, 1995, Atalan ve Holt, 1999). Prostat bezinin parankiması sağlıklı köpeklerde orta derecede ekojeniteye sahiptir ve homojendir. İnce ila orta derecede kaba bir eko dokuya sahiptir. Prostat sınırı tamamen düzdür ve ekojenik bir kapsül ile karakterizedir. Prostat bezinin enine kesitinde ortada loblar arasında ve sagital görünümde ise hipoekoik bir bölge olarak iki prostat lobu arasında prostatik üretra görülür. Prostatın genel şekli ovaldir (Nyland ve Mattoon, 2002, Penninck, 2015).

Prostat parankiminin ultrason görüntüsünde görülen değişiklikler fokal veya diffüz olabilir. Ekojenite açısından ise değişimler hipoekoik, hiperekoik ve miks ekojenitede görülebilirler. Artmış prostat boyutu (prostatomegali) prostat anormalliklerinin çoğunda yaygın ancak spesifik olmayan bir bulgudur. Benzer şekilde, prostat ekojenite değişiklikleri de çoğu prostat bozukluğunda görülebilmektedir. Bununla birlikte, bu değişiklikler belirli bir prostat hastalığını tek başına doğrulamada veya saf dışı bırakmada yeterli olmayabilir (de Souza ve ark, 2017). BPH'de prostat; normal veya genel olarak artmış parankim ekojenitesi ve homojen veya homojen olmayan yayılan çizgili eko doku ile simetrik olarak büyümektedir. Bazı durumlarda prostat küçük kistler içerebilir. Bu kistler başlangıçta yaklaşık 1-2 milimetre çapa sahiptir, ancak hastalık ilerledikçe çapları 2-3 santimetreye kadar büyüyebilir. Bu değişiklikler, sonuç olarak, prostatın ultrasonografik olarak petek tarzında bir yapıda görünmesine neden olabilir (Penninck, 2015, de Souza ve ark, 2017). Ultrasonografi prostat hastalıklarında en sık kullanılan tanı yöntemlerinden biri olmasına rağmen, bu teknik bazı zorluklarla da karşı karşıyadır. Gözlemci deneyimi/bağımlılığı, ultrasonografi kullanımında karşılaşılan en önemli zorluklardandır (Khanbazi ve ark, 2021).

Bu çalışmada, geriatrik köpeklerde prostat hiperplazisinin (BPH) tanısında ultrasonografik ölçüm verileri ve spesifik enzim ölçüm sonuçlarındaki değişim değerlendirildi.

MATERYAL VE METOD

Sunulan çalışmanın materyalini; özel bir Veteriner kliniğe (Pasteur Veteriner Polikliniği, İzmit, Türkiye) getirilen, sahiplerinin verdiği anamnezle paralel olarak, ürolojik problemden şüphelenilen yedi yaş ve üzeri (geriatrik döneme girmiş) 24 erkek köpek (10'u kısırlaştırılmış, 14'ü kısırlaştırılmamış) oluşturdu. Hastalarda öncelikli olarak prostatın ultrasonografik muayenesi gerçekleştirildi. Buna ek olarak tüm hastalarda PSA ölçüldü. PSA analizi için vena cephalica'dan alınan kan örnekleri (serum) kullanıldı. Ölçümler özel bir laboratuvarda (Yüksel Tanı Tıbbi Tahlil Laboratuvarı, İzmit, Türkiye) ve beşeri PSA kiti (Elecsys total PSA/Cobas®, Roche) kullanılarak

yapıldı. Ultrason ölçümleri (Siemens, Affiniti 50) için her köpek dorso-ventral pozisyonda konumlandırıldı ve prostat boyunca longtidunal ve transversal görüntüler alındı. Prostatın kraniokaudal (uzunluk, L), transversal (genişlik, W) ve dorsoventral (derinlik, D) çapları "cm" olarak ölçüldü. Prostatik hacim (cm^3) formüle göre hesaplandı: Volume = Length * Width * Height * 0.523 (Ruel ve ark, 1998).

Sunulan çalışmada ölçümü yapılan ve elde edilen verilerde ortalama ve ortalamanın standart hata değerlerinin hesaplaması ve yanı sıra BPH'lı kısırlaştırılmış ve kısırlaştırılmamış köpekler arası prostat volümü açısından istatistiki değerlendirme (2-Sample t-test) Minitab bilgisayar programı (Versiyon 18.1, Minitab, Inc.) kullanılarak yapıldı.

BULGULAR

Çalışma materyalini oluşturan 10'u kısırlaştırılmış ve 14'ü ise kısırlaştırılmamış, toplam 24 hastanın yaş ortalaması 9.88±0.39 yıl, vücut ağırlıkları ise 13.83±1.31 kg olarak belirlendi. Sadece BPH tanısı konan 14 köpeğin (8'i kısırlaştırılmış, 6'sı ise kısırlaştırılmamış) ise yaş ortalaması 10.29±0.46 yıl ve vücut ağırlıkları ortalaması ise 14.07±1.77 kg'dı. Materyali oluşturan köpeklerde gerçekleştirilen tüm uygulama ve ölçümler rutin klinik faaliyetle ilişkilidir. Gerçekleştirilen ultrasonografik muayene sonuçlarına göre bu hastaların 14'ünde BPH tespit edilirken, buna ek olarak; bir köpekte prostat neoplazisi, dört köpekte prostatitis ve beş köpekte ise prostat kisti belirlendi (n=10). 24 hastanın 16'sında herhangi bir ilave kronik hastalık bulunmazken, sekiz hastada ek olarak; böbrek yetmezliği, diabetes mellitus ve kalp yetmezliği teşhis edildi.

Yapılan ölçümlerde materyali oluşturan 24 köpekte, ortalama değer olarak, prostat boyu 4.88±0.15 cm, prostat eni 3.98±0.11 cm, prostat yüksekliği 3.46±0.13 cm ve prostat hacmi ise 35.18±2.01 cm^3 olarak belirlendi. Sadece BPH tanısı konan köpeklerde ise; ortalama değer olarak, prostat boyu 4.93±0.24 cm, prostat eni 3.74±0.11 cm, prostat yüksekliği 3.63±0.11 cm ve prostat hacmi ise 34.97±2.44 cm^3 olarak tespit edildi. BPH harici, prostat neoplazisi, prostatitis ve prostat kisti tanısı alan 10 köpekte ise, gerçekleştirilen ölçümlerde ortalama prostat boyu (length) 4.80±0.15 cm, ortalama prostat eni (width) 4.34±0.16 cm ve ortalama prostat yüksekliği (height) 3.22±0.25 cm olarak belirlendi. Bu köpeklerde ortalama prostat hacmi (volume) 35.46±3.56 cm^3, ortalama yaş 9.30±0.65 ve ortalama vücut ağırlığı ise 13.50±2.02 kg olarak hesaplandı (Tablo 1). Materyali oluşturan köpeklerde (n=24) gerçekleştirilen PSA analiz sonuçları, tüm köpekler için; <0.006 ng/mL olarak, laboratuvar tarafından belirtilen referans sınırlar (0.000-4.000 ng/mL) dahilinde, belirlendi. Kısırlaştırılmış ve kısırlaştırılmamış BPH'lı köpekler arasında prostat hacmi yönüyle gerçekleştirilen karşılaştırmada ise istatistiki açıdan önem arz eden bir fark tespit edilmedi (p=0.179).

Tablo 1. Çalışma materyalini oluşturan köpeklerde elde edilen veriler (mean±SE).
Table 1. Data obtained in dogs that make up the study material (mean±SE).

	L (cm)	W (cm)	H (cm)	V (cm^3)	Yaş (yıl)	CA (kg)
Total (n=24)	4.88±0.15	3.98±0.11	3.46±0.13	35.18±2.01	9.88±0.39	13.83±1.31
BPH (n=14)	4.93±0.24	3.74±0.11	3.63±0.11	34.97±2.44	10.29±0.46	14.07±1.77
Non-BPH (n=10)	4.80±0.15	4.34±0.16	3.22±0.25	35.46±3.56	9.30±0.65	13.50±2.02

Total; çalışma materyalini oluşturan tüm köpekler, BPH; bening prostat hiperplazi teşhis edilen köpekler, Non-BPH; prostat neoplazisi, kisti veya prostatit teşhis edilen köpekler, CA; vücut ağırlığı, L; ortalama prostat boyu (length), W; ortalama prostat eni (width), H; ortalama prostat yüksekliği (height), V; ortalama prostat hacmi (volume), SE; standart error mean.

TARTIŞMA VE SONUÇ

BPH köpeklerde yaşla orantılı olarak yaygın hale gelmektedir. Geriatrik dönem köpeklerde BPH oranının %90'ın üzerinde olduğu bildirilmiştir (Christensen, 2018). Sunulan çalışmada BPH tespit edilen köpeklerin yaş ortalamasının 10.29±0.46 yıl olması bu sonuçla uyumludur.

Sunulan araştırmada, materyali oluşturan toplam 24 köpeğin 14'ü (%58.33) ultrasonografik olarak BPH tanısı almıştır. Benzer şekilde, köpeklerde prostatla ilişkili hastalıkların %50'den fazlasının BPH ile ilişkili olduğu bildirilmiştir (Krawiec ve ark, 1992).

Amorim ve ark. (2004), sağlıklı köpeklerin serum ve idrar örneklerinde beşeri kit ile PSA ölçmüş ve ortalama serum PSA düzeyini 0.005 ng/dl olarak bildirmiştir. Bu araştırmada 4-6 yaş köpeklerde PSA düzeyi 0.005 ng/dl ve 7-11 yaş köpeklerde ise 0.006 ng/dl olarak belirlenmiştir. PSA konsantrasyonları prostat dokusundaki morfolojik değişikliklerle ilişkilendirilmektedir (Amorim ve ark, 2004). Sunulan çalışmada da, materyali oluşturan 24 ve yine özel olarak BPH tespit edilen 14 köpeğin tamamında serum PSA düzeyi <0.006 ng/mL olarak ölçüldü. Köpek serumunda veya seminal plazmada PSA'nın saptanamamasının bu sıvılardaki PSA konsantrasyonunun kullanılan testin duyarlılığının altında kalması ya da köpek prostat bezi tarafından PSA üretilmemesi ile ilişkili olabileceği rapor edilmiştir (Bell ve ark, 1995). Bell ve ark. (1995), yapmış oldukları bu çalışmada, BPH tespitinde PSA ölçümünün tek başına yeterli olmadığını bildirmiştir. Bu sonuç sunulan çalışma ile uyumlu olup, ultrasonografik olarak BPH tespit edilen köpeklerde PSA ölçümünün tanıda tek başına yeterli olmadığı kanısına varıldı.

Ruel ve ark. (1998) tarafından, ortalama yaşı 4.25 ve ortalama canlı ağırlığı 15 kg olan, 100 sağlıklı köpek üzerinde yürütülen bir çalışmada; ortalama prostat boyu 3.4±1.1 cm, ortalama prostat eni 3.3±0.7 cm, ortalama prostat yüksekliği 2.8±0.8 cm ve ortalama prostat hacmi ise 18.9±15.5 cm^3 olarak ölçülmüştür. Sunulan çalışmada ise ultrasonografik kontrolü yapılan ve BPH tanısı konan 14 köpekte; ortalama değer olarak, prostat boyu 4.93±0.15 cm, prostat eni 3.74±0.11 cm, prostat yüksekliği 3.63±0.11 cm ve prostat hacmi ise 34.97±2.44 cm^3 olarak hesaplandı. Bu 14 köpeğin ortalama yaşı 10.29±0.46 yıl ve ortalama vücut ağırlığı ise 14.07±1.77 kg'dı. Bu sonuçlar BPH'ye bağlı olarak prostatta görülen büyümeye ve yaşa bağlı olarak BPH görülme sıklığındaki artışa olasılıkla (Korodi ve ark, 2008, Lévy ve ark, 2014) işaret etmektedir. Benzer şekilde Russo ve ark. (2012) ve Nizanski ve ark. (2020)'de yaptıkları araştırmada BPH'li köpeklerde prostattaki büyümeyi ortaya koymuştur. Öte yandan bu araştırmada, BPH harici, prostat neoplazisi, prostatitis ve prostat kisti tanısı alan 10 köpekte ortaya konan ultrasonografik ölçüm sonuçları, BPH'a benzer şekilde, prostat boyutlarında ve hacminde (35.46±3.56 cm^3) bir artış olduğunu ortaya koydu. Bu durum; neoplazilerin, kistlerin ve prostatitisin, boyut olarak, total prostat hacminde artışa yol açmasıyla ilişkili olabilir (Galosi ve ark, 2009, Zhang ve ark, 2012, Werner ve Riese, 2021).

Wilson (2011), yaptığı çalışmada kısırlaştırılmamış köpeklerin prostatla ilişkili olguların büyük çoğunluğunu oluşturduğu bildirilmiş olmakla birlikte, sunulan bu çalışmada BPH tanısı konan 14 köpeğin 8'i kısırlaştırılmış ve 6'sı kısırlaştırılmamıştı. Kısırlaştırılmış köpekler BPH teşhis edilen hastaların yaklaşık %57'sini oluşturdu. Öte yandan kısırlaştırılmış ve kısırlaştırılmamış BPH'lı köpekler ortalama prostat hacmi açısından karşılaştırıldığında, sırasıyla, 35.79±4.00 ve 33.87±2.39 cm^3 olarak belirlenen değerler arasında istatistiki açıdan önem arz eden bir fark belirlenmedi (p=0.179).

Sunulan çalışmada elde edilen veriler; prostat hastalıklarının klinik değerlendirilmesinde ultrasonografinin önemli bir enstruman olduğunu, ultrasonografik olarak elde edilen prostat ölçüm verilerindeki (en, boy, yükseklik ve hacim) pozitif yönlü değişimin BPH'ın tanısında önem arz ettiğini ve köpeklerde PSA ölçümlerinin prostat hastalıklarının tanısında, olasılıkla, tek başına belirleyici olmadığını ortaya koydu. Bu bağlamda daha büyük bir örneklem üzerinde yürütülecek benzer çalışmalarla bu araştırmada elde edilen sonuçların desteklenmesi önerilir. Geriatrik dönem erkek köpeklerde, prostat hiperplazisi klinik muayenede mutlaka göz önünde bulundurulmalı ve bu köpeklerin, ilerleyen yaşla birlikte, ultrasonografiyi de içeren düzenli klinik kontrolleri sağlanmalıdır.

Conflict of Interest: The authors declared that there is no conflict of interest.

Ethics Committee Information: This study was carried out after obtaining permission from TC Kocaeli Valiliği, İl Tarım ve Orman Müdürlüğü, No:2746798/2021; AKÜ HADYEK, 22.01.2021, No:141.

KAYNAKLAR

Amorim RL, Moura VMBD, Di Santis GW, Bandarra EP, Padovani, C. Serum and urinary measurements of prostatic acid phosphatase (PAP) and prostatic specific antigen (PSA) in dogs. Arq Bras Med Vet Zootec. 2004; 56(3): 320–324.

Atalan G., Barr FJ, Holt, PE. Comparison of ultrasonographic and radiographic measurements of canine prostate dimensions. Vet Radiol Ultrasound. 1999; 40: 408–412.

Bell FW., Klausner JS, Hayden, DW, Lund EM, Liebenstein BB, Feeney DA, Johnston SD, Shivers JL, Ewing CM, Isaacs WB. Evaluation of serum and seminal plasma markers in the diagnosis of canine prostatic disorders. J Vet Intern Med. 1995; 9(3): 149–53.

Christensen BW. Canine Prostate Disease. Vet Clin North Am Small Anim Pract. 2018; 48(4): 701–719.

De Souza MB, Da Silva LDM, Moxon R, Russo M, England GC. Ultrasonography of the prostate gland and testes in dogs. In Pract. 2017; 39: 21–32.

Dube JY, Lazure C, Tremblay RR. Dog prostate arginine esterase is related to human prostate specific antigen. Clin Invest Med. 1986; 9(1): 51–54.

Edson Pontes J, Ohe H, Watanabe H, Murphy GP. Transrectal ultrasonography of the prostate. Cancer. 1984; 53: 1369–1372.

Evans HE., Christensen GC. The urogenital system, In: Miller's anatomy of the dog, Ed; Evans HE, 3rd Ed., WB Saunders, USA; pp. 514–516.

Galosi AB, Montironi R, Fabiani A, Lacetera V, Galléand G, Muzzonigro G. Cystic Lesions of the Prostate Gland: An Ultrasound Classification with Pathological Correlation. J Urol. 2009; 181: 647-657.

Gunzel-Apel AR, Mohrke C, Nautrup CP. Colour-coded and pulsed Doppler sonography of the canine testis, epididymis and prostate gland: physiological and pathological findings. Reprod Domest Anim. 2001; 36: 236–240.

Korodi G, Igna V, Cernescu H, Mircu C, Frunza I, Knop R. Canine prostate pathology. Lucrari Stiintifice-Universitatea de Stiinte Agricole a Banatului Timisoara. Med Vet. 2008; 41:187–194.

Krawiec D, Heflin D. Study of prostatic disease in dogs: 177 cases (1981-1986). J Am Vet Med Assoc. 1992; 200:1119–1122.

Kutzler M, Yeager A. Prostatic diseases. In: Textbook of Veterinary Internal Medicine, Ed; Ettinger SJ, Feldman EC, 6th Ed., WB Saunders, USA, pp: 1809–1819.

Lévy X, Niżański W, Von Heimendahl A, Mimouni P. Diagnosis of Common Prostatic Conditions in Dogs: an Update. Reprod Domest Anim. 2014; 49: 50–57.

Niżański W, Ochota M, Fontaine C, Pasikowska J. B-Mode and Doppler Ultrasonographic Findings of Prostate Gland and Testes in Dogs Receiving Deslorelin Acetate or Osaterone Acetate. Animals (Basel). 2020; 11;10(12):2379.

Nyland TG, Mattoon JS. Small Animal Diagnostic Ultrasound, 2nd Ed., Elsevier Health Sciences, USA.

Penninck D, D'anjou MA. Atlas of Small Animal Ultrasonography, 2nd Ed., John Wiley & Sons, USA.

Roehrborn CG, Boyle P, Gould AL. Serum prostatespecific antigen as a predictor of prostate volume in men with benign prostatic hyperplasia. Urology. 1999; 53:581-589.

Ruel Y, Barthez PY, Mailles A, Begon D. Ultrasonographic evaluation of the prostate in healthy intact dogs. Vet Radiol Ultrasound. 1998; 39(3): 212–216.

Russo M, Vignoli M, England GCW. B-Mode and Contrast-Enhanced Ultrasonographic Findings in Canine Prostatic Disorders. Reprod Domest Anim. 2012; 47: 238–242.

Smith J. Canine prostatic disease: A review of anatomy, pathology, diagnosis, and treatment. Theriogenology. 2008; 70(3): 375–383.

Werner JRY, Riese TW. Any Correlation Between Prostate Volume and Incidence of Prostate Cancer: A Review of Reported Data for the Last Thirty Years. Res Rep Urol. 2021; 13:749–757.

Wilson JD. The critical role of androgens in prostate development. Endocrinol Metab Clin North Am. 2011; 40: 577–590.

Zhang HT, Xu Y, Chang JW, Zhang ZH, Liu RL, Ma BJ. Correlation of histological prostatitis with PSA, prostate volume, PSAD, IPSS, Qmax and PVR in BPH patients. Zhonghua Nan Ke Xue. 2012; 18(3): 208-211.

Zohil AM, Castellano MC. Prepubic and transrectal ultrasonography of the canine prostate: a comparative study. Vet Radiol Ultrasound. 1995; 36:393–396.

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022) 15(2):194-199

DOI: 10.30607/kvj. 1068222

RESEARCH ARTICLE

Streptococcus minor; Can There Be A Potential Pathogenic Bacterial Agent In Dog Bites?

Streptococcus minor in dog bites

Hafize Tuğba YÜKSEL DOLGUN[1*], Şükrü KIRKAN[1]

[1] *Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, Aydın, Turkey.*

ABSTRACT

Dogs and humans are in constant interaction which can be in the form of close friendship, or sometimes an attack by dogs on people. Dog bite cases are common in the world and *Streptococcus* species are often isolated from these cases and most frequently isolated species is *Streptococcus canis*. *Streptococcus minor* which was described in 2004 has been isolated in dog bite cases. This research was aimed to reveal the presence of *S. minor* in canine oral flora. In this study, 19 Gram-positive cocci were isolated from 50 dog oral swab samples. Of 19 isolates, 17 isolates were catalase-negative and were typed genotypically by PCR and sequencing. Eight isolates were identified as *S. minor*. *S. minor* isolates were found to be resistant to tetracycline at a rate of 75% and susceptible to other antibiotics at various rates. Trimethoprim resistance gene was detected in one *S. minor* isolate and tetracycline resistance gene was found in one *S. minor* isolate. The results of this research, it has been shown that *S. minor* can be isolated from dogs oral flora and it can appear as a potential bacterial pathogen in dog bite cases.

Keywords: Antibacterial Drug Resistance, Dogs, Molecular Sequencing Data, *Streptococcus.*

***

Streptococcus Minor; Köpek Isırıklarında Potansiyel Patojenik Bakteriyel Etken Olabilir Mi?

Köpek ısırıklarında *Streptococcus minor*

ÖZ

Köpekler ve insanlar, yakın arkadaşlık veya bazen köpeklerin insanlara saldırması şeklinde olabilen sürekli bir etkileşim halindedir. Köpek ısırık vakaları dünyada sık görülmektedir. Bu vakalardan sıklıkla *Streptococcus* türleri izole edilir ve en sık izole edilen tür *Streptococcus canis*'tir. 2004 yılında tanımlanan *Streptococcus minor* köpek ısırması vakalarında izole edilmiştir. Bu araştırmada köpek ağız florasında *S. minor* varlığının ortaya konulması amaçlanmıştır. Bu çalışmada, 50 köpek oral svap örneğinden 19 Gram pozitif kok izole edilmiştir. Ondokuz izolattan 17'si katalaz negatif olduğu belirlenmiş ve PCR ve dizileme ile genotipik olarak tiplendirilmiştir. Sekiz izolat *S. minor* olarak tanımlandı. *S. minor* izolatlarının tetrasikline %75 oranında dirençli ve diğer antibiyotiklere çeşitli oranlarda duyarlı olduğu bulunmuştur. Bir *S. minor* izolatında trimetoprim direnç geni, bir *S. minor* izolatında ise tetrasiklin direnç geni saptanmıştır. Bu araştırma sonucunda *S. minor*'un köpeklerin ağız florasından izole edilebileceği ve köpek ısırık vakalarında potansiyel bir bakteriyel patojen olarak ortaya çıkabileceği gösterilmiştir.

Anahtar Kelimeler: Antibakteriyel İlaç Direnci, Köpekler, Moleküler Dizi Verileri, *Streptococcus.*

To cite this article: Yüksel Dolgun H.T. Kırkan Ş. Streptococcus minor; Can There Be A Potential Pathogenic Bacterial Agent In Dog Bites. Streptococcus minor in dog bites. Kocatepe Vet J. (2022) 15(2):194-199

Submission: 04.02.2022 **Accepted**: 19.04.2022 **Published Online**: 17.05.2022

ORCID ID; *HTYD: 0000-0002-1125-5792, ŞK: 0000-0001-5111-8656*

*Corresponding author e-mail: tugba.yuksel@adu.edu.tr

INTRODUCTION

Living with animals enrich the lives of humans and including pets in daily routines ensure social interaction, exercise, emotional supports and social connectedness. Dogs are the closest friends of people, they live in the same home environment with people and they feed on foods of animal origin (Bata et al. 2020). Researches have focused on the subject of microbiota and especially focused on the microbiota of the gastrointestinal tract. Studies have shown that the gastrointestinal microbiota is closely related to the oral cavity microbiota (Zarco et al. 2012). The normal oral flora of dogs contains a large number of microorganisms which includes *Porphyromonas, Fusobacterium, Streptococcus, Capnocytophaga* genera and members of the *Pasteurellaceae* and *Neisseriaceae* families (Sturgeon et al. 2013, Oh et al. 2015, Isaiah et al. 2017, Bell et al. 2020, Ruparell et al. 2020). Some of these microorganisms can form a basic health barrier together with the immune system (Marsh 1994), but some of them may be pathogenic, cause periodontitis, dental caries and systemic disease. Dogs' age, food consumption, health status, and environmental factors influence oral microbiome composition. When dogs' health deteriorates, pathogenic oral bacteria can cause systemic infections (Fowler et al. 2001). However, pathogenic bacteria can show zoonotic properties as a result of the contact of dogs with impaired health and sometimes even biting people (Chen et al. 2010). Dog bite cases seen in humans are one of the important health problems in the world. It starts with common wound infections associated with dog bite and can develop into local and systemic infections if left untreated (Tabaka et al. 2015, Goldstein et al. 2018). It is known that 3-18% wounds of dog bites are infected with the dog's oral flora (Tabaka et al. 2015, Damborg et al. 2016) and wound infections are generally an infection involving anaerobic and aerobic bacteria. *Streptococcal* species are commonly involved in canine bite wounds and infections. *Streptococcus canis* and S*treptococcus pyogenes* are the most common pathogens in dog bite cases. However, *Streptococcus minor* species, which was identified by molecular methods in 2004, has also started to be reported in dog bite cases. Infections caused by *S. minor* can be overlooked due to the facultative anaerobic nature of the organism and the difficulty of identifying α-hemolytic streptococci at the species level with current laboratory techniques and *S. minor* does not react with Lancefield groups A, C, D, F or G antisera. *S. minor* has the potential to be the primary pathogen in dog bites (Vancanneyt et al. 2004, Tre-Hardy et al. 2016).

In this study, it was aimed to reveal the presence of *S. minor* species, which has recently gained importance in dog bite cases, in canine oral flora and its antibiotic susceptibility.

MATERIAL AND METHODS

Sample Collection

Samples were taken with cotton swabs from oral cavities of randomly selected 50 dogs. The oral swab samples were transported at +4°C to the microbiology laboratory.

Phenotypic Identification

Each swab sample was plated on 5-7% Columbia Blood Agar. Plates were incubated for 24-48 h at 37°C microaerophilic condition. After the incubation, plates were examined and small, smooth, translucent and alpha-hemolytic colonies were subcultured to the Triyptic Soy Agar to obtain of pure cultures. When pure colonies were obtained, each colony was isolated according to the Gram staining microscopy, and catalase tests. Gram-positive cocci and catalase-negative isolates were determined and were recorded as suspected *Streptococcus sp* (Razali et al. 2020).

Genotypic Identification

DNA Extraction

DNA extraction were performed from isolates as *Streptococcus sp.* recommended by the manufacturer using the Genomic DNA Purification Extraction Kit (Thermo Fisher Scientific™) for use in PCR. The DNA samples were stored in cryotubes at -20°C up to the PCR.

16S rRNA PCR for *Streptococcus sp.*

For molecular identification of the 16S rRNA genes were amplified using universal primers (27F and 1492R) by SimpliAmp Thermal Cycler Applied Biosystems (Thermo Fisher Scientific™). PCR amplicons were electrophoresed on 2% agarose gel and were visualized on UV transilluminator (Vilber Lourmat). 16S rRNA gene specific bands at 1450 bp were considered positive (Lane 1991).

Purification and Sequencing of PCR Product

PCR amplicons were purified with enzymatic purification kit for sequencing. Purified PCR products concentrations were prepared ~50ng for sequencing PCR. PCR products were sequenced with 1492R PCR primers (3.2 pmol) using the Big Dye Terminator Ready Reaction Mixv 3.1. Nucleotide sequences were run on an ABI Prism 310 Genetic Analyser (Applied Biosystems). The nucleotide sequences of PCR products was analysed using Standard Nucleotide BLAST® NCBI Genomic Reference Sequences. The results obtained were compared electronically with the NCBI Blast® nucleotide sequences and the percent similarity rates were determined (Turner et al. 1999).

Determination of Antimicrobial Susceptibility

For the determination of antibiotic susceptibility pattern of the *Streptococcus minor* isolates were used the Kirby-Bauer disc diffusion method (CLSI 2016). Antibiotic discs were used comprising ampicilline (10µg), streptomycin (300µg), vancomycin (30µg), eritromycin (15µg), florfenicol (30µg), cefotaxime (30µg), cefepime (30µg), trimethoprim (20µg), methicillin (5µg), tetracycline (30µg), sulfamethoxazole-trimethoprim (25µg), amoxicillin-clavulanic acid (30µg), penisilin (10 IU) (Oxoid, Hampshire, England).

Determination of Antibiotic Resistance Genes

For detection of antibiotic resistance genes, PCR protocols were examined by list of references in Table 1. PCR master mix were prepared a total volume of 25 µl; including of 5µl 10X PCR Buffer, 2.5 mM MgCl2, 200 µM dNTP's, 0.5 µM of each primer (F & R), 2U Taq DNA polymerase, 3µl template DNA. The amplification conditions were as follow; an initial denaturation step at 94°C for 8 min; by 32 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 80 s and elongation at 72°C for 2 min; 1 cycle of final elongation at 72°C for 10 min. (Randall et all. 2002, Toro et al. 2005, Mammeri et al. 2005, Van et al. 2008). PCR products were electrophoresed on 2% agarose gel and were performed on Vilber Lourmat UV transilluminator. The PCR product bands were evaluated on target gene product size (Table 1).

Table 1. Antibiotic resistance gene primer sequences

Primers	Sequences (5'-3')	Size of Product (bp)	Target gene	References
aadA1-F	TATCCAGCTAAGCGCGAACT	447	Streptomycin resistance	Randall et al. 2004
aadA1-R	ATTTGCCGACTACCTTGGTC			
tetA-F	GGTTCACTCGAACGACGTCA	577	Tetracycline resistance	Randall et al. 2004
tetA-R	CTGTCCGACAAGTTGCATGA			
tetB-F	CCTCAGCTTCTCAACGCGTG	634	Tetracycline resistance	Randall et al. 2004
tetB-R	GCACCTTGCTGATGACTCTT			
dfrA1-F	GGAGTGCCAAAGGTGAACAGC	367	Trimethoprim resistance	Toro et al. 2005
dfrA1-R	GAGGCGAAGTCTTGGGTAAAAAC			
Qnr-F	GGGTATGGATATTATTGATAAAG	670	Floroquinolone resistance	Mammeri et al. 2005
Qnr-R	CTAATCCGGCAGCACTATTTA			
aac[3]-IV-F	CTTCAGGATGGCAAGTTGGT	286	Gentamicin resistance	Van et al. 2008
aac[3]-IV-R	TCATCTCGTTCTCCGCTCAT			
Sul1-F	TTCGGCATTCTGAATCTCAC	822	Sulfonamide resistance	Van et al. 2008
Sul1-R	ATGATCTAACCCTCGGTCTC			
blaSHV-F	TCGCCTGTGTATTATCTCCC	768	Cephalothin resistance	Van et al. 2008
blaSHV-R	CGCAGATAAATCACCACAATG			
CITM-F	TGGCCAGAACTGACAGGCAAA	462	Ampicillin resistance	Van et al. 2008
CITM-R	TTTCTCCTGAACGTGGCTGGC			
ereA-F	GCCGGTGCTCATGAACTTGAG	419	Erytromycin resistance	Van et al. 2008
ereA-R	CGACTCTATTCGATCAGAGGC			

RESULTS

Phenotypic and Genotypic Identification

In this study, 19 (38%) Gram-positive, cocci were isolated from 50 oral swab samples of dogs. The catalase test was performed on 19 Gram-positive isolates; 2 (10.5%) isolated found to be catalase-positive and 17 (89.5%) isolates found to be catalase-negative. Gram-positive, catalase-negative 17 (89.5%) isolates were evaluated *Streptococcus sp.* 17 (89.5%) *Streptococcus sp.* suspected isolates were passaged on Tryptic soy agar plates and DNA extractions were performed. PCR analysis was performed on obtained DNA using universal primers. All *Streptococcus* PCR products (n=17) were visualised at 1450 bp bands in gel image analysis.

The 17 PCR products showing the band on 1450 bp were subjected to Sanger sequencing. As a result of Sanger sequence analysis, 8 (47%) of 17 isolates were identified as *Streptococcus minor* and other 9 (53%) *Streptococcus* isolates could not be typed by the Sanger sequencing method. Of the 5 (66%) *Streptococcus* isolates were 97% similarity to *Streptococcus minor* strain

B-5-2 AP strain (Accesion Number MT510388.1) and the other 3 (44%) *Streptococcus* isolates were 97% similarity to *Streptococcus minor* strain B-3-MS-7- AP strain (Accession Number MT492055.1).

It was found that the antibiogram results of *Streptococcus minor* isolates were 100% susceptible to ampiciline, vancomycin, cefotaxime, cefepime, sulfamethoxazole-trimethoprim, amoxicillin-clavulanic acid; 87.5% susceptible to streptomycin, florfenicol, trimethoprim, methicillin; 75% sensitive to erythromycin and penicillin; 75% resistant to tetracycline (Table 2).

Table 2. *S. minor* isolates antimicrobial susceptibility profile

S. minor **Isolates**	**AMP (10µg)**	**S (300µg)**	**V (30µg)**	**E (15µg)**	**FFC (30µg)**	**CTX (30µg)**	**CFP (30µg)**	**TMP (20µg)**	**M (5µg)**	**T (30µg)**	**SXT (25µg)**	**AMC (30µg)**	**P (10 IU)**
1	S	S	S	R	S	I	S	S	S	R	S	S	S
2	S	S	S	S	S	S	S	S	S	R	S	S	S
3	S	S	S	R	R	S	S	S	S	R	S	S	S
4	S	R	S	I	S	S	S	S	S	R	S	S	S
5	S	S	S	S	S	S	S	S	S	R	S	S	R
6	S	S	S	S	S	S	S	R	S	R	S	S	S
7	S	S	S	S	S	S	S	S	S	I	S	S	S
8	S	S	S	S	S	I	S	S	R	S	S	S	R
	100% S	87.5% S	100% S	75% S	87.5% S	100% S	100% S	87.5% S	87.5% S	75% R	100% S	100% S	75% S

AMP: Ampicilline, S: Streptomycin, V: Vancomycin, E: Eritromycin, FFC: Florfenicol, CTX: Cefotaxime, CFP: Cefepime, TMP: Trimethoprim, M: Methicillin, T: Tetracycline, SXT: Sulfamethoxazole-trimethoprim, AMC: Amoxicillin-clavulanic acid, P: Penisilin

In the antibiotic resistance gene analyzes, tetracycline resistance gene was found in the one S. minor isolate and the trimethoprim resistance gene was found in the one S. minor isolate. The antibiotic resistance genes were not detected on the other S. minor (n=6) isolates.

DISCUSSION

Dogs come into contact with the environment, there are also dog-to-dog differences in their oral microbiome. Generally, P*roteobacteria, Firmicutes, Bacteroidetes* and *Fusobacteria* are commonly reported in the oral bacterial composition. In addition, *Porphyromonas, Fusobacterium, Streptococcus, Capnocytophagae* and *Pasteurella* species are also found in canine oral microbiomes at varying rates (Sturgeon et al. 2013, Bell et al. 2020, Ruparell et al. 2020).

Oral microbiota is related to the oral health of dogs, but there is an increase in the number of pathogen Gram-positive and Gram-negative bacteria with periodontal diseases. Bacteria found in the oral cavity of dogs appear as potentially dangerous agents in dog bites in humans. *E.coli, Streptococcus, Staphylococcus* and *Klebsiella, Pasteurella* species are among these pathogens. The most common species isolated from dog bites is *Pasteurella* species (50%), while *Streptococcus* species (46%) is the second causative agent (Abrahamian and Golstein 2011). *Streptococcus* species can cause septicemic infections, especially by passing through bite wounds into the circulation. *Streptococcus* species play an important role in infections such as endocarditis, septic arthritis, pharyngitis and cellulitis. *Streptococcus canis* is one of the most important species isolated from bite wounds (Stefanopoulos and Tarantzopoulou 2005). Ohtaki et al. (2013) identified *Streptococcus canis* from the femur fracture site of a 91-year-old woman. Researchers reported that the dog lived in the same house with its owner. It is noteworthy that *Streptococcus canis* was isolated from the wound site, although there were no bite cases. There are literatures about *Streptococcus canis*, which causes bacteremia and ulcers on the skin, such as this case (Bert and Lambert 1997, Takeda et al. 2001, Lam et al. 2007). Takeda et al. (2001) reported that they isolated *Streptococcus canis* from septicemia that occurred 2 weeks after the dog bite in a 75-year-old woman.

In recent years, with the development of molecular diagnostic methods, identification of new *Streptococcus* species has begun. Vancanneyt et al. (2004) were identified *Streptococcus minor* for the first time in canine tonsils. *Streptococcus minor* is also included in the oral *Streptococcus* species. Then, Tre-Hardy et al. (2016) identified *Streptococcus minor* from the bite wound of a 51-year-old woman. Thus, *Streptococcus minor* was isolated for the first time as a wound infection agent originating from dog bite.

CONCLUSION

In this study, it was concluded that with the development of molecular diagnostic techniques, *Streptococcus minor* will play an important role in dog bite cases like other *Streptococcus* species. For this reason, it was important to investigate whether *Streptococcus minor* species exist in canine oral flora. For this purpose, 8 (16%) *Streptococcus minor* identifications out of 50 oral swab samples were made using sequence-based diagnostic methods. In the antibiogram analysis, it was determined that most of the isolates were sensitive to antibiotics, but resistance to tetracycline was 75%. Tetracycline and trimethoprim resistance genes were found to be in only two of these isolates.

As a result, *Streptococcus minor* species have an important potential to become a zoonotic pathogen in dog bite cases in the coming years. In the diagnosis of *Streptococcus minor* infections, it should be investigated whether there is dog contact or not. Septicemic and ulcerative infections can develop within about 2 weeks after dog bites. In these cases, it is recommended that the identification of *Streptococcus* species in isolation from wound infections should be made by molecular methods and that *Streptococcus minor* species, which may be the primary pathogen should be taken into consideration.

Financial Support: There is no financial support for this study.

Authors Contribution Rate: The authors declared that they contributed equally to the article.

Ethics Committee Information: This study is not subject to HADYEK's permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Conflict of Interest: The authors declared that there are no actual, potential or perceived conflicts of interest for this article.

REFERENCES

Abrahamian FM, Goldstein EJ. Microbiology of animal bite wound infections. Clinical microbiology reviews. 2011; 24 (2), 231-246.

Bata SI, Andua OA, Maimadu A, Sabo JA, Mayowa O, Waziri IA. Oral cavities multidrug resistant bacteria colonization in apparently healthy dogs in jos, Plateau State, Nigeria. Science World Journal. 2020; 15 (1), 15-20.

Bell SE, Nash AK, Zanghi BM, Otto CM, Perry EB. An assessment of the stability of the canine oral microbiota after probiotic administration in healthy dogs over time. Front. Vet. Sci. 2020; 7, 616.

Bert F, Lambert-Zechovsky N. Septicemia caused by Streptococcus canis in a human. J Clin Microbiol. 1997; 35, 777–779.

Chen T, Yu WH, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database (Oxford). 2010; Article ID: baq013. doi: 10.1093/database/baq013

Damborg P, Broens EM, Chomel BB. Bacterial zoonoses transmitted by household pets: State-of-the-art and future perspectives for targeted research and policy actions. J. Comp. Pathol. 2016; 155 (1), 27-40.

Fowler EB, Breault LG, Cuenin MF. Periodontal disease and its association with systemic disease. Mil Med. 2001; 166 (1), 85–89.

Goldstein EJC, Citron DM, Tyrrell KL, Leoncio E, Merriam CV. Comparative in vitro activity of omadacycline against dog and cat bite wound isolates. Antimicrob. Agents Chemother. 2018; 62 (4), e02551-17.

Isaiah A, Hoffmann AR, Kelley R, Mundell P, Steiner JM, Suchodolski JS. Characterization of the nasal and oral microbiota of detection dogs. PLoS One. 2017; 12 (9), e0184899.

Lam MM, Clarridge JE, Young EJ, Mizuki S. The other group G Streptococcus: increased detection of Streptococcus canis ulcer infections in dog owners. J Clin Microbiol. 2007; 45 (7), 2327–2329.

Lane DJ. 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. Stackebrandt, E., and Goodfellow, M., eds., John Wiley and Sons, New York, NY, 1991; 115-175.

Mammeri H, Van De Loo M, Poirel L, Martinez-Martinez L, Nordmann P. Emergence of plasmid-mediated quinolone resistance in Escherichia coli in Europe. Antimicrob Agents Chemother. 2005; 49 (1), 71–76.

Marsh PD. Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res. 1994; 8 (2), 263–271.

Oh C, Lee K, Cheong Y, Lee SW, Park SY, Song CS, Choi IS, Lee JB. Comparison of the oral microbiomes of canines and their owners using next-generation sequencing. PLoS One. 2015; 10 (7), e0131468.

Ohtaki H, Ohkusu K, Ohta H, Miyazaki T, Yonetamari J, Usui T, Mori I, Ito H, Ishizuka T, Seishima M. A case of sepsis caused by Streptococcus canis in a dog owner: a first case report of sepsis without dog bite in Japan. J Infect Chemother. 2013; 19 (6), 1206-1209.

Randall LP, Cooles SW, Osborn MK, Piddock LJ, Woodward MJ. Antibiotic resistance genes integrons and multiple antibiotic resistance in thirtyfive serotypes of Salmonella enterica isolated from humans and animals in the UK. J Antimicrob Chemother. 2004; 53 (2), 208–216.

Razali K, Kaidi R, Abdelli A, Menoueri MN, Ait-Oudhia K. Oral flora of stray dogs and cats in Algeria: Pasteurella and other zoonotic bacteria. Veterinary World. 2020; 13 (12), 2806-2814.

Ruparell A, Inui T, Staunton R, Wallis C, Deusch O, Holcombe LJ. The canine oral microbiome: Variation in bacterial populations across different niches. BMC Microbiol. 2020; 20 (1), 1-13.

Stefanopoulos PK, Tarantzopoulou AD. Facial bite wounds: management update. Int. J. Oral Maxillofac. Surg. 2005; 34 (5), 464–472.

Sturgeon A, Stull JW, Costa MC, Weese JS. Metagenomic analysis of the canine oral cavity as revealed by high-throughput pyrosequencing of the 16S rRNA gene. Vet. Microbiol. 2013; 162 (2-4), 891–898.

Tabaka ME, Quinn JV, Kohn MA, Polevoi SK. Predictors of infection from dog bite wounds: Which patients may benefit from prophylactic antibiotics? Emerg. Med. J. 2015; 32 (11), 860-863.

Takeda N, Kikuchi K, Asano R, Harada T, Totsuka K, Uchiyama T, Hosoda S. Recurrent septicemia caused by Streptococcus canis after a dog bite. Scand J Infect Dis. 2001; 33 (12), 927–928.

Toro CS, Farfán M, Contreras I, Flores O, Navarro N, Mora GC, Prado V. Genetic analysis of antibiotic-resistance determinants in multidrugresistant Shigella strains isolated from Chilean children. Epidemiol Infect. 2005; 133 (1), 81–86.

Tré-Hardy M, Saussez T, Yombi JC, Rodriguez-Villalobos H. First case of a dog bite wound infection caused by Streptococcus minor in human. NMNI. 2016; 14, 49-50.

Turner S, Pryer KM, Mıao VPW, Palmer JD. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J. Eukaryot. Microbiol. 1999 46 (4), 327-338.

Van TT, Chin J, Chapman T, Tran LT, Coloe PJ. Safety of raw meat and shellfish in Vietnam: an analysis of Escherichia coli isolations for antibiotic resistance and virulence genes. Int J Food Microbiol. 2008; 124 (3), 217–223.

Vancanneyt M, Devriese LA, De Graef EM. Streptococcus minor sp. nov., from faecal samples and tonsils of domestic animals. IJSEM. 2004; 54 (2), 449–452.

Wayne, PA. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement M100-S26., Clinical and Laboratory Standards Institute. 2016.

Zarco MF, Vess TJ, Ginsburg GS. The oral microbiome in health and disease and the potential impact on personalized dental medicine. Oral Dis. 2012; 18 (2), 109-120.

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022) 15(2): 200-208

DOI: 10.30607/kvj. 1087546

RESEARCH ARTICLE

The Effect of Nisin and Thyme Oil on *Listeria monocytogenes* and *Staphylococcus aureus* Inoculated into Cream During Storage

Hatice ÇAYIR ÜSTÜNDAĞ[1], Halil YALÇIN[2*]

[1]Burdur Mehmet Akif Ersoy University, Institute of Health Sciences, 15030, Burdur, Turkey
[2]Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, 15030, Burdur, Turkey*

ABSTRACT

The main objective of this study was to investigate the effect of nisin and thyme oil on *Listeria monocytogenes* and *Staphylococcus aureus* inoculated in cream. Milk skim was used in the study, a total of 12 groups including *L. monocytogenes* and *S. aureus* were inoculated and the study was divided into two work packages. In the first work package, *L. monocytogenes* were inoculated into each group. Subsequently, 5 mg/kg and 10 mg/kg of nisin and 0.25% and 0.5% thyme oil were added to groups other than bacterial control group. The same procedure was applied in the second work package, which is *S. aureus*. Microbiological follow-up of the prepared samples on the 0, 3, 5 and 7th storage days was performed. In addition, pH, ORP (Oxidation Reduction Potential) and a_w analyzes were performed. Three replications of the experimental groups formed in both work packages were carried out and two parallel samples were analyzed from each sample. In the study, it was determined that using 0.5% thyme oil showed a high antimicrobial effect in samples containing *L. monocytogenes* and *S. aureus* ($p<0.05$). Although thyme oil inhibits bacterial growth, it is observed that the sharp aroma of thyme oil suppresses the unique taste and smell of cream.

Keywords: Cream, *L. monocytogenes*, Nisin, *S. aureus,* Thyme oil

***

Nisin ve Kekik Yağının Kaymağa İnoküle Edilen *Listeria monocytogenes* ve *Staphylococcus aureus* Üzerine Depolama Süresince Etkisi

ÖZ

Bu araştırmanın temel amacı, nisin ve kekik yağının kaymağa inoküle edilen *Listeria monocytogenes* ve *Staphylococcus aureus* üzerine etkisini incelemektir. Araştırmada süt kaymağı kullanılmış, kontrol örneği de dâhil olmak üzere *L. monocytogenes* ve *S. aureus* inoküle edilen toplam 12 grup oluşturulmuş ve çalışma iki iş paketine ayrılmıştır. Birinci iş paketinde her bir gruba *L. monocytogenes* inokule edilmiştir. Daha sonra bakteri kontrol grubu haricindeki gruplara 5 mg/kg ve 10 mg/kg nisin ile %0,25 ve %0,5 kekik yağı eklenmiştir. *S. aureus* olan ikinci iş paketinde de aynı işlemler uygulanmıştır. Hazırlanan örneklerin 0., 3., 5. ve 7. depolama günlerindeki mikrobiyolojik takibi yapılmıştır. Ayrıca pH, ORP (Oksidasyon Redüksiyon Potansiyeli) ve a_w analizleri yapılmıştır. Her iki iş paketinde de oluşturulan deneme gruplarının 3 tekerrürlü üretimi gerçekleştirilmiş ve her bir örnekten 2 paralelli olacak şekilde analiz gerçekleştirilmiştir. Araştırmada %0,5 kekik yağı kullanımının *L. monocytogenes* ve *S. aureus* içeren örneklerde daha fazla antimikrobiyal etki gösterdiği belirlenmiştir ($p<0,05$). Kekik yağının bakteri gelişimini inhibe ettiği belirlenmekle birlikte, kekik yağının keskin aromasının kaymağın kendine has tat ve kokusunu baskıladığı gözlenmiştir.

Anahtar Kelimeler: Kaymak, Kekik yağı, *L. monocytogenes*, Nisin, *S. aureus*

To cite this article: Çayır Üstündağ H. Yalçın H. The Effect of Nisin and Thyme Oil on Listeria monocytogenes and Staphylococcus aureus Inoculated into Cream During Storage. Kocatepe Vet J. (2022) 15(2):200-208

Submission: 14.04.2022 **Accepted**: 19.05.2022 **Published Online**: 30.05.2022
ORCID ID; *HÇÜ: 0000-0003-1567-3729, HY: 0000-0003-2162-2418*
*Corresponding author e-mail: hyalcin@mehmetakif.edu.tr

GİRİŞ

Süt ve ürünleri, insanların sağlıklı, dengeli ve yeterli beslenebilmeleri için gerekli olan yağ, protein, mineral ve vitaminleri barındırmaktadır (Baysal 2004). Kaymak, süt yağından elde edilen yaklaşık %60 oranında yağ içeren bir süt ürünüdür. Afyonkarahisar başta olmak üzere Erzurum, Kilis, Edirne, Kocaeli, İstanbul, Ankara ve Bursa illerinde de geleneksel yöntemler ile kaymak üretimi yapılmaktadır (Hasdoğan 2004). Türkiye'de özellikle manda ve inek sütlerinden kaymak üretimi yaygındır. Ancak yağ ve kuru madde içeriği ile kaymak bağlama özelliği yüksek olduğu için en iyi kaymak manda sütünden elde edilir (İpekçioğlu ve Gürler 2017). Avrupa'nın güneydoğu bölgeleri (Sırbistan, Bosna-Hersek, Karadağ) ile İran, Afganistan ve Hindistan'da da kaymak benzeri ürünler üretilmektedir (Pudja ve ark. 2008).

Gıda kaynaklı hastalıklar gün geçtikçe artmakta ve önemli sağlık sorunları haline gelmektedir. Patojen mikroorganizmaların neden olduğu hastalıklar ise en tehlikeli grupta yer almaktadır (Anyogu ve ark. 2021). Kaymak içerdiği besin öğeleri, yüksek su oranı ve fermente ürün olmamasından dolayı patojen mikroorganizmaların üremeleri için elverişli bir ortamdır (İpekçioğlu ve Gürler 2017).

L. monocytogenes doğada yaygın olarak bulunan, birçok çevresel etmene karşı direnç gösteren, buzdolabı sıcaklığında üreyebilen gıda kaynaklı patojen bir bakteridir (Zamuz ve ark. 2021). Bu özellikleri ile L. monocytogenes et, süt ve süt ürünleri, deniz ürünleri ve sebzeler gibi çeşitli gıdalarda doğal olarak bulunabilmekte ve gelişme gösterebilmektedir (EFSA 2019). Türk Gıda Kodeksi Mikrobiyolojik Kriterler Yönetmeliği'ne göre; L. monocytogenes için tolerans yoktur, limit değeri "Bulunmamalı/25g" şeklindedir (Anonim 2011).

Stafilokoklar insanlarda ve hayvanlarda çeşitli enfeksiyonlara ve intoksikasyonlara neden olan bakterilerdir. Koagulaz negatif ve koagulaz pozitif stafilokoklar enfeksiyon oluşturabilmektedirler ancak gıda intoksikasyonlarının en önemli etmeni S. aureus'tur (Cheung ve ark. 2021). S. aureus geniş pH ve sıcaklık aralığında gelişme gösterebilmekte ve birçok gıda maddesinde canlılığını koruyabilmektedir (Abdeen ve ark. 2020). Süt ve süt ürünlerindeki en önemli kontaminasyon kaynağı mastitisli ineklerin sütleri ile sağlıklı ineklerden elde edilen temiz sütlerin birbirine karışması ve işletme hijyenine dikkat edilmemesidir (Küplülü ve ark. 2002). Gıda işletmelerindeki personelden hapşırma, öksürme gibi refleksler ile kontaminasyon meydana gelebilmektedir. Bununla birlikte enfekte olmuş deriden de gıdalara geçebilmektedir (Bergdoll ve Wong 2006).

Bakteriyosinlerin gıdaları korumak amacıyla kullanılmalarının en önemli nedenlerden biri GRAS (Generally Recognized As Safe) maddeler listesinde yer almasıdır. Diğer sebepler ise sıcaklık ve pH'ya karşı dirençli olmaları, patojen mikroorganizmalara karşı antimikrobiyal etki göstermeleridir (İbrahim ve ark. 2021). Nisin, lantibiyotikler grubunda bulunan, laktik asit bakterilerinden biri olan Lactococcus lactis'in ürettiği 1. sınıf bakteriyosinler arasında yer alan bir bileşiktir (Cheigh ve Pyun 2005). Ülkemizde peynir, kaymak, irmik, puding, pastörize sıvı yumurta gibi ürünlerde 3-12,5 mg/kg aralığında değişen dozlarda kullanımına izin verilmektedir (Anonim 2013). Diğer bakteriyosinlere oranla nisinin antibakteriyel etki alanı daha fazladır. Gram pozitif bakterilerin neredeyse tamamı üzerinde etkilidir (Arauz ve ark. 2009). Şelat oluşturucu ajanlarla birlikte kullanıldığında gram negatif bakteriler üzerinde de etkin olduğu belirtilmiştir (Boziaris ve Adams 1999). Nisin gıda endüstrisinde bakteriyel kontrol için değişik dozlarda (100-2000 IU g−1) yaygın olarak kullanılmakta olup (Khelissa ve ark. 2021) L. monocytogenes'e karşı yüksek etkili olduğu belirtilmiştir (Zhang ve ark. 2021). Nisin hedef hücre zarında porlar oluşturarak hücre için gerekli mikro moleküllerin sızmasına sebep olur. Hedef membran ile ilk temastan nisinin C-terminal bölgesi sorumludur. Nisin C-terminal bölgesi ile hedefteki fosfatidilgliserole bağlanır, müteakiben gözenek oluşumu ve ani hücre ölümü gerçekleşir (Liu ve ark. 2022).

Kekik, Labiateae ailesine ait odunsu ve aromatik bir bitkidir. Kekik yağı ise 60'dan fazla maddeyi yapısında bulundurmaktadır. Kekik yağının en önemli bileşikleri timol (%44-60), karvakrol (%2,2-4,2), p-simen (%18,5-23,5), monoterpen hidrokarbonlar ve c-terpinen (%16,1-18,9)'dir (Baranauskiene ve ark. 2003). Bunlardan karvakrol ve timolün antimikrobiyal etkinliğinin yüksek olduğu bildirilmiştir (Benli ve Yiğit 2005). Kekik yağında bulunan fenolik bileşiklerin mikroorganizmaların hücre zarındaki fosfolipit tabakasını uyarıp, hücre içinde bulunan yaşamsal öneme sahip yapıların geçirgenliğini artırarak veya mikroorganizmaların enzim sistemlerini bozarak etki ettiği belirtilmektedir (Helander ve ark. 1998; Lambert ve ark. 2001).

Bu çalışmada, kaymağa inoküle edilen Listeria monocytogenes ve Staphylococcus aureus üzerine nisin ve kekik yağının depolama süresince etkisi araştırılmıştır.

MATERYAL ve METOT

Materyal

Araştırma kapsamında yerel bir süt işletmesinin, inek sütünden ürettiği kaymaklar kullanılmıştır. Süt kaymağı orijinal ambalajında ve soğuk zincir kırılmadan laboratuvara getirilmiş, zaman geçmeden işleme alınmıştır. Çalışmada kullanılan kaymakların araştırmaya konu olan patojenleri içerip içermediği kaymak kontrol grubu oluşturularak incelenmiştir. Araştırmada Listeria monocytogenes RSKK 02028 ve Staphylococcus aureus ATCC 25923 suşları kullanılmıştır.

Çalışma Gruplarının Hazırlanması

Her bakteri için 2 adet kaymak kontrol, 2 adet bakteri kontrol, 5 mg/kg ve10 mg/kg nisin (Merck, Almanya) ile %0,25 ve %0,5 kekik yağı (Origanum onites, Manolya Doğal Aromatik Ürünler Gıda San. ve Tic. Ltd. Şti. İzmir) eklenerek toplam 12 grup oluşturulmuştur. Bakteri kontrol gruplarında sadece ilgili bakteri kullanılmıştır. Üç tekerrürlü ve iki paralelli olarak yürütülen çalışmanın 0., 3., 5. ve 7. depolama günlerinde mikrobiyolojik analizler yapılmıştır. Ayrıca pH değeri (WTW pH 3110 Set 2), Oksidasyon Redüksiyon Potansiyeli-ORP (WTW Sentix ORP Elektrodu) ve su aktivitesi değeri-aw (Testo F 650) ölçülmüştür.

Kontaminasyon Sıvısının Hazırlanması

Ayrı çalışma günlerinde olmak üzere -80 °C'de bulunan suşlardan 40 µl alınarak CASO Broth (Merck, Almanya) besiyerine aktarılmıştır. Daha sonra *L. monocytogenes* 30 °C'de, *S. aureus* 37 °C'de 24 saat inkübe edilmiştir. İnkübasyon sonrası suşlar 5000 rpm'de 5 dakika santrifüj (Eppendorf Centrifuge 5810 R) edilmiştir. Süpernatant uzaklaştırılarak peletlerin üzerine %0,9'luk steril fizyolojik tuzlu sudan 10 ml eklenerek vorteks aracılığı ile pelet dağıtılmıştır ve santrifüj işlemi tekrarlanmıştır. Bu işlem 3 defa yapıldıktan sonra suşların bulunduğu peletler %0,1'lik peptonlu su içinde süspanse edilerek birleştirilmiştir (Yalçın ve Arslan 2011).

Örneklere Bakteri ve Antimikrobiyal Madde Eklenmesi

Araştırma kapsamındaki kaymak kontrol grubu haricindeki her bir çalışma grubu tek bir bakteri ile kontamine edilmiştir. Kontaminasyon düzeyi 0,5 McFarland ile ayarlama ve sonrasında seri dilüsyonlar yapılarak 5 log KOB/ml olacak şekilde ayarlanmış ve bakteri adaptasyonu için 30 dakika beklenmiştir. Nisin gruplarını oluşturmak için 500 ppm nisin çözeltisinden C ve M gruplarına 1 gram, D ve N gruplarına 2 gram eklenmiştir. Kekik yağı gruplarını oluşturmak için ise E ve P gruplarına 250 µl, F ve R gruplarına ise 500 µl kekik yağı eklenmiştir. Tüm ekleme aşamalarından sonra örnekler steril cam baget ile manuel olarak 2 dakika sürecince aseptik şartlarda aynı kişi tarafından karıştırılarak homojenizasyon sağlanmıştır. Bu aşamayı takiben 30 dakika daha beklenip 0. gün analizleri yapılmıştır. Hazırlanan gruplar depolama sürecince buzdolabında (+4 °C) muhafaza edilmiştir.

Mikrobiyolojik Analizler

L. monocytogenes inoküle edilen örneklerin mikrobiyolojik analizleri Yalçın ve Arslan'ın (2011), belirttiği yönteme göre yapılmıştır. Seri dilüsyonlar hazırlanarak PALCAM Agara (Merck, Almanya) ekimler yapılmış ve petriler 30±2 °C'de 24-48 saat inkübe edilip siyah haleli ve gri olan koloniler sayılmıştır. *S. aureus* inoküle edilen örneklerin mikrobiyolojik analizleri Burnham ve ark. (2008), belirttiği yöntem ile yapılmıştır. Baird Parker Agara (BPA-Merck, Almanya) ekimler yapılmış ve petriler 37±2 °C'de 24-48 saat inkübe edilip şeffaf zonlu, 1-2 mm çaplı parlak siyah renkli koloniler sayılmıştır. Her iki bakterinin sayım sonuçları log10 kob/g olarak hesaplanmıştır.

İstatistiksel Analiz

Araştırmada elde edilen veriler SPSS (Statistical Package for Social Sciences) Windows 22.0 programı kullanılarak analiz edilmiştir. Gruplar arasındaki fark Kruskall hallis, grup içerisindeki tekrarlı ölçümler arasındaki fark Friedman ve Wilcoxon testi ile incelenmiştir.

BULGULAR

Kontrol grubu kaymakta *L. monocytogenes* ve *S. aureus* araştırılmış ancak herhangi bir bulguya rastlanmamıştır.

L. monocytogenes ile kontamine edilen kaymaklarda saptanan bakteri sayıları Tablo 1'de verilmiştir. Herhangi bir inhibisyon uygulaması yapılmayan B grubunda depolama sonunda 2.41 log artış belirlenmiştir. Bunun yanında 5 mg/kg nisin uygulanan C grubunda 2.71, 10 mg/kg nisin uygulanan D grubunda ise 2.75 log çoğalma belirlenmiştir. Diğer yandan %0,25 kekik yağı kullanılan grupta 2.39 ve %0,5 kekik yağı ilave edilen grupta 2.30 log çoğalma tespit edilmiştir. Her iki maddenin de çalışmamızda kullanılan dozlarının kaymakta *L. monocytogenes* üzerine inhibisyon etkisi belirlenmemiştir. Gruplar içerisinde *L. monocytogenes'e* karşı en etkili olan %0,5 kekik yağıdır.

L. monocytogenes kullanılan gruplarda aw değeri depolama süresince dalgalı seyir göstermiştir. Tüm gruplar içerisinde en düşük değerin 79,05±0,49 ile A grubunun 3. depolama gününde, en yüksek değerin ise 96,35±0,89 ile B grubunun 0. gününde olduğu belirlenmiştir (Tablo 2). ORP değerlerine baktığımızda en düşük değerin 2,43±0,24 (D grubu, 0. gün), en yüksek değerin ise 104,03±2,4 (A grubu, 3. gün) olduğu tespit edilmiştir (Tablo 3). Tüm gruplarda pH değerlerinin 0. güne kıyasla depolama sonunda düştüğü ve bu düşüşün istatistiki olarak önemli olduğu ($p<0,05$) ortaya konulmuştur (Tablo 4).

S. aureus ile kontamine edilen kaymaklarda saptanan bakteri sayıları Tablo 5'te verilmiştir. Nisin ve kekik yağı kaymağa inoküle edilen S. aureus'u depolama süresince inhibe etmiştir. Kekik yağı kullanılan gruplarda bakteri sayısındaki azalmanın istatistiki olarak önemli olduğu ($p<0,05$) belirlenmiştir (Tablo 5).

S. aureus kullanılan gruplarda aw değerinin depolamanın 0. günü ile son günü arasındaki farkların istatistiki olarak önemli olduğu ($p<0,05$) belirlenmiştir (Tablo 6). ORP sonuçlarını incelediğimizde en düşük değer 3,73±0,21 (N grubu-10mg/kg nisin, 0. gün), en yüksek değer ise aynı grubun 5. gününde 215,67±0,57 olarak tespit edilmiştir (Tablo 7). N grubu hariç diğer

araştırma gruplarında pH değerlerinin 0. güne kıyasla depolama sonunda düştüğü ve bu düşüşün istatistiki olarak önemli olduğu ($p<0,05$) bulunmuştur (Tablo 8).

Tablo 1. Nisin ve kekik yağı ilave edilmiş kaymaklardaki L. monocytogenes sayıları (log10 kob/g)

Grup	Depolama Süresi (Gün)			
	0	3	5	7
B	$5,42\pm0,09^{Aa}$	$7,58\pm0,09^{Ba}$	$7,67\pm0,06^{Ba}$	$7,83\pm0,06^{Ca}$
C	$5,36\pm0,21^{Aa}$	$7,68\pm0,01^{Ba}$	$7,73\pm0,02^{Bab}$	$8,07\pm0,03^{Ca}$
D	$5,28\pm0,00^{Aa}$	$7,61\pm0,16^{Ba}$	$7,83\pm0,04^{Bb}$	$8,03\pm0,21^{Ca}$
E	$5,47\pm0,07^{Aa}$	$7,39\pm0,03^{Bb}$	$7,59\pm0,05^{Bc}$	$7,86\pm0,33^{Ba}$
F	$5,46\pm0,08^{Aa}$	$7,34\pm0,09^{Bb}$	$7,46\pm0,10^{Bc}$	$7,76\pm0,09^{Ba}$

^{abc}: Aynı sütunda (↓) farklı harflerle gösterilen veriler arasında $p<0,05$ düzeyinde istatistiksel fark bulunmaktadır.
^{ABC}: Aynı satırda (→) farklı harflerle gösterilen veriler arasında $p<0,05$ düzeyinde istatistiksel fark bulunmaktadır.
Veriler Aritmetik ortalama ± Standart sapma olarak verilmiştir.
(B: Bakteri kontrol grubu, C: 5 mg/kg nisin eklenmiş grup, D: 10 mg/kg nisin eklenmiş grup, E: %0,25 kekik yağı eklenmiş grup, F: %0,5 kekik yağı eklenmiş grup)

Tablo 2. *L. monocytogenes* ile kontamine kaymak gruplarında a_w değerleri

Grup	Depolama Süresi (Gün)			
	0	3	5	7
A	$82,42\pm0,32^{Cd}$	$79,05\pm0,49^{Dd}$	$92,52\pm0,21^{Ab}$	$86,47\pm0,26^{Bd}$
B	$96,35\pm0,89^{Aa}$	$88,05\pm0,36^{Cb}$	$89,72\pm0,45^{Bd}$	$84,67\pm0,43^{De}$
C	$95,63\pm0,96^{Aa}$	$86,22\pm0,59^{Cc}$	$88,48\pm0,32^{Be}$	$86,67\pm0,27^{Cd}$
D	$88,82\pm0,87^{Bb}$	$86,60\pm0,37^{Cc}$	$91,43\pm0,41^{Ac}$	$89,47\pm034^{Bc}$
E	$87,72\pm0,47^{Cc}$	$86,63\pm0,20^{Dc}$	$93,52\pm0,30^{Aa}$	$91,73\pm0,28^{Bb}$
F	$87,80\pm0,50^{Dc}$	$89,43\pm0,19^{Ca}$	$91,48\pm0,39^{Bc}$	$93,47\pm0,16^{Aa}$

^{abc}: Aynı sütunda (↓) farklı harflerle gösterilen veriler arasında $p<0,05$ düzeyinde istatistiksel fark bulunmaktadır.
^{ABC}: Aynı satırda (→) farklı harflerle gösterilen veriler arasında $p<0,05$ düzeyinde istatistiksel fark bulunmaktadır.
Veriler Aritmetik ortalama ± Standart sapma olarak verilmiştir.
(A: Herhangi bir uygulama yapılmayan grup, B: Bakteri kontrol grubu, C: 5 mg/kg nisin eklenmiş grup, D: 10 mg/kg nisin eklenmiş grup, E: %0,25 kekik yağı eklenmiş grup, F: %0,5 kekik yağı eklenmiş grup)

Tablo 3. *L. monocytogenes* ile kontamine kaymak gruplarında ORP değerleri

Grup	Depolama Süresi (Gün)			
	0	3	5	7
A	$12,82\pm0,40^{Cd}$	$104,03\pm2,4^{Aa}$	$8,73\pm0,29^{Df}$	$76,77\pm0,36^{Bb}$
B	$33,37\pm0,40^{Aa}$	$20,42\pm0,33^{Cf}$	$15,82\pm0,32^{Dd}$	$22,47\pm0,39^{Be}$
C	$17,15\pm0,59^{Cc}$	$81,17\pm0,83^{Ab}$	$69,60\pm0,31^{Ba}$	$12,55\pm0,32^{Df}$
D	$2,43\pm0,24^{Df}$	$47,57\pm0,48^{Be}$	$44,83\pm0,65^{Cb}$	$127,27\pm0,51^{Aa}$
E	$18,63\pm0,56^{Cb}$	$58,60\pm0,64^{Dd}$	$9,60\pm0,42^{Ae}$	$57,33\pm0,20^{Bc}$
F	$10,12\pm0,38^{De}$	$72,03\pm0,74^{Ac}$	$18,33\pm0,26^{Cc}$	$36,68\pm0,27^{Bd}$

^{abc}: Aynı sütunda (↓) farklı harflerle gösterilen veriler arasında $p<0,05$ düzeyinde istatistiksel fark bulunmaktadır.
^{ABC}: Aynı satırda (→) farklı harflerle gösterilen veriler arasında $p<0,05$ düzeyinde istatistiksel fark bulunmaktadır.
Veriler Aritmetik ortalama ± Standart sapma olarak verilmiştir.
(A: Herhangi bir uygulama yapılmayan grup, B: Bakteri kontrol grubu, C: 5 mg/kg nisin eklenmiş grup, D: 10 mg/kg nisin eklenmiş grup, E: %0,25 kekik yağı eklenmiş grup, F: %0,5 kekik yağı eklenmiş grup)

Tablo 4. *L. monocytogenes* ile kontamine kaymak gruplarında pH değerleri

Grup	Depolama Süresi (Gün)			
	0	3	5	7
A	6,26±0,03^{Bb}	6,28±0,02^{Ba}	6,77±0,03^{Aa}	5,54±0,03^{Ca}
B	6,33±0,03^{Ba}	5,90±0,02^{Cb}	6,57±0,02^{Ab}	5,56±0,02^{Da}
C	5,75±0,03^{Ac}	5,54±0,03^{Be}	5,25±0,03^{Ce}	4,46±0,02^{De}
D	5,66±0,02^{Bd}	5,78±0,03^{Ac}	5,55±0,04^{Cc}	5,43±0,03^{Db}
E	5,75±0,04^{Ac}	5,73±0,03^{Ad}	5,29±0,03^{Be}	4,76±0,03^{Cd}
F	5,44±0,03^{Ce}	5,75±0,04^{Ad}	5,47±0,03^{Bd}	5,25±0,03^{Dc}

^{abc}: Aynı sütunda (↓) farklı harflerle gösterilen veriler arasında p<0,05 düzeyinde istatistiksel fark bulunmaktadır.
^{ABC}: Aynı satırda (→) farklı harflerle gösterilen veriler arasında p<0,05 düzeyinde istatistiksel fark bulunmaktadır.
Veriler Aritmetik ortalama ± Standart sapma olarak verilmiştir.
(A: Herhangi bir uygulama yapılmayan grup, B: Bakteri kontrol grubu, C: 5 mg/kg nisin eklenmiş grup, D: 10 mg/kg nisin eklenmiş grup, E: %0,25 kekik yağı eklenmiş grup, F: %0,5 kekik yağı eklenmiş grup)

Tablo 5. Nisin ve kekik yağı ilave edilmiş kaymaklardaki *S. aureus* sayıları (log_{10} kob/g)

Grup	Depolama Süresi (Gün)			
	0	3	5	7
L	5,59±0,11^{Aa}	5,68±0,02^{Aa}	5,72±0,00^{Aa}	5,70±0,01^{Aa}
M	5,36±0,27^{Ab}	5,30±0,16^{Ab}	5,38±0,20^{Ab}	5,35±0,20^{Ab}
N	5,56±0,09^{Aa}	5,47±0,01^{Ac}	5,48±0,00^{Ab}	5,52±0,04^{Ab}
P	5,81±0,08^{Ac}	5,67±0,03^{Aa}	5,68±0,06^{Aa}	5,47±0,30^{Bb}
R	5,75±0,15^{Ac}	5,62±0,21^{Aa}	5,40±0,23^{Bc}	5,30±0,18^{Bb}

^{abc}: Aynı sütunda (↓) farklı harflerle gösterilen veriler arasında p<0,05 düzeyinde istatistiksel fark bulunmaktadır.
^{ABC}: Aynı satırda (→) farklı harflerle gösterilen veriler arasında p<0,05 düzeyinde istatistiksel fark bulunmaktadır.
Veriler Aritmetik ortalama ± Standart sapma olarak verilmiştir.
(L: Bakteri kontrol grubu, M: 5 mg/kg nisin eklenmiş grup, N: 10 mg/kg nisin eklenmiş grup, P: %0,25 kekik yağı eklenmiş grup, R: %0,5 kekik yağı eklenmiş grup)

Tablo 6. *S. aureus* ile kontamine kaymak gruplarında a_w değerleri

Grup	Depolama Süresi (Gün)			
	0	3	5	7
A	82,42±0,32^{Ce}	79,05±0,49^{De}	92,52±0,21^{Ab}	86,47±0,26^{Bc}
L	94,52±0,35^{Aa}	83,37±0,25^{Cd}	91,47±0,28^{Bd}	83,42±0,38^{Cd}
M	90,75±0,49^{Cc}	89,22±0,30^{Dc}	94,55±0,40^{Aa}	92,58±0,27^{Ba}
N	91,80±0,47^{Cb}	92,62±0,20^{Ba}	94,78±0,38^{Aa}	90,67±0,29^{Db}
P	89,42±0,36^{Dd}	90,88±0,34^{Cb}	94,50±0,28^{Aa}	92,50±0,33^{Ba}
R	89,52±0,33^{Bde}	89,55±0,46^{Bc}	92,47±0,24^{Ac}	92,72±0,46^{Aa}

^{abc}: Aynı sütunda (↓) farklı harflerle gösterilen veriler arasında p<0,05 düzeyinde istatistiksel fark bulunmaktadır.
^{ABC}: Aynı satırda (→) farklı harflerle gösterilen veriler arasında p<0,05 düzeyinde istatistiksel fark bulunmaktadır.
Veriler Aritmetik ortalama ± Standart sapma olarak verilmiştir.
(A: Herhangi bir uygulama yapılmayan grup, L: Bakteri kontrol grubu, M: 5 mg/kg nisin eklenmiş grup, N: 10 mg/kg nisin eklenmiş grup, P: %0,25 kekik yağı eklenmiş grup, R: %0,5 kekik yağı eklenmiş grup)

Tablo 7. *S. aureus* ile kontamine kaymak gruplarında ORP değerleri

Grup	Depolama Süresi (Gün)			
	0	3	5	7
A	12,82±0,40^{Ce}	104,03±2,40^{Ab}	8,73±0,29^{De}	76,77±0,36^{Be}
L	26,12±0,59^{Ba}	26,03±0,44^{Be}	92,78±2,27^{Ac}	18,30±0,34^{Cf}
M	19,13±0,52^{Dd}	86,33±1,27^{Cc}	134,95±2,64^{Ab}	112,62±0,38^{Bd}
N	3,73±0,21^{Df}	52,15±1,84^{Cd}	215,67±0,57^{Aa}	209,33±0,75^{Ba}
P	23,32±0,64^{Cc}	10,50±0,37^{Df}	76,65±0,43^{Bd}	116,57±0,28^{Ac}
R	24,78±0,68^{Cb}	114,67±0,77^{Ba}	5,32±0,23^{Df}	171,62±0,23^{Ab}

^{abc}: Aynı sütunda (↓) farklı harflerle gösterilen veriler arasında p<0,05 düzeyinde istatistiksel fark bulunmaktadır.
^{ABC}: Aynı satırda (→) farklı harflerle gösterilen veriler arasında p<0,05 düzeyinde istatistiksel fark bulunmaktadır.
Veriler Aritmetik ortalama ± Standart sapma olarak verilmiştir.
(A: Herhangi bir uygulama yapılmayan grup, L: Bakteri kontrol grubu, M: 5 mg/kg nisin eklenmiş grup, N: 10 mg/kg nisin eklenmiş grup, P: %0,25 kekik yağı eklenmiş grup, R: %0,5 kekik yağı eklenmiş grup)

Tablo 8. *S. aureus* ile kontamine kaymak gruplarında pH değerleri

Grup	Depolama Süresi (Gün)			
	0	3	5	7
A	6,26±0,03^{Ba}	6,28±0,02^{Ba}	6,77±0,03^{Aa}	5,54±0,03^{Ca}
L	6,17±0,02^{Ab}	5,79±0,04^{Bc}	5,45±0,03^{Cb}	4,49±0,07^{De}
M	5,62±0,05^{Ad}	5,52±0,04^{Be}	5,33±0,03^{Cc}	4,75±0,04^{Dd}
N	5,44±0,04^{Be}	5,95±0,02^{Ab}	5,24±0,03^{Cd}	5,42±0,03^{Bb}
P	5,65±0,03^{Ad}	5,65±0,04^{Ad}	5,46±0,03^{Bb}	4,47±0,03^{Ce}
R	5,76±0,03^{Ac}	5,54±0,03^{Be}	5,47±0,05^{Bb}	4,90±0,05^{Cc}

^{abc}: Aynı sütunda (↓) farklı harflerle gösterilen veriler arasında p<0,05 düzeyinde istatistiksel fark bulunmaktadır.
^{ABC}: Aynı satırda (→) farklı harflerle gösterilen veriler arasında p<0,05 düzeyinde istatistiksel fark bulunmaktadır.
Veriler Aritmetik ortalama ± Standart sapma olarak verilmiştir.
(A: Herhangi bir uygulama yapılmayan grup, L: Bakteri kontrol grubu, M: 5 mg/kg nisin eklenmiş grup, N: 10 mg/kg nisin eklenmiş grup, P: %0,25 kekik yağı eklenmiş grup, R: %0,5 kekik yağı eklenmiş grup)

TARTIŞMA

Kaymakların mikrobiyal yükü elde edildikleri hayvan, işletmelerin hijyenik koşulları, hatalı yapılan ısıl işlemler, üretim sonrasında meydana gelen kontaminasyonlar ve muhafaza aşamasında yapılan hatalara bağlı olarak artmaktadır (Karapınar ve Gönül 1999).

Nisinin düşük pH değerlerinde daha aktif olduğu, düşük protein ve yağ oranlarına sahip gıdalarda ve pH<6,0'da en etkili olduğu (Okereke ve Montville 1991) ve aktivitesinin yüksek yağ oranlarında büyük ölçüde azaldığı (Jung ve ark. 1992) belirtilmiştir. Buna rağmen yağ oranının ve pH'nın nisin üzerine, dolayısıyla da bakterilerin inhibisyonu üzerine etkisi, özellikle kullanılan nisin konsantrasyonuna bağlı olarak değişmektedir (Carminati ve ark. 1989; Jung ve ark.1992). Ayrıca nisinin yaygın kullanımının *L. monocytogenes*'te rastgele ve suşa göre değişebilen direnç gelişimine neden olabileceği belirtilmiştir (Gravesen ve ark. 2002).

Bruno ve ark. (1992), 2,5 µg/ml nisinin (pH 6,5) *L. monocytogenes* suşlarının membran potansiyelini bozarak 5 log'luk bir azalmaya neden olduğunu saptamışlardır. *L. monocytogenes*'in nisine karşı duyarlılığının saptandığı bir çalışmada, 10 µg/ml düzeyindeki nisinin *L. monocytogenes* ATCC 19115, UAL500 ve Scott A suşlarının 10^9 kob/ml olan başlangıç populasyonunda 6-7 log'luk bir düşüş meydana getirdiği ve ortama tuz ilavesinin nisinin bakterisit etkisini arttırdığı bildirilmiştir (Harris ve ark. 1991). Tarafımızdan yapılan çalışmada 5 mg/kg ve 10 mg/kg dozlarında kullanılan nisin 7 günlük depolama süresince kaymaktaki bakteri sayısında herhangi bir azalma meydana getirmemiştir. Bunun nedeni ise kaymaktaki yüksek yağ oranının (%60) nisinin etkisini azaltması olabilir.

Boussouel ve ark. (2000), tarafından yağsız sütlerle yapılan bir çalışmada, 100 ve 200 IU'lik nisinin *L. monocytogenes*'i inhibe ettiği belirtilmiştir. Bhatti ve ark. (2004), homojenize, pastörize ve çiğ sütlere ilk olarak 10^4 kob/ml oranında *L. monocytogenes* ve 0-500 IU/ml aralığındaki farklı dozlarda nisin ilave etmişlerdir. Yapılan çalışma sonucunda *L. monocytogenes* sayısının azaldığını tespit etmişlerdir. Kim ve ark. (2008), sütte sarımsak suyu ve nisinin *L. monocytogenes* üzerindeki etkinliğini araştırmak için 62,5, 125, 250 ve 500 IU/ml nisin kullanmışlar ve bu oranların güçlü antilisterial

etkisinin olduğu sonucuna varmışlardır. Abdala ve ark. (1993), pastörize süte 10^4-10^5 kob/ml düzeyinde *L. monocytogenes* Scott A ve 25 µg/ml nisin ekleyerek ürettikleri peynirlerde, 60 günlük depolama süresince *L. monocytogenes*'in inhibe olmadığını saptamışlar ve buna neden olarak da peynirlerin pH değerlerinin yüksek olmasından dolayı nisinin etkili olamadığını belirtmişlerdir. *L. monocytogenes*'e karşı nisinin etkisinin araştırıldığı çalışma gruplarımızda pH değerlerinin 5-6 aralığında olmasının nisinin bu bakteriye karşı etkinliğini sınırlandırdığı düşünülmektedir.

Aktürkoğlu ve Erol (1999), beyaz peynirde 30 µg/ml nisin kullanımının depolamanın 60. gününde *L. monocytogenes*'i tamamen yok ettiğini ifade etmişlerdir. Yapmış olduğumuz araştırmada ise 5 mg/kg ve 10 mg/kg dozlarında kullanılan nisinin 7 günlük depolama süresince *L. monocytogenes* üzerine herhangi bir bakterisidal etkisi gözlenmemiştir. Ürün tipi, pH'ı ve içeriği, depolama ya da kullanılan antimikrobiyal maddenin doz arttırımı ile gıdalarda *L. monocytogenes* varlığının değişkenlik gösterebileceği düşünülmektedir. Araştırmamızda hem nisinin hem de kekik yağının kullanıldığı gruplarda su aktivitesi değerinin düşük olması bunun yanında ORP değerinin dalgalı seyir göstermesi bu maddelerinin kaymak ortamında *L. monocytogenes*'e karşı etkinlik göstermesini engellemiş olabilir.

Bitkisel antimikrobiyal maddeler Gr (+) bakterilere Gr (-) bakterilerden daha etkilidir (Yalçın ve Uyanık 2019). Çalışmada kullanılan dozların Gram (+) bakteri olan *L. monocytogenes*e karşı etki etmediği ancak diğer bir Gram (+) bakteri olan *S. aureus*'a karşı istatistiki öneme sahip ($p<0,05$) azalma sağladığı belirlenmiştir.

Rasooli ve ark. (2006), 250 ppm dozundaki kekik esansiyel yağının, kültür ortamında *L. monocytogenes*'i 20 dk. içerisinde 7 log düzeyinden tespit limitinin altına düşürdüğünü ifade etmişlerdir. El-Zehery ve ark. (2021), kekiğin *Listeria monocytogenes* (ATCC 19116) suşunda kültür ortamında 24.5 mm'lik inihibisyon zonu oluşturduğunu belirtmiştir. Fenolik bileşiklerin *L. monocytogenes*'e karşı etkinliğinin doza, bakteri suşuna ve ürünün yapısına göre değişebileceği belirtilmiştir (Zamuz ve ark. 2021). Karvakrol, timol, nisin ve ögenolün farklı doz ve kombinasyonlarının sütte kontrol grubuna göre *L. monocytogenes* sayısında değişik düzeylerde azalma sağladığı ifade edilmiştir (Alves ve ark. 2016). Gıdalardaki bakteri gelişimini kontrol altına almak için çoklu bariyer teknolojisi kullanılmaktadır (Zamuz ve ark. 2021). Bütil gallat ile kombine edilen nisinin 5 ayrı *L. monocytogenes* suşunu inhibe ettiği belirtilmiştir (Li 2017). Araştırmamızdaki maddelerin doz kombinasyonlarının farklı etkinlik göstereceği varsayılmaktadır.

Jamuna ve ark. (2005), nisinin faklı Gram (-) ve Gram (+) mikroorganizmalar üzerine etkisini incelemişlerdir. Nisinin 40 IU/ml ve üzeri konsantrasyonlarda sıvı besi yerinde kullanımı *L. monocytogenes*, *S. aureus*, *C. sporogenes* üzerine etkili olmuş; Gram (-) bakterilerden *E. coli* dirençli bulunmuş, *Pseudomonas* ise daha yüksek (320 IU/ml) konsantrasyonlarda inhibe edilmiştir. Araştırmamızda kullanılan nisin miktarları kaymağa inoküle edilen *S. aureus* üzerine bakterisidal etki yapmamış ($p>0,05$) ancak bakteri üzerinde baskılayıcı etki göstermiştir.

Pires ve ark. (2008), natamisin, nisin ve nisin+natamisin içeren selüloz esaslı filmler üreterek dilimlenmiş mozzarella peynirindeki *S. aureus* ATCC 6538 üzerine antimikrobiyel etkinliğini 12±2 °C'de 15 gün süreyle araştırmışlardır. Nisin içeren selüloz filmlerin *S. aureus* ATCC 6538 üzerine antimikrobiyel etki göstermediğini ifade etmişlerdir. Pinto ve ark. (2011), nisinin serro peynirinde 100 IU/ml ve 500 IU/ml dozlarında kullanımının *S. aureus* sayısını olgunlaşmanın 7. gününde sırasıyla 1,2 ve 2,0 log azalttığını tespit etmişlerdir. Ultra filtrasyondan geçirilen sütten üretilen ve 8 °C'de depolanan peynirlerde, 2 µg/g nisinin *S. aureus* sayısını 4 log azalttığı belirtilmiştir (Mohammadi ve Jodeiri 2014). Çalışmamızda kullanılan nisin dozlarının +4 °C'de 7 günlük depolama süresince kaymakta *S. aureus* sayısında herhangi bir azalma oluşturmadığı ancak bakteri gelişimini baskıladığı belirlenmiştir. Nisinin *S. aureus*'a karşı etkinliğinin az olması, dozuna ve kaymaktaki yağ oranına bağlanabilir.

Gıda intoksikasyonlarında en çok görülen toksin tipinin, toksisitesi en yüksek olan SEA olduğu, bunu SEB ve SED tiplerinin takip ettiği bildirilmektedir (Yıldırım ve ark. 2016). Araştırmada kullandığımız *S. aureus* ATCC 25923 A tipi enterotoksin oluşturmaktadır. Soejima ve ark. (2007), yağsız süte 1-2 log kob/ml *S. aureus* inoküle edip çalkalayarak 35 ºC'de inkübe etmişlerdir. Bu koşulların *S. aureus* gelişimini ve SEA üretimini hızlandırdığını bildirmişlerdir. Kaymakta enterotoksin üreten *S. aureus* gelişimini önlemek amacıyla yaptığımız deneysel çalışmada kullanılan 5 mg/kg ve 10 mg/kg nisinin bakteri sayısını azaltıcı yönde bir etkisi olmamış ancak bakteri gelişimini baskılamıştır. Kekik yağının %0,25 oranında kullanılması 7. depolama gününde, %0,5 oranında kullanılması ise 5. depolama gününden itibaren *S. aureus* sayısını azaltıcı yönde etki göstermiştir. Gram (+) bakterilere karşı etkinliği bilinen (Üstündağ ve Yalçın, 2017) nisinin her ikisi de Gram (+) olan *L. monocytogenes* ve *S. aureus*'a karşı kaymak ortamında etkisiz kaldığı belirlenmiştir.

SONUÇ

L. monocytogenes eklenen gruplarda kullanılan antimikrobiyal maddeler ile miktarlarının depolama süresince bakteri üzerinde herhangi bir baskılayıcı etkisi gözlenmemiştir. Bunun tersine depolama sonunda bakteri sayısının arttığı ($p<0,05$) belirlenmiştir. *S. aureus* eklenen gruplarda ise kullanılan nisin miktarlarının olumlu etkisi olmamış ancak %0,25 kekik yağı 7., % 0,5 kekik yağı ise 5. depolama gününde 0. güne oranla inhibisyon sağlamıştır. Oluşan bu etkilerin ise istatistiki açıdan önemli ($p<0,05$) olduğu sonucuna varılmıştır. Tüm deneme gruplarında yapılan a_w, ORP ve pH analiz

sonuçlarına bakıldığında bakterilerin ürün ve depolama şartlarına göre yaşamsal farklılıklar gösterdikleri tespit edilmiştir.

Kaymağın raf ömrünün kısa olmasından dolayı 7 gün olarak belirlenen depolama süresi, sonraki çalışmalarda daha uzun tutularak kullanılan maddelerin ve miktarlarının etkinliği araştırılabilir. Ayrıca daha yüksek dozların kullanımının nasıl etki edeceğine yönelik çalışmalar da yapılabilir. Ancak nisinin gıdalarda kullanımı için belirlenen limit değerlerin aşılmaması gerekmektedir. Kekik yağı için ise belirlenen limit değer yoktur. Kaymak gibi kendine özgü tat ve aromaya sahip bir üründe, baskın tat ve kokuya sahip bir maddenin kullanımı, kaymağın tüketilebilirliğini olumsuz yönde etkileyebilir.

Çıkar Çatışması: Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

Yazarların Katkı Oranı: Yazarlar makaleye eşit oranda katkı sağlamış olduklarını beyan etmişlerdir.

Etik izin: Bu çalışma "Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esaslarına Dair Yönetmelik" Madde 8 (k) gereği HADYEK iznine tabi değildir.

Finansal Destek: Bu çalışmada herhangi bir kurumdan maddi destek alınmamıştır. Yazarlar öz kaynakları ile çalışmayı finanse etmişlerdir. Beyan ederiz.

Teşekkür: Bu çalışmada desteklerini veren Dr. Ali SOYUÇOK'a teşekkürlerimizi sunarız.

Açıklama: Bu çalışma ilk isim yazarın tezinden üretilmiştir. Bu çalışma daha önce herhangi bir kongre/sempozyumda sunulmamıştır. Yayınlanması için herhangi bir dergiye gönderilmemiştir. Beyan ederiz.

KAYNAKLAR

Abdala OM, Davidson PM, Christen GL. Survival of selected pathogenic bacteria in white pickled cheese made with lactic acid bacteria or antimicrobials. J Food Protect. 1993; 56:972-976.

Abdeen EE, Mousa WS, Salam SYA, Al-Maary KS, Mubarak AS, Moussa IM, Hemeg HA, Almuzaini AM, Alajaji AI, Alsubki RA, Elbehiry A. Antibiogram and phylogenetic diversity of enterotoxigenic *Staphylococcus aureus* strains from milk products and public health implications. Saudi J Bio Sci. 2020; 27(8):1968-1974.

Aktürkoğlu E, Erol İ. Beyaz peynir üretiminde nisin kullanımı ile *L. monocytogenes*'in inhibisyonu. Turk J Vet Anim Sci. 1999; 23:785-792.

Alves FCB, Barbosa LN, Andrade BFMT, Albano M, Furtado FB, Marques Pereira AF, et al. Inhibitory activities of the lantibiotic nisin combined with phenolic compounds against *Staphylococcus aureus* and *Listeria monocytogenes* in cow milk. J Dairy Sci. 2016; 99(3):1831–1836. https://doi.org/10.3168/jds.2015-10025

Anonim. Türk Gıda Kodeksi Mikrobiyolojik Kriterler Yönetmeliği. Kanun 5996. Resmi Gazete 29.12.2011-28157, 2011.

Anonim. Türk Gıda Kodeksi Gıda Katkı Maddeleri Yönetmeliği Resmi Gazete 30.16.2013-28693, 2013.

Anyogu A, Olukored A, Anumudu C, Onyeaka H, Areo E, Adewale O, Odimba JN, Nwaiwu O. Microorganisms and food safety risks associated with indigenous fermented foods from Africa. Food Cont. 2021; 129: 108227.

Arauz LJ, Jozala AF, Mazzola PG, Penna TCV. Nisin biotechnological production and application: A review. Trends Food Sci Tech. 2009; 20:146-154.

Baranauskiene R, Venskutoni SPR, Viskelis P, Dambrauskiene E. Influence of nitrogen fertilizers on the yield and composition of thyme (*Thymus vulgaris*). J Agr Food Chem. 2003; 51:7751–7758.

Baysal A. Beslenme. 10. Baskı, Hatiboğlu Yayınları, Ankara, Türkiye. 2004; s, 287-288.

Benli M, Yiğit N. Ülkemizde yaygın kullanımı olan kekik (*Thymus vulgaris*) bitkisinin antimikrobiyal aktivitesi. Orlab On-line Mik Derg. 2005; 3:1-8.

Bergdoll MS, Lee-Wong AC. Staphylococcal intoxications, In: Foodborne Infections and Intoxications, Eds; Rieman HP, Cliver DO. Academic Press, Elsevier Inc., San Diego, California, USA. 2006; p, 523-562.

Bhatti M, Veeramachaneni A, Shelef LA. Factors affecting the antilisterial effects of nisin in milk. Int J Food Microbiol. 2004; 97:215-219.

Boussouel N, Mathieu F, Junelles RAM, Milliere JB. Effects of combinations of lactoperoxidase system and nisin on the behaviour of *L. monocytogenes* ATCC 15313 in skim milk. Int J Food Microbiol. 2000; 61:169-175.

Boziaris IS, Adams MR. Effect of chelators and nisin produced in situ on inhibition and inactivation of Gram negatives. Int J Food Microbiol. 1999; 53:105-113.

Bruno MEC, Kaiser A, Montville TJ. Depletion of proton motive force by nisin in *Listeria monocytogenes* cells. Appl Environ Microbiol. 1992; 58:2255-2259.

Burnham G, Hanson DJ, Koshick CM, Ingham SC. Death of *Salmonella* serovars, *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Listeria monocytogenes* during the drying of meat: A case study using biltong and droewors. J Food Safety. 2008; 28:198-209.

Carminati D, Giraffa G, Bossi MG. Bacteriocin-like inhibitors of *Streptococcus lactis* against *Listeria monocytogenes*. J Food Protect. 1989; 52:614-617.

Cheigh C, Pyun Y. Nisin biosynthesis and its properties. Biotechnol Lett. 2005; 27:1641-1648.

Cheung GY, Bae JS, Otto M. Pathogenicity and virulence of *Staphylococcus aureus*. Virulence. 2021; 12(1):547-569.

EFSA. The European Union one health 2018 zoonoses report. EFSA J. 2019; 17(12): e05926.

El-Zehery HR, Zaghloul RA, Abdel-Rahman HM, Ahmed SA, El-Dougdoug KH. Novel strategies of essential oils, chitosan, and nano-chitosan for inhibition of multi-drug resistant: *E. coli* O157: H7 and *Listeria monocytogenes*. Saudi J Bio Sci. 2021. https://doi.org/10.1016/j.sjbs.2021.12.036.

Gravesen A, Jydegaard Axelsen AM, Mendes da Silva J, Hansen TB, Knøchel S. Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. App Environ Microbiol. 2002; 68:756–764.

Harris LJ, Fleming HP, Klaenhammer TR. Sensitivity and resistance of *Listeria monocytogenes* ATCC 19115, Scott A and UAL500 to nisin. J Food Protect. 1991; 54:836-840.

Hasdoğan H. Van ili kahvaltı salonlarında tüketime sunulan kaymakların bazı kimyasal ve mikrobiyolojik özelliklerinin belirlenmesi. Yüksek Lisans Tezi, Yüzüncü Yıl Üniversitesi Sağlık Bilimleri Enstitüsü, Van, 2004.

Helander IM, Alakomi HL, Latva-Kala K, Mattila-Sandhom T, Pol I, Smid EJ, Gorris LGM, von Wright A. Characterization of the action of selected essential oil components on gram negative bacteria. J Agric Food Chem. 1998; 46:3590-3595.

İbrahim SA, Ayivi RD, Zimmerman T, Siddiqui SA, Altemimi AB, Fidan H, Esatbeyoğlu T, Bakhshayesh RV. Lactic acid bacteria as antimicrobial agents: Food safety and microbial food spoilage prevention. Foods. 2021; 10(12):1-13.

İpekçioğlu V, Gürler Z. Afyonkarahisar'da tüketime sunulan Afyon kaymaklarında bazı patojen bakterilerin aranması. Kocatepe Vet J. 2017; 10(4):354-357.

Jamuna M, Babusha ST, Jeevaratnam K. Inhibitory efficacy of nisin and bacteriocins from *Lactobacillus* isolates against food spoilage and pathogenic organisms in model and food systems. Food Microbiol. 2005; 22:449-454.

Jung D, Bodyfelt FW, Daeschel MA. Influence of fat and emulsifiers on the efficacy of nisin in inhibiting *Listeria monocytogenes* in fluid milk. J Dairy Sci. 1992; 75:387-393.

Karapınar M, Gönül Ş. Hububat ve hububat ürünlerinde mikrobiyolojik bozulmalar, patojen mikroorganizmalar ve muhafaza yöntemleri. Kitap: Gıda Mikrobiyolojisi, Ed; Ünlütürk A, Turantaş F, 2. Basım, Mengi Tan Basımevi, İzmir, Türkiye. 1999; s. 369-384.

Kim EL, Choi NH, Bajpai VK, Kang SC. Synergistic effect of nisin and garlic shoot juice against *Listeria monocytogenes* in milk. Food Chem. 2008; 110:375-382.

Khelissa S, Chihib NE, Gharsallaoui A. Conditions of nisin production by *Lactococcus lactis* subsp. *lactis* and its main uses as a food preservative. Archives of Microbiol. 2021; 203(2), 465-480.

Küplülü Ö, Sarımehmetoğlu B, Kaymaz G. Pastörize sütlerde ELISA tekniği ile stafilokokal enterotoksin varlığının belirlenmesi. Turk J Vet Anim Sci. 2002; 26:631-637.

Lambert R, Skandamis PN, Coote P, Nychas GJ. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J Appl Microbiol. 2001; 91:453-62.

Li KL. Inhibition of *Listeria monocytogenes* growth and biofilm production by nisin and phenolic compounds. Master thesis, University of Alberta Environmental Health Sciences, Alberta, Canada, 2017.

Liu G, Nie R, Liu Y, Mehmood A. Combined antimicrobial effect of bacteriocins with other hurdles of physicochemic and microbiome to prolong shelf life of food: A review. Sci. Total Environ. 2022; 825:1-16, 154058.

Mohammadi K, Jodeiri H. Effects of nisin and temperature on behavior of enterotoxigenic *Staphylococcus aureus* in model cheeses. Kafkas Univ Vet Fak Derg. 2014; 20:461-464.

Okereke A, Montville TJ. Bacteriocin-mediated inhibition of *Clostridium botulinum* spores by lactic acid bacteria at refrigeration and abuse temperatures. Appl Environ Microb. 1991; 57:3423-3428.

Pinto MS, Fernandes de Carvalho A, Pires ACS, Campos-Souza AA, Fonseca da Silva PH, Sobral D. The effects of nisin on *Staphylococcus aureus* count and the physicochemical properties of Traditional Minas Serro cheese. Int Dairy J. 2011; 21:90-96.

Pires ACS, Soares NFF, Andrade NJ, Silva LHM, Camilloto GP, Bernardes PC. Development And Evaluation Of Active Packaging For Sliced Mozzarella Preservation. Packag Technol Sci. 2008; 21:375-383.

Pudja P, Djerovski J, Radovanovic M. An autochthonous Serbian product-Kajmak characteristics and production procedures. Dairy Sci Tech. 2008; 88:163-172.

Rasooli I, Rezaei MB, Allameh A. Ultrastructural studies on antimicrobial efficacy of thyme essential oils on *Listeria monocytogenes*. Int J Infectious Dis. 2006; 10(3):236-241.

Soejima T, Nagao E, Yano Y, Yamagata H, Kag H, Shinagawa K. Risk evaluation for staphylococcal food poisoningin processed milk produced with skim milk powder. Int J Food Microbiol. 2007; 115:29-34.

Üstündağ HÇ, Yalçın H. Bakteriyosinler ve Gıdalarda Kullanımı. MAKÜ Sag Bil Enst Derg. 2017; 5(1):53-65.

Yalçın H, Arslan A. *Escherichia coli* O157:H7 ve *Listeria monocytogenes* ile kontamine edilmiş broyler karkaslarında laktik asit, cetylpyridinium chloride ve trisodyum fosfatın tekil ve kombine etkilerinin incelenmesi. Kafkas Univ Vet Fak Derg. 2011; 17:625-630.

Yalçın H, Uyanık İ. Bölüm: Kekik Esansiyel Yağlarının *Listeria monocytogenes ve Salmonella spp*. Üzerine Antimikrobiyal Etkisi. Kitap: Beslenme ve Diyetetikte Güncel Yaklaşımlar. Ed; Yahya Özdoğan. IKSAD Uluslararası Yayınevi, Ankara, Türkiye. 2019; s. 151-178.

Yıldırım T, Sırıken B, Yavuz C. Çiğ süt ve peynirlerde koagulaz pozitif Stafilokoklar. Vet Hekim Der Derg. 2016; 87:3-12.

Zamuz S, Munekata PE, Dzuvor CK, Zhang W, Sant'Ana AS, Lorenzo, JM. The role of phenolic compounds against *Listeria monocytogenes* in food. A review. Trends in Food Sci Technol. 2021; 110:385-392.

Zhang X, Wang S, Chen X, Qu C. Review controlling *Listeria monocytogenes* in ready-to-eat meat and poultry products: An overview of outbreaks, current legislations, challenges, and future prospects. Trends Food Sci Technol. 2021; 116:24-35.

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022) 15(2)209-216

DOI: 10.30607/kvj.1070754

RESEARCH ARTICLE

An Immunohistochemical Investigation of The Effect of Sambucus Nigra on Chymase-, Tryptase- and Ghrelin- Positive Cells in Rat Lung

Tuğrul Ertuğrul[1]*, Gökçen Sevilgen[2]

[1]Ondokuz Mayis University, Faculty of Veterinary Medicine, Department of Histology and Embryology, Samsun, Turkey
[2]Recep Tayyip Erdogan University, Faculty of Medicine, Department of Thoracic Surgery, Rize, Turkey

ABSTRACT

Sambucus nigra (S.nigra) is used in the treatment of many diseases and disorders thanks to its antioxidant, anticarcinogenic, immunostimulating, antiallergic, antiviral, and antibacterial properties. Ghrelin has anti-inflammatory effect on oxidative damage in various organs and cell types. The aim of this study was to immunohistochemically examine the chymase, tryptase, and ghrelin in rat lung after S. nigra administration. A total of 16 male rats were used in the study. The rats were assigned to two groups, control and S. nigra. 1st control group (n=8): No application was made. 2nd S. nigra group (n=8): S. nigra extract was administered at 15 mg/kg by oral gavage for 14 days. Tryptase, chymase, and ghrelin-positive cells were found in the lung tissue in a spindle-shaped, round, or oval shape. When the groups were evaluated within themselves, a significant increase in the number of tryptase, chymase, and ghrelin positive cells was observed in the S. nigra treated group. This study showed that S. nigra, which has an immunomodulatory and antioxidant effect, increases the expression of chymase-, tryptase- and ghrelin-positive cells in the lungs. Additionally, based on our findings, it can be said that mast cells can produce, store and release ghrelin.
Keywords: Chymase, ghrelin, lung, Sambucus nigra, tryptase

***

Sıçan Akciğerinde Sambucus Nigra'nın Kimaz, Triptaz ve Ghrelin Pozitif Hücrelerin Üzerindeki Etkisinin İmmünohistokimyasal Olarak İncelenmesi

ÖZ

Sambucus nigra (S. nigra) antioksidan, antikanserojenik, immün sistemi uyarıcı, antialerjik, antiviral ve antibakteriyel özellikleri sayesinde birçok hastalık ve rahatsızlığın tedavisinde kullanılmaktadır. Ghrelin, çeşitli organlarda ve hücre tiplerinde oksidatif hasar üzerinde anti-inflamatuar etkilere sahiptir. Bu çalışmanın amacı, S. nigra uygulamasından sonra sıçan akciğerinde kimaz, triptaz ve ghrelin immünopozitif hücrelerinin immünohistokimyasal olarak incelenmesidir. Çalışmada toplam 16 erkek sıçan kullanıldı. Sıçanlar kontrol ve S. nigra olmak üzere iki gruba ayrıldı. 1. kontrol grubu (n=8): herhangi bir uygulama yapılmadı. 2. S. nigra grubu (n=8): S. nigra ekstresi 14 gün süreyle oral gavaj yoluyla 15 mg/kg dozunda uygulandı. Akciğer dokusunda yuvarlak, oval veya mekik şeklinde triptaz, kimaz ve ghrelin pozitif hücreler bulundu. İki grup kendi içinde değerlendirildiğinde S. nigra uygulanan grupta triptaz, kimaz ve ghrelin pozitif hücre sayısında önemli artış gözlendi. Bu çalışma, immünomodülatör ve antioksidan etkiye sahip olan S. nigra'nın akciğerde kimaz, triptaz ve ghrelin pozitif hücrelerin ekspresyonunu arttırdığını göstermiştir. Ayrıca mast hücrelerinin diğer bağışıklık hücreleri gibi ghrelin üretebildiği, depolayabildiği ve serbest bırakabildiği açıklanmaya çalışılmıştır.
Anahtar kelimeler: Akciğer, ghrelin, kimaz, Sambugus nigra, triptaz

To cite this article: Ertuğrul T. Sevilgen G. The Immunohistochemical Investigation of Chymase-, Tryptase- and Ghrelin-Positive Cells on The Lung of Rat After an Application of Sambucus Nigra
Kocatepe Vet J. (2022) 15(2)209-216
Submission: 09.02.2022 **Accepted**: 18.04.2022 **Published Online**: 30.05.2022
ORCID ID; *TE: 0000-0002-9310-1200, GS: 0000-0003-4638-8007.*
*Corresponding author e-mail: tugrulertugrul06@hotmail.com

INTRODUCTION

Sambucus nigra (S. nigra) is a widespread species of the Caprifoliaceae family that grows in most of Europe, western Asia, northern Africa, and the United States (Fazio et al. 2013). Because of its antioxidant, anticarcinogenic, immune system boosting, antiallergic, antiviral, and antibacterial characteristics, S. nigra has been used in folk medicine for generations to treat a variety of ailments and problems (Oniszczuk et al. 2016). It contains high levels of polyphenols, especially flavonols, phenolic acids and anthocyanins. At the same time, these compounds are known as radical scavengers, which protect the body against oxidative stress and lipid peroxidation (Duymuş et al. 2014). Herbal supplements, including S. nigra, have been known to be used to boost immunity against respiratory diseases (Wieland et al. 2021). Moreover, it has been suggested that S. nigra may help in the treatment of upper respiratory tract symptoms and shorten the duration of the common cold or flu (Hawkins et al. 2018).

Mast cells (MCs) are tissue-resident sentinel cells with densely packed secretory granules. (Metcalfe et al. 1997). MCs are capable of secreting a variety of biologically active mediators, cytokines, and chemokines. MC-derived mediators can affect the biological activities of adjacent cells and tissues (Mukai et al. 2018). These cells are multifunctional effector cells involved in innate immunity, host defense, hypersensitivity, and allergic disease (Da Silva et al. 2014). MCs exhibit substantial heterogeneity based on their granule content and protease expression patterns (Dwyer et al. 2016). Based on protease content, two types of MCs have been identified immunohistochemically: tryptase positive mast cells (MC_T) and chymase positive mast cells (MC_{TC}) (Tütüncü et al. 2020). Tryptase is stored in the secretory granules of MCs, from which it is released after degranulation following cell stimulation (Schwartz et al. 1981). Under various physiological and pathological settings, tryptase is involved in the activation, proliferation, and migration of many mesenchymal cells, including endothelial cells and fibroblasts (Sonneck et al. 2006). Chymase is an intracellular, granular-associated, neutral serine protease produced mainly by MCs. It plays a role in regulating extracellular matrix proteolysis, which promotes tissue remodeling (Hamada et al. 1999). In addition, MC_{TC} can promote vascular proliferation, atherosclerosis and tissue fibrosis (Miyazaki et al. 2006). In experimental studies, chymase has been shown to reduce fibrosis in lung tissue (Tomimori et al. 2003, Sakaguchi et al. 2004).

Ghrelin is an endogenous peptide that interacts with the growth hormone secretagogue receptor 1a (GHSR1a) (Kojima and Kangawa, 2005). The presence of ghrelin and its receptor in a wide range of tissues has been determined by gene expression studies in humans and rats (Akalu et al. 2020). Ghrelin regulates growth hormone secretion, cell proliferation, appetite increase, and inflammation through GHSR1a (Nakazato et al. 2001). It has anti-inflammatory effects on oxidative damage in various organs and cell types (Raghay et al. 2020). The expression of ghrelin can be modulated by factors such as peptide hormones, neurotransmitters, glucose, fatty acids, neurotransmitters and enzymes (Akalu et al. 2020). It has been reported that ghrelin is expressed in immune tissues and modulates immune function (Chowen and Argente. 2017). Furthermore, it is stated that ghrelin may have an effect on hematological parameters, which may increase lymphocyte count by stimulating lymphopoiesis (Narin and Çetin, 2010). Moreover, Stefanov et al. (2017) suggested that MCs in rat stomachs can produce, store, and release ghrelin like other immune cells.

The aim of this study was to investigate the effect of S. nigra on the immunohistochemical distribution of chymase-, tryptase- and ghrelin-positive cells in rat lung. Also, the lack of data on the ability of MCs to express, store and release ghrelin motivated this study.

MATERIAL and METHODS

Animals

All procedures were approved by the Ethical Committee of Ondokuz Mayıs University (Decision no: 11.03.2020, number 15).

In this study, 16 male rats, weighing 250-300 g, that were used. The rats were kept in a standard cage with 12 hours of light and 12 hours of darkness in a 22°C ambient temperature environment and were given ad libitum and tap water.

Experiment Groups

1st control group (n=8): there was no application made. 2nd S. nigra group (n=8): S. nigra extract was administered at a dose of 15 mg/kg by oral gavage for 14 days (Bidian et al. 2021). Then, cervical dislocation was performed under anesthesia and lung tissue samples were collected for immunohistochemistry. The lung tissue samples were fixed for 24 h in a 10% formaldehyde solution, and tissue sections were cut from the prepared paraffin blocks with a thickness of 5 μm.

Immunohistochemistry

The lung sections were stained immunohistochemically with Streptavidin biotin complex. Immunopositive cells were determined using anti-rabbit polyclonal chymase (1/200 dilution, Biorbyt, orb11030), mouse monoclonal tryptase

(1/200 dilution, Abcam, ab2378), and rabbit polyclonal anti-ghrelin antibody (1/400 dilution, Abcam, ab129383) (True, 1990). As a secondary antibody, Histostain Plus (Zymed kit: 85-6743) was used. Following deparaffinization, sections were heated in a 700-watt microwave oven in a citrate buffer (pH=6) solution for proteolysis. The tissues were treated in a 3 % hydrogen peroxide solution to inhibit endogenous peroxidase activity. To prevent nonspecific protein binding in sections, serum in the kit was instilled after washing with phosphate buffer solution (PBS). The primary antibody was applied to the samples, which were then kept at +4 0C overnight. The negative control group's tissues were treated with only PBS solution. Following the washing procedure, sections were treated with a biotinylated secondary antibody and incubated with streptavidin-horseradish peroxidase complex. Finally, 3, 3'-diaminobenzidine (DAB) was utilized as chromogen, and the samples were counterstained with hematoxylin and then coated with entellan.

Microscopical Evaluation and Positive Cell Counts

Following histochemical and immunohistochemical staining, tissue samples were examined under an microscope (Nikon Eclipse 50i) in terms of immunoreactivity. Chymase-, tryptase- and ghrelin-positive cell distribution was evaluated semiquantitatively. The following criteria were employed in semiquantitative evaluation: no positive cell in the scanned area (-), 1-2 cells (±), 3-4 cells (+), and 5-6 cells (++) (Ertuğrul et al. 2021).

The immunopositive cells were scored from 0 to 3 semi-quantitatively (Samrao et al. 2012) as follows. A histoscore was derived from the immunopositive cell distribution, 0: no positive cell in the scanned area (-), +1: 1-2 cells (±), +2: 3-4 cells (+), +3: 5-6 cells (++).

Statistical Analysis

The IBM SPSS Statistics Version 22.0 statistical software program was used for all statistical analyses. The Shapiro-Wilk W test was used to determine whether the distribution was normal. Depending on the normality of the data, comparisons between control and S. nigra groups were performed by independent Student's t-test for parametric data. The findings were presented as mean ± SEM (standard error of the mean), and statistical significance was accepted at $p < 0.05$.

RESULTS

Tryptase- and Chymase- Immunopositive Cells

In the light microscopic examination, MC_T and MC_{TC} with positive immune reactions were clearly distinguished in brown color. Tryptase and chymase positive cells were found in the lung tissue in a spindle-shaped, round, or oval shape. (Figures 1A, 2A). MC_T and MC_{TC} were observed around the sacculus alveolaris, in the visceral pleura of the lung, the bronchial wall, and connective tissue in the terminal and respiratory bronchioles' walls (Figures 1B, 2B). Also, MC_{TC} and MT_C were observed to be mostly located near the blood vessels and bronchus-associated lymphatic tissue (BALT). When the two groups are evaluated among themselves, a significant increase in the number of MC_T and MC_{TC} was observed in the S. nigra treated group. MC_{TC} and MT_C were seen individually or in groups in the lung tissue (Table 1).

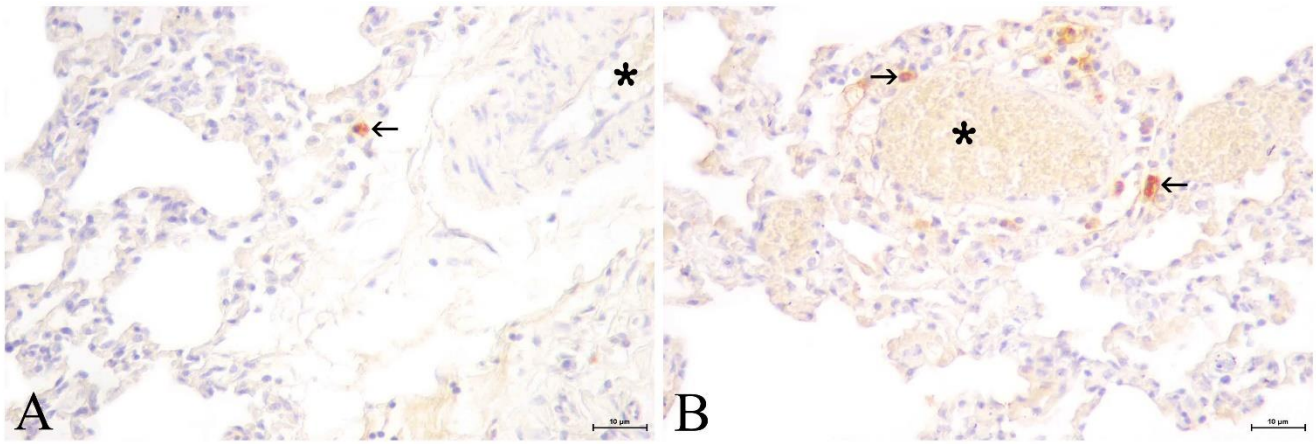


Figure 1: Lung tissue immunostained with antibodies against tryptase; (A) Control group, (→): tryptase immunopositive cell, (asterix): blood vessel, (B) S. nigra group, (→): tryptase immunopositive cell, (asterix): blood vessel, original magnification X40; range bar, 10 µm.

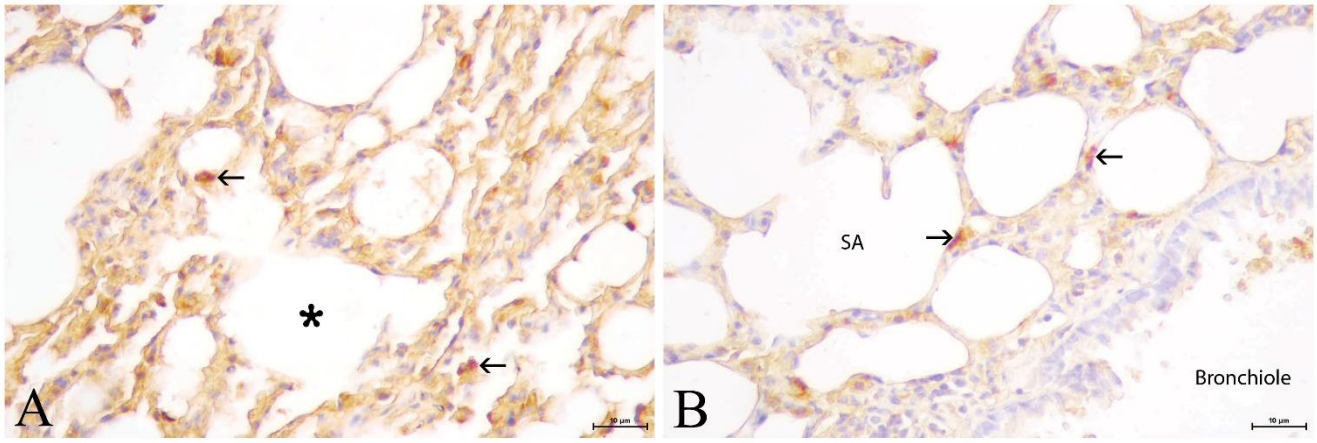


Figure 2: Lung tissue immunostained with antibodies against chymase; (A) Control group, (→): chymase immunopositive cell, (asterix): alveolar space, (B) S. nigra group, (→): chymase immunopositive cell, (SA): sacculus alveolaris, original magnification X40; range bar, 10 μm.

Table 1. Tryptase-. chymase- and ghrelin- immunopositive cell numerical density

	Control group (mean ± SEM)	S. nigra group (mean ± SEM)
Tryptase immunopositive cell	0.73 ± 0.06	1.28 ± 0.09**
Chymase immunopositive cell	0.72 ± 0.05	1.31 ± 0.11**
Ghrelin immunopositive cell	1.82 ± 0.07	2.15 ± 0.11*

* $p<0.05$ and ** $p<0.001$

Ghrelin- Immunopositive Cells

Immunohistochemistry revealed that brown ghrelin-positive cells were scattered throughout the lung tissue, including perivascular areas. In lung tissue, ghrelin-positive cells were spindle-shaped, round, or oval. Ghrelin-positive cells were observed around the sacculus alveolaris, in the interalveolar septal connective tissue, on the periphery of the bronchi, and around blood vessels (Figure 3A). Ghrelin-positive cells were also observed inside and at the margin of the bronchus-associated lymphatic tissue in the lungs. In the lung tissue, ghrelin-positive cells were mostly seen separately or in groups (Figure 3B).

A significant increase in ghrelin positive cells was observed after S. nigra administration compared to the control group (Table 1).

When we compared ghrelin-positive cells with MC_T and MC_{TC}, we also found that ghrelin-positive cells had similar morphology (similar size and shape) and localization to MC_T and MC_{TC}, which reacted positively to immune staining. In addition, ghrelin-positive cell density increase after S. nigra application allowed us to suggest that MCs can produce, store and release ghrelin in rat lung.

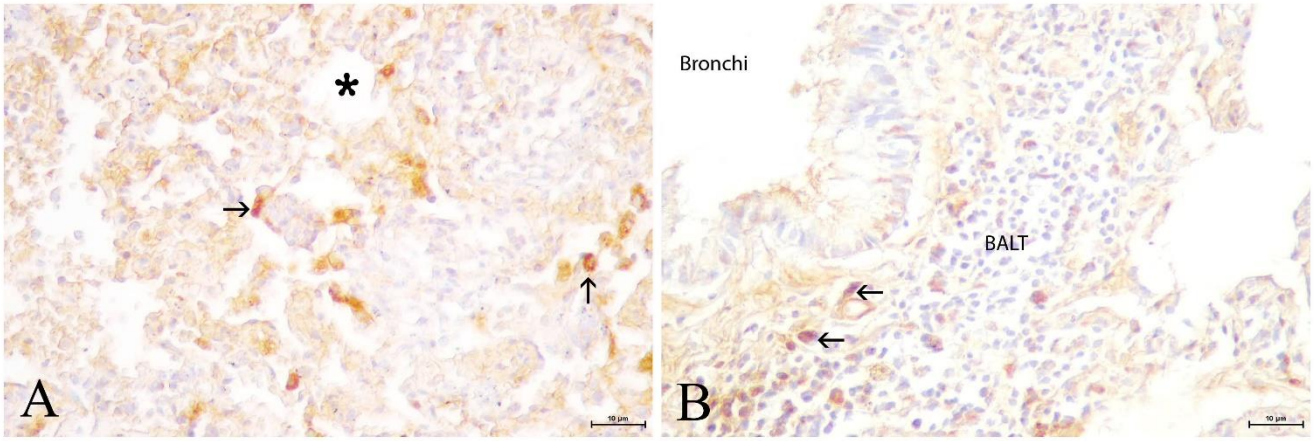


Figure 3: Lung tissue immunostained with antibodies against ghrelin; (A) Control group, (→): ghrelin immunopositive cell, (asterix): alveolar space, (B) S. nigra group, (→): ghrelin immunopositive cell, (BALT): bronchus-associated lymphatic tissue, original magnification X40; range bar, 10 μm.

DISCUSSION

Numerous food components, due to their immunomodulatory properties, have been shown to activate MCs as well as modulate the synthesis of MC mediators (Uranga et al. 2020). Cells of the immune system are highly sensitive to changes in metabolism status. They can affect by changes in circulating hormones, which can affect immune responses and cytokine expression (Baatar et al. 2011).

Chymase plays an important role in the regulation of coagulation by activating and catalyzing the degradation of thrombin and plasmin (Dell'Italia LJ and Husain, 2002). MC_{TC} has been shown to contribute to tissue remodeling, fibroblast mitogenicity, and angiogenesis in lung tissue (Mitani et al. 1999). Increased numbers of MC_{TC} have been found in chronic asthma (Van der Velden et al. 2012), lungs with interstitial pneumonia (Hirata et al. 2007), and blunt lung trauma (Tütüncü at al. 2020). It is also known to cause an increase in MC_{TC} in many conditions such as viral infections, asthma, chronic obstructive pulmonary syndrome, pulmonary hypertension, and fibrosis in the lungs (Kosanovic et al. 2015). However, although there are studies on some active substances with protective effects on the organism in the literature, we have not found any studies on the effects of S. nigra on chymase expression in the lung. Because of this, our study is the first one conducted in this field. There are studies in the literature on the effects of other active ingredients on the number of MC_{TC}s. For example, in a study evaluating the effects of thymoquinone (TQ) application method and dose on the expression of the cytokines in the rat spleen, it was observed that TQ, which has an immunomodulatory effect, did not directly affect the number of MC_{TC} (Ertuğrul et al. 2021). However, Hayıroğlu et al. (2016) emphasized that chymase expression increases in metabolic diseases such as diabetes and hormonal changes. Moreover, it was found in the study conducted by Tütüncü et al. (2020) that MC counts were closely related to polyphenolic antioxidants with anti-inflammatory effects. It was reported that there is a close relationship between increased chymase expression and resveratrol application. In this study, we found that S. nigra, an immune system stimulating product, may have positive effects on MC_{TC} in the lung. It is well known that MCs play a key role in lung pathophysiology. We think that increased MC_{TC} with S. nigra application may have a beneficial effect on possible lung disorders.

Tryptase is a neutral protease that plays an important role in allergic diseases, cytokine release, adhesion molecule expression, and smooth muscle bronchi contraction (Pejler et al. 2010). Also, tryptase is a known potent growth factor for epithelial and smooth muscle cells of the airways in lung tissue (Payne and Kam, 2004). It has been observed that the number of MC_{T}s increases in diseases acute lung injury, sepsis, pneumonia (Zhao et al. 2014). Montelukast (Çetinel et al. 2011), which is used in the treatment of asthma, and ketamine (Li et al. 2014), which can be used for pain relief and sedation, have been shown in studies to cause an increase in the number of MC_{T}s. Furthermore, resveratrol, an active element in the structure of many plants that has an antibacterial effect and can be utilized against infections, has been shown to increase MC_{T}s (Tütüncü et al. 2020). Additionally, it was demonstrated that capsaicin increased the density of tryptase-positive cells. (Tutuncu and Ertuğrul, 2019). Immunostaining against tryptase found in the granules of MCs is one of the most effective methods for identifying MCs. Parallel to the above studies, it has been demonstrated that MC_{T}s behavior can vary according to different active substances. In this study, it was observed that S. nigra, effectiveness was investigated recently can also affect the number of MC_{T}s. It is known that MCs are multifunctional

effector cells involved in host defense. Based on the study's findings, it can be said that S. nigra may be effective in protecting the lung tissue indirectly by increasing the number of MCTs.

Ghrelin regulates many cellular functions and physiological processes, including apoptosis, vascular permeability, and both innate and adaptive immunity. It also contributes to the healing of various lung diseases such as pulmonary edema, emphysema, cystic fibrosis, and pneumonia (Chen et al. 2008, Schwenke et al. 2008). Ghrelin is known to regulate the expression of inflammatory cytokines and plays an important role in immune cell function (Xia et al. 2004). Ghrelin-producing cells are found in various tissues such as the hypothalamus, pituitary, stomach, heart, lung, pancreas, intestine, kidney, testes, and ovaries (Gnanapavan et al. 2002). Volante et al. (2002) observed by immunochemistry using a specific antibody that ghrelin-producing cells have a polygonal or elongated shape and are found in small clusters in fetal, infant, and adult human lungs. It has also been shown that ghrelin-positive cells in the lung are found mainly around the bronchi, in the bronchiolar wall, and in the alveolar septa (Ivanova et al. 2021). Stefanov et al. (2017) reported that in double immunofluorescence staining, both ghrelin and tryptase are expressed by the same MCs in rat stomachs. Additionally, it was demonstrated that ghrelin-positive cells have a similar morphology and location to MCTs. Moreover, co-localization with tryptase immunoreactivity in serial sections from the porcine bile duct suggested that most ghrelin-positive cells might also be MCTs (Stefanov, 2021). Stefanov et al. (2021) used immunohistochemistry to investigate the distribution of ghrelin cells and MCTs in the domestic pig and found that the numbers of ghrelin and tryptase immunoreactive positive cells both varied in parallel to the tunicas of the common hepatic duct. Furthermore, it was shown that the percentages of ghrelin- and tryptase-positive cells increased in parallel with age in the interalveolar septa of the rat lung (Ivanova and Stefanov, 2021). Our data showed that S. nigra increased ghrelin immunopositive cell expression. In addition, an increase in tryptase immunopositive cells was also observed in our study. Also, we found that ghrelin- and tryptase-positive cells were morphologically similar to each other. This approach does not allow for precise identification but considering in our study, ghrelin, tryptase positive cells, which increased in density with S. nigra application, and previous studies, it might be concluded that MCs can produce and secrete ghrelin.

CONCLUSION

This study showed that S. nigra, which has an immunomodulatory and antioxidant effect, increases the expression of chymase-, tryptase- and ghrelin-positive cells in lung. Also, it has been tried to explain that MCs can produce, store and release ghrelin like other immune cells. In conclusion, we think that the findings we obtained in this study will contribute to the literature on the potential role of S. nigra, the MC, and ghrelin in the respiratory and immune systems.

Conflict of Interest: The authors declare that there is no conflict of interest for this article and no financial support has been received.

Ethics Committee Information: The tissue samples used in our study were obtained from the project named "Sambucus Nigranın diyabetli rat dalağında mast hücre ve Vasküler Endotelial Büyüme faktörü (VEGF) üzerine etkilerinin histokimyasal ve immunohistokimyasal olarak incelenmesi" which is approved by the Animal Ethics Committee of Ondokuz Mayıs University (Decision no: 11.03.2020, number 15).

Financial Support: --

Acknowledgment: --

Description: --

Authors Contribution Rate: TE:%65, GS%35

REFERENCES

Akalu Y, Molla MD, Dessie G, Ayelign B. Physiological effect of ghrelin on body systems. Int J Endocrinol. 2020; 25:1385138. doi: 10.1155/2020/1385138.

Baatar D, Patel K, Taub DD. The effects of ghrelin on inflammation and the immune system. Mol Cell Endocrinol. 2011; 340(1): 44-58. doi: 10.1016/j.mce.2011.04.019.

Bidian C, Mitrea DR, Tatomir C, Perdeschrepler M, Lazăr C, Chetan I, Bolfa P, David L, Clichici S, Filip GA, Mureşan M, Micle O. Vıtıs Vınıfera L. And Sambucus Nıgra L. extracts attenuate oxıdatıve stress and ınflammatıon ın femoral ıschemıa. Farmacia. 2021; 69:1. **doi.org/10.31925/farmacia.2021.1.8.**

Chen J, Liu XJ, Shuo QL, Li SQ, Luo FM. Ghrelin attenuates lipopolysaccharide-induced acute lung injury through no pathway. Med Sci Monitor. 2008; 14(7): 141-146.

Chowen JA, Argente J. Ghrelin: A Link Between Energy Homeostasis and the Immune System. Endocrinology. 2017; 158(7): 2077-2081. doi: 10.1210/en.2017-00350.

Çetinel S, Çanıllıoğlu YE, Çikler E, Sener G, Ercan F. Leukotriene D4 receptor antagonist montelukast alleviates protamine sulphate-induced changes in rat urinary bladder. BJU Int. 2011; 107(8): 1320-1325. doi: 10.1111/j.1464-410X.2010.09532.x.

Da Silva EZM, Jamur MC, Oliver C. Mast cell function: a new vision of an old cell. J Histochem Cytochem. 2014; 62(10): 698-738. doi: 10.1369/0022155414545334.

Dell'Italia LJ, Husain A. Dissecting the role of chymase in angiotensin II formation and heart and blood vessel diseases. Curr Opin Cardiol. 2002; 17(4): 374-379. doi: 10.1097/00001573-200207000-00009.

Dwyer DF, Barrett NA, Austen KF. Expression profiling of constitutive mast cells reveals a unique identity within the immune system. Nat Immunol. 2016; 17(7):878-887. doi: 10.1038/ni.3445.

Duymuş HG, Göger F, Başer KHC. In vitro antioxidant properties and anthocyanin compositions of elderberry extracts. Food Chem. 2014; 155: 112-119. **doi: 10.1016/j.foodchem.2014.01.028.**

Ertuğrul T, Tutuncu Ş, Ozdemır B, Delıce N. Possible effect of thymoquinone on mast cell number and chymase, IL-4 and IFN-γ expression in rat spleen. Med Weter 2021; 77(10): 484-490. **doi.org/10.21521/mw.6580.**

Fazio A, Plastina P, Meijerink J, Witkamp RF, Gabriele B. Comparative analyses of seeds of wild fruits of Rubus and Sambucus species from southern Italy: fatty acid composition of the oil, total phenolic content, antioxidant and anti-inflammatory properties of the methanolic extracts. Food Chem. 2013; 140(4): 817-824. doi: 10.1016/j.foodchem.2012.11.010.

Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J Clin Endocrinol Metab. 2002; 87(6): 2988. doi: 10.1210/jcem.87.6.8739.

Hamada H, Terai M, Kimura H, Hirano K, Oana S, Niimi H. Increased expression of mast cell chymase in the lungs of patients with congenital heart disease associated with early pulmonary vascular disease. Am J Respir Crit Care Med. 1999; 160(4): 1303-1308. doi: 10.1164/ajrccm.160.4.9810058.

Hawkins J, Baker C, Cherry L, Dunne E. Black elderberry (Sambucus nigra) supplementation effectively treats upper respiratory symptoms: a metaanalysis of randomized, controlled clinical trials. Complement Ther Med. 2019; 42:3 61-65. doi.org/10.1016/j.ctim.2018.12.004.

Hayıroğlu AE, Karaca T, Demirtaş S. Streptozotosin ile deneysel diyabet oluşturulan sıçanlarda östrus siklusunun değişik evrelerinde ovaryum ve uterus dokularında mast hücrelerinin dağılımının histokimyasal ve immünohistokimyasal olarak incelenmesi. Kafkas J Med Sci. 2016; 6(1):29–37. doi: 10.5505/kjms.2016.30074.

Hirata K, Sugama Y, Ikura Y, Ohsawa M, Inoue Y, Yamamoto S, Kitaichi M, Ueda M. Enhanced mast cell chymase expression in human idiopathic interstitial pneumonia. Int J Mol Med. 2007; 19(4): 565-570.

Ivanova K, Stefanov I, Ivanova I, Ananiev J, Gulubova M. Ghrelın expressıon in mast cells of infant lung with respıratory distress syndrome. Acta Med. Bulg. 2021; 48: 40-45. doi.org/10.2478/amb-2021-0006.

Ivanova IG, Stefanov IS. Tryptase- and ghrelin positive mast cells in the interalveolar septa of rat's lung. Bulg J Vet Med. 2021; 24(4): 469-477.

Kojima M, Kangawa K. Ghrelin: structure and function. Physiol Rev. 2005; 85(2): 495-522. doi: 10.1152/physrev.00012.2004.

Kosanovic D, Luitel H, Dahal BK, Cornitescu T, Janssen W, Danser AH, Garrelds IM, De Mey JGR, Fazzi G, Schiffers P, Iglarz M, Fischli W, Ghofrani HA, Weissmann N, Grimminger F, Seeger W, Reiss I, Schermuly RT. Chymase: a multifunctional player in pulmonary hypertension associated with lung fibrosis. Eur Respir J. 2015; 46(4): 1084-1094. doi: 10.1183/09031936.00018215.

Li M, Yang K, Wang X, Xu X, Zhu L, Wang H. Mast cells infiltration and decreased E-cadherin expression in ketamine-induced cystitis. Toxicol Rep. 2014; 8(2): 205-209. doi: 10.1016/j.toxrep.2014.12.003.

Metcalfe DD, Baram D, Mekori YA. Mast cells. Physiol Rev. 1997; 77(4):1033-1079. doi: 10.1152/physrev.1997.77.4.1033.

Miyazaki M, Takai S, Jin D, Muramatsu M. Pathological roles of angiotensin II produced by mast cell chymase and the effects of chymase inhibition in animal models. Pharmacol Ther. 2006; 112(3):668-676. doi: 10.1016/j.pharmthera.2006.05.008.

Mitani Y, Ueda M, Maruyama K, Shimpo H, Kojima A, Matsumura M, Aoki K, Sakurai M. Mast cell chymase in pulmonary hypertension Thorax. 1999; 54(1): 88-90. doi: 10.1136/thx.54.1.88.

Mukai K, Tsai M, Saito H, Galli SJ. Mast cells as sources of cytokines, chemokines, and growth factors. Immunol Rev. 2018; 282(1): 121-150. doi: 10.1111/imr.12634.

Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A rolefor ghrelin in the central regulation of feeding. Nature. 2001**; 409(6817):194-8. doi: 10.1038/35051587.**

Narin N, Çetin E. Effect of ghrelin administration on some hematological parameters in rats. J Health Sci. 2010; 19(3): 202-208.

Oniszczuk A, Olech M, Oniszczuk T, Wojtunik-Kulesza K, Wójtowicz A. Extraction methods, LC-ESI-MS/MS analysis of phenolic compounds and antiradical properties of functional food enriched with elderberry flowers or fruits. Arabian Journal of Chemistry. 2016; 12(8): 4719-4730. doi.org/10.1016/j.arabjc.2016.09.003.

Payne V, Kam PC. Mast cell tryptase: a review of its physiology and clinical significance. Anaesthesia 2004; 59(7): 695-703. doi: 10.1111/j.1365-2044.2004.03757.x.

Pejler G, Ronnberg E, Waern I, Wernersson S. Mast cell proteases: multifaceted regulators of inflammatory disease. Blood. 2010; 115(24): 4981-4990. doi: 10.1182/blood-2010-01-257287.

Raghay K, Akki R, Bensaid D, Errami M. Ghrelin as an anti-inflammatory and protective agent in ischemia/ reperfusion injury. Peptides. 2020; 124:170226. doi: 10.1016/j.peptides.2019.170226.

Sakaguchi M, Takai S, Jin D, Okamoto Y, Muramatsu M, Kim S, Miyazaki M. A specific chymase inhibitor, NK3201, suppresses bleomycin-induced pulmonary fibrosis in hamsters. Eur J Pharmacol. 2004; 493(1-3): 173-176. doi: 10.1016/j.ejphar.2004.04.024.

Samrao D, Wang D, Ough F, Lin YG, Liu S, Menesses T, Yessaian A, Turner N, Pejovic T, Mhawech-Fauceglia P. Histologic parameters predictive of disease outcome in women with advanced stage ovarian carcinoma treated with neoadjuvant chemotherapy. Transl Oncol. 2012; 5(6): 469-474. doi: 10.1593/tlo.12265.

Schwartz LB, Lewis RA, Seldin D, Austen KF. Acid hydrolases and tryptase from secretory granules of dispersed human lung mast cells. J Immunol. 1981; 126(4): 1290-1294.

Schwenke DO, Tokudome T, Shirai M, Hosoda H, Horio T, Kishimoto, Kangawa K. Exogenous ghrelin attenuates the progression of chronic hypoxia-induced pulmonary hypertension in conscious rats. Endocrinology. 2008; 149(1): 237-44. doi: 10.1210/en.2007-0833.

Sonneck K, Florian S, Böhm A, Krauth MT, Kondo R, Hauswirth AW, Gleixner KV, Aichberger KJ, Derdak S, Pickl WF, Sperr WR, Schwartz LB, Valent P. Evaluation of biologic activity of tryptase secreted from blast cells in acute myeloid leukemia. Leuk Lymphoma. 2006; 47(5): 897-906. doi: 10.1080/10428190500513652.

Stefanov IS, Ananiev JR, Ivanovab KV, Tolekovac AN, Vodenicharovd AP, Gulubova MV. Distribution of ghrelin-positive mast cells in rat stomach. Biotechnol Biotechnol Equip. 2017; 31(4): 774–781. doi.org/10.1080/13102818.2017.1326013.

Stefanov I. Mast cell distribution in porcine common bile duct with special reference to ghrelin. Bulg J Vet Med. 2021; (online first). doi: 10.15547/bjvm.2020-0127.

Stefanov I, Vodenicharov A, Atanassova P, Hrischev P, Vulkova I, Stoyanov D, Tsandev N, Hristov HA. Mast cell density in domestic swine common hepatic duct. Bulg. J. Vet. Med. 2021; (online first). doi: 10.15547/bjvm.2020-0145.

Tomimori Y, Muto T, Saito K, Tanaka T, Maruoka H, Sumida M, Fukami H, Fukuda Y. Involvement of mast cell chymase in bleomycin-induced pulmonary fibrosis in mice. Eur J Pharmacol. 2003; 478(2-3): 179-185. doi: 10.1016/j.ejphar.2003.08.050.

True LD. Principles of immunohistochemistry, Gower Medical Publishing, New York. 1990; p. 67-75.

Tutuncu S, Ertuğrul T. Immunohistochemical expression of tryptase-chymase and mast cell heterogeneity in capsaicin-treated rat ovaries. IJVAR. 2019; 2(2): 25-31.

Tütüncü Ş, Torun AÇ, Ertuğrul T. The role of resveratrol on mast cell and chymase and tryptase expression in blunt-chesttrauma-induced acute lung injury in rats. Turk J Vet Anim Sci. 2020; 44: 1260-1268. doi:10.3906/vet-2005-23.

Xia Q, Pang W, Pan H, Zheng Y, Kang JS, Zhu SG. Effects of ghrelin on theproliferation and secretion of splenic T lymphocytes in mice. Regul Pept. 2004; 122(3):173-178. doi: 10.1016/j.regpep.2004.06.016.

Uranga JA, Martínez V, Abalo R. Mast cell regulation and ırritable bowel syndrome: effects of food components with potential nutraceutical use. Molecules. 2020; 25(18): 4314. doi: 10.3390/molecules25184314.

Van der Velden J, Barker D, Barcham G, Koumoundouros E, Snibson K. Increased mast cell density and airway responses to allergic and non-allergic stimuli in a sheep model of chronic asthma. PLoS One. 2012; 7(5): e37161. doi: 10.1371/journal.pone.0037161.

Volante M, Fulcheri E, Allìa E, Cerrato M, Pucci A, Papotti M. Ghrelin expression in fetal, infant, and adult human lung. J Histochem Cytochem. 2002; 50(8): 1013-1021. doi: 10.1177/002215540205000803.

Wieland LS, Piechotta V, Feinberg T, Ludeman E, Hutton B, Kanji S, Seely D, Garritty C. Elderberry for prevention and treatment of viral respiratory illnesses: a systematic review. BMC Complement Med Ther. 2021; 21(1): 112. doi: 10.1186/s12906-021-03283-5.

Zhao W, Gan X, Su G, Wanling G, Li S, Hei Z, Yang C, Wang H. The interaction between oxidative stress and mast cell activation plays a role in acute lung injuries induced by intestinal ischemia-reperfusion. J Surg Res. 2014; 187 (2): 542-552. doi.org/10.1016/j.jss.2013.10.033.

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022) 15(2):217-222

DOI: 10.30607/kvj. 1081105

RESEARCH ARTICLE

Effect of Chondrogenic Differentiation Medium Supplemented with BMP-9 and TGF-ß3 on Hypertrophy in Transwell Co-Culture

Elif Ece AKGÜN[1]*, Eda DEMİRTAŞ[2], Özlem ÖZDEN AKKAYA[1], Metin ERDOĞAN[2], Korhan ALTUNBAŞ[1]

[1]*Afyon Kocatepe University, Veterinary Faculty, Histology and Embryology Department, 03200, Merkez, Afyonkarahisar, Turkey*
[2]*Afyon Kocatepe University, Veterinary Faculty, Genetics Department, 03200, Merkez, Afyonkarahisar, Turkey*

ABSTRACT

Mesenchymal stem cells are widely used in the treatment of many diseases, including osteoarthritis, due to their ability to differentiate into cartilage. The high chondrogenic differentiation potential of synovial fluid-derived mesenchymal stem cells increases the importance of these cells in osteoarthritis treatments. Addition of BMP-9 and TGF-ß3 into chondrogenic differentiation medium, increases chondrogenic differentiation and they also cause hypertrophic effects on chondrocytes. In our study, it was aimed to demonstrate the effects of BMP-9 and TGF-ß3 on cell hypertrophy by adding them into the chondrogenic basal medium during in vitro chondrogenic differentiation. In the study, stem cells in passage 5 and chondrocytes in passage 1 were cultured in a transwell co-culture system and six experimental groups were formed. Cell hypertrophy was demonstrated by examining MMP-13 and RUNX -2 gene expressions, in stem cells where chondrogenesis were induced in transwell co-culture. Although the addition of BMP-9 and TGF-ß3 to the chondrogenic medium increased hypertrophic gene expressions in experimental groups compared to control, the results were not statistically significant. The addition of BMP-9 and TGF -ß3, separately or in combination, during the chondrogenic differentiation of stem cells does not cause significant chondrocyte hypertrophy.

Keywords: Cartilage, Chondrogenesis, Hypertrophy, Synovial Fluid, Transwell Co-culture

***

BMP-9 ve TGF-ß3 Eklenmiş Kondrojenik Farklılaşma Medyumunun Transwell Ko-kültürde Hipertrofi Üzerine Etkisi

ÖZ

Mezenkimal kök hücreler, kıkırdağa farklılaşma yetenekleri nedeniyle osteoartrit dahil birçok hastalığın tedavisinde yaygın olarak kullanılmaktadır. Sinoviyal sıvı kökenli mezenkimal kök hücrelerinin kondrojenik farklılaşma potansiyellerinin fazla olması bu hücrelerin osteoartrit tedavilerindeki önemini artırmaktadır. Kondrojenik farklılaşma medyumuna ilave edilen BMP-9 ve TGF-ß3 büyüme faktörleri kondrojenik farklılaşmayı artırır ve aynı zamanda kondrositlerde hipertofik etkilere sebep olur. Çalışmamızda in vitro kondrojenik farklılaşma esnasında kondrojenik bazal medyuma ilave edilen BMP-9 ve TGF-ß3'ün hücre hipertrofisi üzerine etkilerinin gösterilmesi amaçlandı. Çalışmada transwell ko-kültür sisteminde 5. pasajdaki kök hücreler ve pasaj 1'deki kondrositler birlikte kültüre edildi ve altı deney grubu oluşturuldu. Hücre hipertrofisi, transwell ko-kültüründe kondrogenezin indüklendiği kök hücrelerde MMP-13 ve RUNX-2 gen ekspresyonları incelenerek gösterildi. BMP-9 ve TGF-ß3'ün kondrojenik ortama eklenmesi, kontrol grubuna göre deney gruplarında hipertrofik gen ekspresyonlarını artırmasına rağmen bu artış istatistiksel olarak anlamlı değildi. Kök hücrelerin kondrojenik farklılaşması esnasında BMP-9 ve TGF-ß3'ün ayrı ayrı veya birlikte kullanılması önemli derecede kıkırdak hücresi hipertrofisine neden olmamaktadır.

Anahtar Kelimeler: Hipertrofi, Kıkırdak, Kondrogenezis, Sinoviyal Sıvı, Transwell Ko-kültür

To cite this article: Akgün E.E. Demirtaş E. Özden Akkaya Ö. Erdoğan M. Altunbaş K. Effect of Chondrogenic Differentiation Medium Supplemented with BMP-9 and TGF-ß3 on Hypertrophy in Transwell Co-Culture. Kocatepe Vet J. (2022) 15(2):217-222

Submission: 01.03.2022 **Accepted**: 27.05.2022 **Published Online**: 31.05.2022

ORCID ID; *EEA: 0000-0002-2982-0307, ED: 0000-0002-3061-009, ÖÖA: 0000-0001-6372-9155, ME: 0000-0003-0975-1989, KA: 0000-0001-7580-3412*
*Corresponding author e-mail: eakgun@aku.edu.tr

INTRODUCTION

Synovial fluid mesenchymal stem cells (SFMSCs) have gained importance due to their potential for treatment of osteoarthritis in recent years (Sekiya et al. 2021). It is known that SFMSCs have good therapeutic potential due to their immunosupressive functions (Lee et al. 2015). Besides, they have ability to differentiate into adipocytes, osteocytes and chondrocytes (Jones et al. 2008). Regarding chondrogenic potential of mesenchymal stem cells, it has been suggested that SFMSCs are a better choice compared to other stem cell sources such as bone marrow and adipose tissue (Jones et al. 2008, Murata et al. 2014). Chondrogenesis has been triggered by means of transforming growth factor beta (TGF-ß) family proteins including bone morphogenetic proteins (BMPs) (Kurth et al. 2007, Scarfì 2016). It has been reported that TGF-ß2 and TGF-ß3 have better potential to form cartilage than TGF-ß1 (Barry et al. 2001). TGF-ß3 with dexamethasone on chondrogenic medium enhances aggrecan, type II collagen and cartilage oligomeric matrix protein (COMP) gene expressions on chondrogenesis. (Derfoul et al. 2006). Moreover, TGF-ß3 blocks the terminal differentiation and triggers the initial stages of chondrogenesis (Van der Kraan et al. 2009). Kurth et al. (2007) also demonstrated that this growth factor enhances extracellular matrix production of cartilage. However, Kubosch et al. (2016) showed that TGF-ß3 added culture medium also increases hypertrophy in transwell system which includes synovial mesenchymal stem cells and chondrocytes.

BMP-9 has also higher chondrogenic potential compared to BMP-6 and BMP-2 (Cheng et al. 2016). Nonetheless, BMP-9 not only stimulates chondrogenic differentiation but also causes hypertrophy (Blunk et al. 2003). While TGF-ß activates Smad 2/3 pathway, BMP activates Smad 1/5/8 (Massagué and Wotton 2000). BMPs can induce Runt Related Transcription Factor (RUNX)-2 activity, which is a transcription factor of osteogenesis, via that pathway (Tang et al. 2009). Matrix Metalloproteinase-13 (MMP- 13) is considered as a hypertrophy marker since it is related with calcification (D'angelo et al. 2000). Besides, it was showed that in osteoarthritis (OA) cartilage, upregulation of RUNX-2 gene can increase expression of MMP-13 (Wang et al. 2004). In transwell co-culture, where there is no cell-cell contact, it is possible to observe the cellular changes mediated by the paracrine secretions among the cells, which provides an advantage over other culture systems (Renaud and Martinoli 2016). Moreover, it mimics in vivo cartilage environment when chondrocytes and tissues cultured in it (Aung et al. 2011). It was shown that bone marrow mesenchymal stem cells and chondrocytes which were cultured in transwell system with chondrogenic differentiation medium (containing 10 ng/ml TGF-ß3), decreased Collagen X gene expression which is considered as a hypertrophy marker (Ahmed et al. 2007). However, it is unknown whether addition of TGF-ß3 with BMP-9 or BMP -9 alone to differentiation medium in transwell co-culture induce hypertrophy in the cells during chondrogenesis.

In this study, we established a transwell co-culture system which include SFMSCs and bovine chondrocytes (BCs). We cultured SFMSCs and BCs using chondrogenic differentiation medium supplemented with BMP- 9 and TGF-ß3 in transwell co-culture and detected hypertrophic markers of MMP-13 and RUNX-2.

MATERIALS AND METHODS

Cell Culture of SFMSCs

All procedures were approved by the Ethical Committee of Afyon Kocatepe University, Turkey (AKÜHADYEK-06-22; 27.01.2022).

Bovine joints were brought from the slaughterhouse (n=6) for collecting synovial fluid and articular cartilage. For SFMSCs isolation, approximately 3-5 mL of synovial fluid was collected from the metatarsophalangeal joint. High glucose (hg)-DMEM (Sigma-Aldrich, USA) with 10% fetal bovine serum (FBS) (Biowest, South America) was diluted with synovial fluid at a ratio of 1:2 in a 15 mL centrifuge tube. It was centrifuged at 300 g for 10 min. After discarding the supernatant, the cell pellet was suspended with DMEM-hg (Sigma-Aldrich, USA) containing 10% FBS (Biowest, South America), 1% L- Glutamin (Gibco, UK), 1% penicillin - streptomycin (Gibco, UK), 0.1% amphotericin B (Biochrom, Germany) and cultured in an incubator with 5% CO_2 at 37 ℃ in a 25 cm^2 culture flask. After reaching 70-80% confluence, the cells were passaged. In the 4th passage, the cells were seeded in 6-well plates for transwell co-culture.

Cell Culture of BCs

BCs were obtained from the metatarsophalangeal joint of bovine (n=6). The cartilage was cut with a scalpel blade and put into phosphate buffer saline with 1% pen-streptomycin. For enzymatic tissue digestion, minced cartilage pieces incubated in hg-DMEM containing 0.3% collagenase type II (Sigma,USA) for 25 minutes and then in hg-DMEM containing 0.06 % collagenase type II for 24 hours at 37℃. On the following day, chondrocytes were filtered through a 70 µm cell strainer and centrifuged at 400 g for 10 min (Bernstein et al 2009). Subsequently, cell pellet was suspended with hg-DMEM containing 10% FBS, 0.1% amphotericin B, 1% penicillin -streptomycin and seeded into 25cm^2 flasks. As soon as reaching 70-80 % confluence, the cells were used for transwell co-culture.

Use of BMP-9 and TGF-ß3 with Transwell Co-culture

For chondrogenic induction, 6 groups were planned and shown in table 1. While SFMSCs were used at passage (P) 4, chondrocytes were used at P1. SFMSCs were seeded as 15.000 cells per cm^2 into the surface of 6-well plate then chondrocytes were placed as 15.000 cells per cm^2 at the top the wells using 0.4 μm transwell inserts (Millipore, USA). As SFMSCs basal medium, hg-DMEM with 10% FBS, 1% penicillin - streptomycin and 0.1% amphotericin B was used. For basal chondrogenic medium, hg-DMEM consisting of 5% FBS, 0.1 mM dexamethasone (Sigma, Belgium), 50 mM L-ascorbic acid (Dr. Ehrenstorfer GMbH, Germany), 1% ITS-Premix (Gibco, USA), 1 mM sodium pyruvate (Lonza, Belgium), 0.35 mM proline, 1% non -essential amino acid (Lonza, Belgium) and 1% penicillin-streptomycin (Gibco, UK) was prepared. Chondrogenic differentiation was performed for 21 days according to groups given in table 1. Negative control group (1st group) and 3rd group were cultured with SFMSCs basal medium and other groups were cultured with basal chondrogenic medium supplemented with TGF-ß3, BMP-9 or both. Medium was changed every 3 days. Chondrogenic differentiation protocol was modified from the method of Mackay et al (1998).

Real Time PCR

After culturing BCs together with SFMSCs in the chondrogenic differentiation medium for 21 days in transwell coculture, the cells were collected and stored at -80℃ for real time PCR analysis. A commercial RNA isolation kit (TRIzol Reagent, Thermo, USA) was used for extracting total RNA by following the instructions of the manufacturer and the amount of total RNA was measured with nanodrop. Afterwards, cDNA synthesis was performed according to the kit's instructions (A.B.T., Turkey). 2x qPCR SYBR Green Master Mix kit (A.B.T., Turkey) was used for Real-Time PCR analysis. RUNX-2 and MMP-13 were demonstrated as PCR markers, and GAPDH was included as the housekeeping gene (Table 2).

Statistics

$2^{-\Delta\Delta ct}$ method was used for analysis of real time PCR results and statistics were performed with SPSS 22 software. Shapiro-Wilk univariate normality test was used in order to evaluate the normality of the data. Due to the absence of normal distribution, a non parametric testing was performed using Kruskal-Wallis tests. Data were presented as mean and standard error values in table 3. Kruskal-Wallis test was used to perform multiple comparisons between the groups. Groups were compared with first group which is negative control (Table 3). Statistical significance was determined as $p \leq 0.05$.

Table 1. Experimental Groups

Groups
1st Group: SFMSCs+ SFMSC Basal Medium
2nd Group: SFMSCs+ Chondrogenic Basal Medium with 10ng/ml TGF-ß3
3rd Group: SFMSCs+ BCs+ SFMSC Basal Medium in Transwell System
4th Group: SFMSCs+ BCs+ Chondrogenic Basal Medium with 10ng/ml TGF-ß3 Transwell System
5th Group: SFMSC+ BCs+ Chondrogenic Basal Medium with 10ng/ml BMP-9 in Transwell System
6th Group: SFMSC+ BCs+ Chondrogenic Basal Medium with 10ng/ml BMP-9 and 10ng/ml TGF-ß3 in Transwell System

Table 2. Real Time PCR Primers

Genes	Forward	Reverse	Tm
RUNX-2	CCGGCAGTCGGCTTCRTCGA	AGGGTGGAAATGAGGGGCGA	64 °C
MMP-13	GACCCAGGAGCACTCATGTT	GGTCTTCATCTCCTGGACCATA	64 °C
GAPDH	TGGGCAAGGTCATCCCTGAGC	TCCACAACAGACACGTTGGGA	64 °C

Table 3. Demonstration of MMP-13 and RUNX2 gene expressions. **MMP-13:** Matrix Metalloproteinase-13, **RUNX-2:** Runt Related Transcription Factor-2 **n:** sample size, **x:** mean, **Sx:** standard error, **p:** significance, **ns:** not significant

Groups	n	x ± Sx
1st Group RUNX-2	6	0.91±0.28
2nd Group RUNX-2	6	2.10±1.48
3rd Group RUNX-2	6	2.27±1.40
4th Group RUNX-2	6	3.11±0.84
5th Group RUNX-2	6	2.25±0.40
6th Group RUNX-2	6	4.22±1.21
P		**NS**
1st Group MMP-13	6	1.86±0.67
2nd Group MMP-13	6	2.10±0.67
3rd Group MMP-13	6	3.40±2.62
4th Group MMP-13	6	3.55±0.80
5th Group MMP-13	6	2.74±1.00
6th Group MMP-13	6	3.70±1.05
P		**NS**

RESULTS AND DISCUSSION

It is known that co -culture system is superior for chondrogenesis than monolayer culture (Xu et al. 2018). Kubosch et al. 2016 indicated that paracrine effects of chondrocytes increased chondrogenic induction of synovial membrane derived stem cells in transwell culture. In this study, we also tried to establish an in vitro transwell co -culture system by combining BCs and SFMSCs to better mimic the environment of natural articular cartilage.

In the beginning of chondrogenic differentiation of the stem cells, Smad 2/3 and Smad 1/5/8 phosphorylation are necessary. Whereas TGF-ß activates Smad 2/3 phosphorylation and blocks terminal differentiation, BMP activates Smad 1/5/8 phosphorylation and triggers chondrogenic hypertrophy (López-Ruiz et al. 2018). TGF-ß3 and BMP-9 are added into condrogenic differentiation medium to differentiate stem cells into chondrocytes TGF- ß3 at a concentration of 10ng/ml has been widely used for chondrogenesis in both co-culture systems (Qing et al. 2011, Wu et al. 2012) and monolayer culture (Sekiya et al. 2012). Kubosch et al. 2016) showed that transwell culture which includes chondrocytes and synovial mesenchymal stem cells treated with 10 ng/ml TGF-ß3 into culture medium increased Collagen X(COL X) expression which is a hypertrophy gene whereas Ahmed et al. (2007) indicated that bone marrow mesenchymal stem cells and chondrocytes which were cultured in same culture system with same amount of TGF -ß3 decreased COL X gene expression. In our study, we also observed that compared to negative group, TGB-ß3 upregulated both RUNX2 (2.10±1.48) and MMP-13 (2.10±0.67) expressions. Besides, compared to group 2, in transwell co-culture of synovial fluid derived stem cells with chondrocytes, TGB-ß3 also increased RUNX2 (3.11±0.84) and MMP-13 (3.55±0.80) expressions more in group 4. In other words, the paracrine effect of chondrocytes increased the chondrogenic hypertrophy of SFMSCs in transwell culture. But the statistically significance was not seen between these groups (table 3).

BMP-9 has high chondrogenic potential on alginate and pellet culture when used at a concentration of 100 ng/ml (Majumdar et al. 2001, Morgan et al. 2020). However, it has been showed that 10 ng/mL BMP-9 possess higher chondrogenic potential than 100ng/ml BMP-9 on C3H10T1/2 mesenchymal stem cells in micro mass culture (Cheng et al. 2016). Also, the hypertrophic potential of BMP- 9 on chondrogenesis has been lesser investigated. In the study of Morgan et al. (2020), it was revealed that the doses of 50, 100 and 200 ng/ml BMP-9 were highly effective in chondrogenic differentiation of chondroprogenitor cells but that doses also caused high hypertrophy. Also, it was determined that the doses of 6, 12.5 and 25ng/ml showed to lead neither high chondrogenic differentiation nor high hypertrophy in that study. In another study, it was showed that 25ng/ml BMP -9 had a hypertrophic effect inactivated by 0.1 ng/ml TGF-ß1 in chondrocytes.

In our study, the highest hypertrophy was observed in 6th group for both MMP-13 (3.70±1.05) and RUNX-2 (4.22±1.21) mRNA levels and the lowest was seen in the 2nd group for both MMP -13 (2.10±0.67) and RUNX-2 (2.10±1.48) compared to negative group. The 5th group which was supplemented with 10ng/ml BMP-9 was observed to have higher gene expressions rates than 2nd group for MMP-13 (2.74±1.00) and RUNX-2 (2.25±0.40) gene expressions. In addition, the 4th group which was supplemented with 10ng/ml TGF -ß3 also showed more hypertrophic condition with higher MMP-13 (3.55±0.80) and RUNX-2 (3.11±0.84) gene expressions than the 5th group. With these results, we could suggest that 10ng/ml TGF-ß3 is more effective than 10 ng/ml BMP-9 to induce hypertrophy in transwell co-culture. When we compare the 3rd group with the 4th group, we think that addition of TGF-ß3 to transwell culture may have been triggered hypertrophy as Kubosch et al. (2016) stated. Even if we see an increase in the gene expressions indicating the cell hypertrophy from data, compared with negative group the observed the increase in the experimental groups was not statistically significant ($p>0.05$) (Table 3).

CONCLUSION

In conclusion, the hypertrophic effect of these doses of BMP-9 and TGF-ß3 during chondrogenesis is limited. The fact that paracrine effects of chondrocytes with these growth factors can induce chondrogenesis with minimal hypertrophy indicates that they are potential candidates for chondrogenesis. In addition, the investigation of the possible hypertrophic effects of different doses of these growth factors at distinct time periods of cell culture during chondrogenesis will be significant for future researches.

Conflict of interest: The authors declared that there is no conflict of interest.

Authors Contribution Rate: 1EEA: %50, ED: %20, ÖÖA: %15, ME: %10, KA:%5

Ethical Approval: This study has received permission with AKU HADYEK number 06-22 and 27.01.2022. The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

REFERENCES

Ahmadi MR, Derakhshandeh A, Shirian S, Daneshbod Y, Ansari-Lari M, Nazifi S. Detection of **Ahmed N, Dreier R, Göpferich A, Grifka J, Grässel S**. Soluble signalling factors derived from differentiated cartilage tissue affect chondrogenic differentiation of rat adult marrow stromal cells. Cell Physiol Biochem. 2007;20(5):665-78.

Aung A, Gupta G, Majid G, Varghese S. Osteoarthritic chondrocyte-secreted morphogens induce chondrogenic differentiation of human mesenchymal stem cells. Arthritis Rheum. 2011 Jan;63(1):148-158.

Barry F, Boynton RE, Liu B, Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res. 2001 Aug 15;268(2):189-200.

Bernstein P, Dong M, Corbeil D, Gelinsky M, Günther KP, Fickert S. Pellet culture elicits superior chondrogenic redifferentiation than alginate-based systems. Biotechnol Prog. 2009 Jul-Aug;25(4):1146-52.

Blunk T, Sieminski AL, Appel B, Croft C, Courter DL, Chieh JJ, Goepferich A, Khurana JS, Gooch KJ. Bone morphogenetic protein 9: a potent modulator of cartilage development in vitro. Growth Factors. 2003 Jun;21(2):71-7.

Cheng A, Gustafson AR, Schaner Tooley CE, Zhang M. BMP-9 dependent pathways required for the chondrogenic differentiation of pluripotent stem cells. Differentiation. 2016 Dec;92(5):298-305.

D'Angelo M, Yan Z, Nooreyazdan M, Pacifici M, Sarment DS, Billings PC, Leboy PS. MMP-13 is induced during chondrocyte hypertrophy. J Cell Biochem. 2000 Apr;77(4):678-93.

Derfoul A, Perkins GL, Hall DJ, Tuan RS. Glucocorticoids promote chondrogenic differentiation of adult human mesenchymal stem cells by enhancing expression of cartilage extracellular matrix genes. Stem Cells. 2006 Jun;24(6):1487-95.

Jones EA, Crawford A, English A, Henshaw K, Mundy J, Corscadden D, Chapman T, Emery P, Hatton P, McGonagle D. Synovial fluid mesenchymal stem cells in health and early osteoarthritis: detection and functional evaluation at the single-cell level. Arthritis Rheum. 2008 Jun;58(6):1731-40.

Kubosch EJ, Heidt E, Bernstein A, Böttiger K, Schmal H. The trans-well coculture of human synovial mesenchymal stem cells with chondrocytes leads to self-organization, chondrogenic differentiation, and secretion of TGFβ. Stem Cell Res Ther. 2016 Apr 26;7(1):64.

Kurth T, Hedbom E, Shintani N, Sugimoto M, Chen FH, Haspl M, Martinovic S, Hunziker EB. Chondrogenic

potential of human synovial mesenchymal stem cells in alginate. Osteoarthritis Cartilage. 2007 Oct;15(10):1178-89.

Lee WJ, Hah YS, Ock SA, Lee JH, Jeon RH, Park JS, Lee SI, Rho NY, Rho GJ, Lee SL. Cell source-dependent in vivo immunosuppressive properties of mesenchymal stem cells derived from the bone marrow and synovial fluid of minipigs. Exp Cell Res. 2015 May 1;333(2):273-288.

López-Ruiz E, Jiménez G, Kwiatkowski W, Montañez E, Arrebola F, Carrillo E, Choe S, Marchal JA, Perán M. Impact of TGF-β family-related growth factors on chondrogenic differentiation of adipose-derived stem cells isolated from lipoaspirates and infrapatellar fat pads of osteoarthritic patients. Eur Cell Mater. 2018 Apr 13;35:209-224.

Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng. 1998 Winter;4(4):415-28.

Majumdar MK, Wang E, Morris EA. BMP-2 and BMP-9 promotes chondrogenic differentiation of human multipotential mesenchymal cells and overcomes the inhibitory effect of IL-1. J Cell Physiol. 2001 Dec;189(3):275-84.

Massagué J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. EMBO J. 2000 Apr 17;19(8):1745-54.

Morgan BJ, Bauza-Mayol G, Gardner OFW, Zhang Y, Levato R, Archer CW, van Weeren R, Malda J, Conlan RS, Francis LW, Khan IM. Bone morphogenetic protein-9 Is a potent chondrogenic and morphogenic factor for articular cartilage chondroprogenitors. Stem Cells Dev. 2020 Jul;29(14):882-894.

Mueller MB, Fischer M, Zellner J, Berner A, Dienstknecht T, Prantl L, Kujat R, Nerlich M, Tuan RS, Angele P. Hypertrophy in mesenchymal stem cell chondrogenesis: effect of TGF-beta isoforms and chondrogenic conditioning. Cells Tissues Organs. 2010;192(3):158-66.

Murata D, Miyakoshi D, Hatazoe T, Miura N, Tokunaga S, Fujiki M, Nakayama K, Misumi K. Multipotency of equine mesenchymal stem cells derived from synovial fluid. Vet J. 2014 Oct;202(1):53-61.

Qing C, Wei-ding C, Wei-min F. Co-culture of chondrocytes and bone marrow mesenchymal stem cells in vitro enhances the expression of cartilaginous extracellular matrix components. Braz J Med Biol Res. 2011 Apr;44(4):303-10.

Renaud, J., & Martinoli, M. G. Development of an insert co-culture system of two cellular types in the absence of cell-cell contact. Journal of visualized experiments: JoVE. 2016 (113), 10.3791/54356.

Scarfi S. Use of bone morphogenetic proteins in mesenchymal stem cell stimulation of cartilage and bone repair. World J Stem Cells. 2016 Jan 26;8(1):1-12.

Sekiya I, Katano H, Ozeki N. Characteristics of MSCs in synovial fluid and mode of action of intra-articular injections of synovial MSCs in knee osteoarthritis. Int J Mol Sci. 2021 Mar 11;22(6):2838

Sekiya I, Ojima M, Suzuki S, Yamaga M, Horie M, Koga H, Tsuji K, Miyaguchi K, Ogishima S, Tanaka H, Muneta T. Human mesenchymal stem cells in synovial fluid increase in the knee with degenerated cartilage and osteoarthritis. J Orthop Res. 2012 Jun;30(6):943-9.

Tang QO, Shakib K, Heliotis M, Tsiridis E, Mantalaris A, Ripamonti U, Tsiridis E. TGF-beta3: A potential biological therapy for enhancing chondrogenesis. Expert Opin Biol Ther. 2009 Jun;9(6):689-701.

Van Caam A, Blaney Davidson E, Garcia de Vinuesa A, van Geffen E, van den Berg W, Goumans MJ, ten Dijke P, van der Kraan P. The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFβ1. Osteoarthritis Cartilage. 2015 Jun;23(6):985-95.

Van der Kraan PM, Blaney Davidson EN, Blom A, van den Berg WB. TGF-beta signaling in chondrocyte terminal differentiation and osteoarthritis: modulation and integration of signaling pathways through receptor-Smads. Osteoarthritis Cartilage. 2009 Dec;17(12):1539-45.

Xu L, Wu Y, Liu Y, Zhou Y, Ye Z, Tan WS. Non-contact coculture reveals a comprehensive response of chondrocytes induced by mesenchymal stem cells Through Trophic Secretion. Tissue Eng Regen Med. 2017 Sep 27;15(1):37-48.

Wang X, Manner PA, Horner A, Shum L, Tuan RS, Nuckolls GH. Regulation of MMP-13 expression by RUNX2 and FGF2 in osteoarthritic cartilage. Osteoarthritis Cartilage. 2004 Dec;12(12):963-73.

Wu L, Prins HJ, Helder MN, van Blitterswijk CA, Karperien M. Trophic effects of mesenchymal stem cells in chondrocyte co-cultures are independent of culture conditions and cell sources. Tissue Eng Part A. 2012 Aug;18(15-16):1542-51.

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022) 15(2):223-232

DOI: 10.30607/kvj. 1100512

RESEARCH ARTICLE

The Efficacy of Clicker Method During Desensitising Horse

Özlem Hacan[1*], Sibel Danışan[2], Ceyhan Özbeyaz[3]

[1] Department of Animal Science, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03200, Afyonkarahisar, Turkey
[2] Horse Breeding and Coaching Programme, Department of Plant and Animal Production, Mahmudiye Horse Breeding Vocational School, Eskisehir Osmangazi University, Eskişehir, Turkey.
[3] Department of Animal Science, Faculty of Veterinary Medicine, Ankara University, 06110, Ankara, Turkey

ABSTRACT

Encouraging horses to do tasks willingly during training relating to their welfare is important. Horses are trained for desensitization using de-spooking tracks. In this study, the efficacy of using the clicker method during desensitization to obstacles and novel objects is investigated. Fourteen Arabian horses participated in the study. Their success in completing the tasks, as well as their heart rate and behaviour were examined. The average achievement for the hanging pool noodle door task was significantly higher ($P<0.05$) in the clicker group (100%) than in the control group (43%). Average heart rate is highly significant ($P<0.01$) in the clicker group (139.28 pcs/minute) than the control group (109.42 pcs/minute). In the scope of frightening behaviours, "trot" was determined highly significant ($P<0.01$) in the control group than the clicker group. Clicker training appears to provide an advantage due to its ease of application, low cost, and fast learning by horses. The findings suggest that this method is advisable because of its efficacy during desensitising of horses using the de-spooking track. Fulfilling tasks willingly during training is also important for the horse's welfare and trainer's safety.
Key Words: Behavioural training, clicker method, desensitising, horse, learning theory

***

Clicker Yönteminin Atlarda Duyarsızlaştırma Eğitimine Etkisi

ÖZ

Eğitim sırasında verilen görevi kendi istekleriyle yerine getirmeleri, atların refahı ve başarıları açısından oldukça önemlidir. Bu amaçla atların temel eğitimleri sırasında ürkekliklerinin azaltılması ve çeşitli seslere, nesnelere karşı alışmalarının sağlanması için duyarsızlaştırma eğitimlerinde bazı parkurlardan yararlanılmaktadır. Bu araştırmada atların farklı zemin üzerinde yürüme ve nesneler arasından geçme (dar alan, top, şemsiye, halka, branda, yandan engelli kapı ve üstten engelli kapı) gibi bazı görevleri yerine getirmesi sırasında clicker metodunun kullanımının atın parkurdaki başarısına etkisi araştırılmıştır. Bu amaçla 14 baş Arap kısraktan yararlanılmıştır. Atların parkurda görevleri yerine getirme başarıları, kalp atım hızları ve davranışları incelenmiştir. Üstten engelli kapı görevini başarma ortalaması, clicker uygulanan grupta (%100) kontrol grubuna (%43) göre önemli düzeyde ($P<0.05$) yüksek bulunmuştur. Ortalama kalp atım hızı clicker uygulanan grupta (139.28 adet/dk) kontrol grubuna (109.42 adet/dk) göre önemli düzeyde ($P<0.01$) yüksek olmuştur. Kontrol grubunda clicker uygulanan gruba göre; ani durma, süratli veya dörtnala kalkma davranışlarının önemli derecede ($P<0.01$) yüksek olduğu belirlenmiştir. Sonuç olarak, clicker yönteminin atlar tarafından hızla öğrenilen, uygulaması kolay ve maliyeti düşük bir yöntem olması ve aynı zamanda görevlerin başarılmasındaki etkinliği nedeniyle at eğitiminde kullanılması tavsiye edilebilir. Bununla birlikte, clicker metodu kullanılarak atın görevleri kendi isteğiyle yerine getirmesinin hem antrenörün güvenliği hem de hayvan refahı açısından önemli olduğu söylenebilir.
Anahtar Kelimeler: At, clicker metodu, davranış temelli eğitim, duyarsızlaştırma, öğrenme teorisi

To cite this article: Hacan Ö. Danışan S. Özbeyaz C. The Efficacy of Clicker Method During Desensitising Horse. Kocatepe Vet J. (2022)15(2):223-232

Submission: 08.04.2022 **Accepted**: 27.05.2022 **Published Online**: 31.05.2022
ORCID ID; *ÖH: 0000-0001-6340-1117, SD: 0000-0002-2621-4555, CÖ: 0000-0002-3748-9992*
*Corresponding author e-mail: , gucuyener@aku.edu.tr

INTRODUCTION

Today, horses used for sports are expected to perform very different movements and tasks. Horses learn these behaviors through training. At the end of a proper and effective training process, horses can successfully sustain the effective behaviors learned for many years. However, if the relationship between horse and human is formed as a result of pressure and is not based on mutual trust, the horse may not feel safe and may exhibit some instinctive behaviors such as running away, resisting and fighting. This situation may endanger the safety of horse specialists (farrier, trainer, veterinarian, rider, etc.) and can also lead the loss of horses through injury or behavioural problems. For these reasons, behavior-based training methods based on learning theory are recommended.

Learning becomes more effective with the development of techniques in the field of animal training. The training process itself becomes more efficient through the application of learning theory, which is rooted in the psychology, of animal behaviour (Breland and Breland 1951, 1966, Skinner 1938, 1951). Training processes are based on communication between the trainer and the animal.

Learning can be defined as a process of adaptive changes in individual behaviour as a result of experience (Thorpe 1963). Learning theory is non-associative learning, which includes habituation and sensitisation processes, and associative learning, which includes classical and operant conditioning processes. One of the main learning processes involved in the training of horses involves operant conditioning, also known as instrumental learning (McLean and Christensen 2017). The use of positive reinforcers within the scope of learning theory gives favourable results in horse training. Clicker training, in which positive reinforcers are used, started to be preferred in the 1990s and continues to be popular today (Kurland 2001). Various forms of clicker training have been used to teach a variety of tasks to horses (Flannery 1997, Ferguson and Rosales-Ruiz 2001, Williams et al. 2004).

Skinner's theory of operant conditioning (1938) proposes that animals learn to "operate" their world based on the consequences of their behaviours. According to this theory, behaviors followed immediately by a desirable consequence (reinforcement) become more likely to occur again, while behaviors followed immediately by an undesirable consequence (punishment) become less likely to occur. In behavior-based training, the animal is taught step by step to exhibit a certain behaviour in response to a specific stimulus. During the operant conditioning process, the trainer provides the desired response to the animal by making a "click" sound or by giving an auditory stimulus with a specific word after the hores exhibits the desired behaviour (Skinner 1951, Pryor 2005).

With the clicker method, the trainer gives the animal a reinforcement with rewarding words or food after the auditory stimulus. In this way, the animal learns which behaviour is rewarded through trial and error. In the shaping procedures, the aim is to teach complex tasks by dividing them into parts; in this way, complex actions can be simplified (Lindsay 2000). Training can become more effective in a short time with the use of the clicker method. It is reported by Pryor (1999) that the use of the clicker method in the training of animals speeds up the learning of new tasks. Pryor explains this effect of training with three mechanisms. First, the clicker acts on the initially neutral stimulus, pairing with the repeated primary reinforcer, as a conditional and secondary reinforcer. Second, the clicker acts as a marking signal by enabling the animal to distinguish a particular behaviour by reaching the primary reinforcer as a result of an event. Finally, the clicker acts as a bridge between behaviour and primary reinforcer, indicating that the primary reinforcer will come (Pryor 1999, Pryor 2005, Skinner 1938, Williams 1994). The potential of the clicker stimulus to improve animal learning may not only increase the rate of behaviour acquisition but also reduce animal frustration and further enhance the relationship between trainer and animal (Bartlett 2010, Lindsay and Wood 2007, Bornhede 2010, Ferguson and Rosales-Ruiz 2001, Danışan and Özbeyaz 2021). Today, the clicker method is successfully applied in the training of many animal species such as dogs (Lindsay and Wood 2007, D'Onofrio 2015), pigs (Paredes-Ramos et al. 2020) mice (Leidinger et al. 2017), goats (Langbein et al. 2007) and horses (Danışan and Özbeyaz 2021).

Desensitization is studied under non-associative learning. Four main Desensitization techniques can be derived from the applied animal behaviour literature: systematic Desensitization, counter-conditioning, overshadowing; and response prevention (McLean 2008, Mills et al. 2010). Systematic Desensitization technique is used in the modification of some behaviors in horses to prevent behavioural problems. Within the scope of the training, a stimulus is given to the horse, which gradually increases. The horse is rewarded for giving the desired response to the stimulus and the stimulus level is increased. With this technique, the response threshold is increased gradually (McLean and Christensen 2017). For example, police horses are often systematically desensitised to noise, smoke, flags, rapidly advancing people and objects. Also, therapy horses can be desensitised to objects, peoples behaviour patterns and some therapathic games etc.

In this study, horses were passed through the de-spooking track which was prepared for desensitization training by using the clicker method. The effect of using this method on the success of the horse on the track was investigated while it performed tasks such as passing over novel grounds

and between static novel objects (road cones, ball, umbrella, ring, tarp, and lateral and hanging pool noodle doors).

MATERIAL and METHODS

Animals

The animal material of the research consisted of 14 Arabian mares (4-24 years old) bred in the Mahmudiye district of Eskişehir province in Turkey. This study was carried out in December 2016.

Study Design

The horses (n=14) were divided into the clicker group (n=7) and the control group (n=7). Desensitization training was used twice for all horses in the clicker groups (clicker1 and clicker 2) and the control groups (control 1 and control 2). There were in seven static novel objects (road cones, ball, umbrella, ring, tarp, laterally pool noodle door, hanging pool noodle door) on the de-spooking track in the test arena. Horses passed over or between the objects.

The horses in the control group were given an audible stimulus to walk with the trainer. If they did not walk, negative reinforcement was applied via pressure to the halter. The horses in the clicker group were given an audible stimulus and used a target stick to walk with the trainer. The target stick were introduced to the horses and within three seconds after the horses touched the stick, the "click" sound was made with the help of a clicker device. A small pieces of carrot was given to the horses as a positive reinforcement.

The horses' behaviour and heart rate were recorded by a Polar Equine M400 device, which was fastened to the left side of the horse just behind the front leg with an elastic girth. Horses were recorded on video using a Go-Pro Action Camera when they do tasks.

The behavioural responses of the horses were evaluated using an equine ethogram (Table 1) within the scope of curiosity, fright and threat behaviours. The types of behaviour included were: alert, nibble, sniffing/licking (curiosity behaviours); snort, neigh, head high, balk, vigilance, trot (fright behaviours); ear laid back, paw, stomp, kick (threat behaviours). The data on the behavioural and physiological responses of the horses were obtained only in their first trial on the de-spooking track. In both groups, ethological analyses were made according to whether seven horses showed behaviors in with seven different novel objects (7x7=49 behaviour).

In statistical analysis, differences between the groups in terms of accomplishing tasks and behaviors were analysed with the Chi-square method, and differences between the groups in terms of heart rate were analysed with the t-test. SPSS 14.01 (license number: 9869364) package program was used for statistical analysis (Anonymous 2022).

RESULTS

Comparison of the success rate for the tasks on the de-spooking track between control-1, control-2, clicker-1 and clicker-2 groups are given in Table 2. In all groups, the success rate of navigating narrow spaces (with road cones), ball, umbrella and lateral pool noodle door tasks was 100%. While the success rate of the ring task was 71% in the control-1 group and the clicker-1 group, it was 100% in the control-2 group and the clicker-2 group. In the control-1, control-2, clicker-1 and clicker-2 groups, the success rate of the walking on the tarp task was determined as 43%, 71%, 71% and 100%, respectively. In the hanging pool noodle door task, the rate of accomplishing the task was found as 43%, 57%, 100% and 100% in the control-1, control-2, clicker-1 and clicker-2 group, respectively. Average heart rate was 139.28 pcs/minute in the clicker group and 109.42 pcs/minute in the control group.

Results on curiosity, fright and threat behaviors are given in Table 3. From curiosity behaviours; alert, sniffing/licking and nibble behaviors were 0%, 6%; 55%, 45% and 41%, 45% in control-1 and clicker-1 groups respectively. Sniffing/licking behaviors were not observed in both groups. From fright behaviours; snort, neigh (vocalisation), head high, balk, vigilance and trot behaviors were 24%, 14%; 2%, 0%; 16%, 16%; 35%, 8%; 12%, 8% and 35%, 8% in control-1 and clicker-1 groups respectively. From threat behaviours, the ears laid back (pinned) was 14% and 10% in control-1 and clicker-1 groups respectively. Paw was 2% in the control-1 group and stomp and kick behaviour were not observed in both groups.

Table 1. Behaviors evaluated within the scope of the research

Behaviour		Description
Curiosity Behaviour	Alert	Rigid stance with the neck elevated and the head oriented toward the object or animal of focus. The ears are held stiffly upright and forward and the nostrils may be slightly dilated. (McDonnell and Haviland 1995).
	Nibble	With jaws closed the upper lip is moved upward and downward against an object, typically without dental contact of the object. Comments: Nibbling of an object is typically one of the first play responses associated with an investigative approach of the object (McDonnell and Poulin 2002).
	Sniffing/Licking	Sniffing and/or licking an inanimate object may be as if to investigate the odor, texture, shape, taste, and size of an object. Sniffing and licking of a herd mate sometimes precedes and appears to initiate mutual grooming (Keiper 1985).
Fright Behaviour	Snort	Short explosive exhalations from nostrils (Boyd and Houpt 1994).
	Neigh (Vocalisation)	A high amplitude call of long duration that fluctuates in frequency and is given on expiration (Boyd and Houpt 1994).
	Head high	Nose above the withers (Hall et al. 2014).
	Balk	Stopping suddenly while walking (McGreevy et al. 2009).
	Vigilance	Standing still with elevated neck, intently orientated head and ears (Le Scolan et al. 1997).
	Trot	A two-beat gait (Seaman et al. 2002).
Threat Behaviour	Ears laid back	Ears pressed caudally against the head and neck (McDonnell and Haviland 1995).
	Paw	Striking a vertical or horizontal surface, or the air with a forelimb (Seaman et al. 2002).
	Stomp	One foreleg is raised and lowered, sharply and firmly striking the ground, usually repeatedly (McDonnell and Haviland 1995).
	Kick	One or both hind legs lift off the ground and rapidly extend backwards toward another stallion, with apparent intent to make contact (McDonnell and Haviland 1995).

Road cones

Umbrella

Ball

Ring

Tarp

Lateral pool noodle door

Hanging pool noodle door

Figure 1: Study Design

Table 2. Comparison of task success rates in groups

Groups	Road cones			Ball			Umbrella			Ring			Tarp			Laterally pool noodle door			Hanging pool noodle door		
	Passed	Not passed	%	Passed	Not passed	%	Passed	Not passed	%	Passed	Not passed	%	Passed	Not passed	%	Passed	Not passed	%	Passed	Not passed	%
Control 1	7	0	100	7	0	100	7	0	100	5	2	71	3	4	43	7	0	100	3	4	43
Control 2	7	0	100	7	0	100	7	0	100	7	0	100	5	2	71	7	0	100	4	3	57
P			-			-			-			-			-			-			-
Clicker 1	7	0	100	7	0	100	7	0	100	5	2	71	5	2	71	7	0	100	7	0	100
Clicker 2	7	0	100	7	0	100	7	0	100	7	0	100	7	0	100	7	0	100	7	0	100
P			-			-			-			-			-			-			-
Control 1	7	0	100	7	0	100	7	0	100	5	2	71	3	4	43	7	0	100	3	4	43
Clicker 1	7	0	100	7	0	100	7	0	100	5	2	71	5	2	71	7	0	100	7	0	100
P			-			-			-			-			-			-			*
Control 2	7	0	100	7	0	100	7	0	100	7	0	100	5	2	71	7	0	100	4	3	57
Clicker 2	7	0	100	7	0	100	7	0	100	7	0	100	7	0	100	7	0	100	7	0	100
P			-			-			-			-			-			-			-

*: Significant (P<0.05), -: Insignificant

Table 3. Comparison between behaviors in the de-spooking track (7 horses x 7 novel objects = 49 behaviors were evaluated in each group)

	Curiosity Behaviours											
Groups	**Alert**			**Sniffing**			**Touching**			**Licking-Nibbling**		
	Did	**Did not**	**%**	**Did**	**Did not**	**%**	**Did**	**Did not**	**%**	**Did**	**Did not**	**%**
Control 1	0	49	0	27	22	55	20	29	41	0	49	0
Clicker 1	3	46	6	22	27	45	22	27	45	0	49	0
P			-			-			-			-

	Fright Behaviours																	
Groups	**Snort**			**Neigh**			**Head high**			**Balk**			**Step back-sideways**			**Trot-Gallop**		
	Did	**Did not**	**%**	**Did**	**Did not**	**%**	**Did**	**Did not**	**%**	**Did**	**Did not**	**%**	**Did**	**Did not**	**%**	**Did**	**Did not**	**%**
Control 1	12	37	24	1	48	2	8	41	16	17	32	35	6	43	12	17	32	35
Clicker 1	7	42	14	0	49	0	8	41	16	4	45	8	4	45	8	4	45	8
P			-			-			-			**			-			**

	Threat Behaviours								
Groups	**Ears laid back**			**Stomp**			**Paw-Kick**		
	Did	**Did not**	**%**	**Did**	**Did not**	**%**	**Did**	**Did not**	**%**
Control 1	7	42	14	1	48	2	0	49	0
Clicker 1	5	44	10	0	49	0	0	49	0
P			-			-			-

**: Significant (P<0.01), -: Insignificant

DISCUSSION

In this study, when the findings in Table 2 are evaluated in general, it can be seen that the second trials are more successful than the first ones and the clicker groups are more successful than the control groups. In the second trial of the clicker group, it was determined that all horses were successful in all tasks. This situation shows that the horses' success rate for the tasks is higher on a known track than on a track encountered for the first time. In addition, it can be said that the clicker method increases the success rate of the horses in achieving the tasks.

Negative reinforcement, a traditional method, is widely used in horse training. However, there has been an increase in the use of positive reinforcement in recent years. In positive reinforcement studies related to the loading of horses onto a trailer; it has been reported that positive reinforcement shortens the loading time and reduces associated stress. Thus, it is effective in eliminating negative behaviors during loading (Ferguson and Rosales-Ruiz 2001, Dai et al. 2019, Hendriksena et al. 2011). Sankey et al. (2010) state that horses trained with positive reinforcement also show more positive behaviors in their subsequent relationships with people. In training studies on horses, it has been reported that positive reinforcement supports learning by motivating horses' behaviour (Hockenhulla and Creighton 2013), increases the welfare of horses (Bornhede 2010), is a safer method because it increases the welfare of horses (Slater and Dymond 2011), they have exhibit less frightened and threatening behaviors (Danışan and Özbeyaz 2021) and they are more willing to participate in training (Innes and McBride 2008). Freymond et al. (2014) also report that in horses they trained for various exercises, negative reinforcement groups were more nervous and experienced more negative emotions than with positive reinforcement. Similarly, in this study, pausing and trotting behaviors resulting from fear behaviors were found to be significantly higher ($P<0.01$) in the control group than in the clicker group. Reducing fear and threat behaviors with the use of positive reinforcement will make training easier and safer for both the rider and the horse.

The clicker method, which is applied as a secondary reinforcer in positive reinforcement, has been applied in different animal species and in the fulfillment of different tasks in recent years. Lindsay and Wood (2007) report in their study on dogs that the clicker method reduces training time and food supplements required to learn the behaviour. Similarly, D'Onofrio (2015) states in his study that the clicker is an effective method in dog training. On the other hand, Smith and Davis (2008) state in their study that there was no difference between clicker and the control groups in terms of learning the behaviour, but the clicker group forgot the learned behaviour later than others. Paredes-Ramos et al. (2020) report that the clicker method reduces the number of repetitions required to learn to bring an object among piglets and enables faster learning. Leidinger et al. (2017) states that mice trained with the clicker method learn quickly and perform all tasks successfully. Langbein et al. (2007) states that the clicker method facilitates learning in goats, with the animals being more willing to fulfill the task and performing the task in a shorter time and with fewer trials, and with the incidence of abnormal behaviour reduced. These findings show that the clicker method can be used effectively in different species and different tasks.

There are so far few clicker studies in horses. Bartlett (2010) reports that clicker training provides a basis for creating a positive partnership between the trainer and the horse to take by encouraging an active role in the learning process. Flannery (1997) states in his study that the rate of performing the tasks correctly was higher in the clicker group. Danışan and Özbeyaz (2021) who compared three different training methods in horses in their study, found that obedience behaviour and the rate of success in the task were higher and startle-threat behaviors were lower with the clicker method. McCall and Burgin (2002) and Williams et al. (2004) reported in their study on horses that the clicker method did not reduce training time compared with using only food as a primary reinforcer, and there was no difference between the groups in terms of the forgetting time of the behaviour. However, McCall and Burgin (2002) state that horses that have received secondary reinforcement in the past respond more accurately than other horses in a new training. In this study, we determined that the rate of achievement of the tasks was higher in the clicker group. In the second trial of the clicker group, it was determined that all horses were successful in all tasks. In addition, fear behaviors were found to be significantly higher in the control group than in the clicker group. These findings, suggest that the clicker method increases the welfare of horses by reducing negative emotions and increases the success rate of tasks.

Some trainers are discouraged from using food rewards in training programmes based on positive reinforcement because of concerns that hand feeding will lead to undesirable oral exploratory behaviour (Waran et al. 2002, Hart 2008). Hockenhulla and Creighton (2010) report in their study that there was no relationship between clicker training and undesirable oral behaviour of horses, and that the risk factors for these behaviors may have arisen from outside this practice. In our study, there was no finding that food-based positive reinforcement was associated with undesirable oral behaviors in horses. These findings suggest that horse owners should not be discouraged from using food-based positive reinforcement techniques with their horses.

From their training study in horses, Sankey et al. (2010) report that the mean heart rate in the negative reinforcement group was higher than the positive reinforcement group. Innes and McBride (2008) determined that the heart rate of horses that were given positive reinforcement in the later stages of the trial was significantly higher than that of horses given negative reinforcement. In the study, the increase in heart rate was associated with increased adrenaline/noradrenaline release during training in anticipation of food reward, as horses in the positive reinforcer group appeared to be highly motivated to the task. Similarly, in this study, the average heart rate was found to be significantly higher ($P<0.01$) in the clicker group (139.28 pcs/minute) than the control group (109.42 pcs/minute). This may be because of the food reward excites the horses.

CONCLUSION

It is seen that training based on learning theory is more efficient for horses. It has been observed that horses voluntarily fulfill tasks thanks to the positive reinforcements used in operant conditioning processes. This reduces the frequency of behavioural responses related to fright and threat in horses, thus minimising risks in management and training.
As a result, clicker training provides an advantage in desensitization training of horses due to its ease of application, low cost, and fast learning by horses. Research findings show that this method is advisable due to its effectiveness during the desensitization of horses on the de-spooking track.

Conflict of interest: The authors declared that there is no conflict of interest.

Ethical Approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k).
The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

Description: This study was presented as a summary paper at the 1st International Mediterranean Science and Engineering Congress (IMSEC 2016).

Authors Contribution Rate: The authors declare that they have contributed equally to the article.

REFERENCES

Anonymous. SPSS Statistical Package in Social Sciences for Windows. Statistical Innovations Inc. Chicago, USA, 2022.

Bartlett K. Clicker Basics: What is equine clicker training and how do you apply it to horses? http://www.equineclickertraining.com/articles/plus_r_training_page_new.html, 2009.

Bornhede M. Training techniques used in training horses (Equus Caballus) - with a focus on positive reinforcement. Swedish University of Agricultural Sciences, Department of Animal Environment and Health Ethology and Animal Welfare programme, Student report 329, Skara, 2010.

Boyd L, Houpt KA. Prezwalski's Horse- The history and biology of an endangered species. State University of New York Press, New York, 1994.

Breland K, Breland M. A field of applied animal psychology. American Psychologist, 1951; 6: 202-204.

Breland K, Breland M. Animal behavior. New York: The MacMillan Company. Dennison, 1966.

Dai F, Dalla Costa A, Bonfanti L, Caucci C, Di Martino G, Lucarelli R, Padalino B, Minero M. Positive reinforcement-based training for self-loading of meat horses reduces loading time and stress-related behavior. Frontiers in Veterinary Science, 2019; 6: 1-6.

Danışan S, Özbeyaz C. The effect of the combination of various training methodologies in horse training on the learning performances of Arabian horses. Livestock Studies, 2021; 61(2): 66-76.

D'Onofrio J. Measuring the efficiency of clicker training for service dogs. Master Thesis. The Pennsylvania State University, Department of Education Psychology, Counseling, and Special Education, Pennsylvania, 2015.

Ferguson DL, Rosales-Ruiz J. Loading the problem loader: The effects of target training and shaping on trailer-loading behavior of horses. Journal of Applied Behavior Analysis, 2001; 34: 409–424.

Flannery B. Relational discrimination learning in horses. Applied Animal Behaviour Science, 1997; 54: 267–280.

Freymond SB, Briefer EF, Zollinger A, Gindrat-von Allmen Y, Wyss C, Bachmann. Behaviour of horses in a judgment bias test associated withpositive or negative reinforcement. Applied Animal Behaviour Science, 2014; 158: 34–45.

Hall C, Kay R, Yarnell K. Assessing ridden horse behavior: Professional judgment and physiological measures. Journal of Veterinary Behavior, 2014; 9: 22-29.

Hart B. The art and science of clicker training for horses. Souvenir Press, London, 2008.

Hendriksena P, Elmgreena K, Ladewigb J. Trailer-loading of horses: Is there a difference between positive and negative reinforcement concerning effectiveness and stress-related signs? Journal of Veterinary Behavior, 2011; 6: 261-266.

Hockenhulla J, Creighton E. Unwanted oral investigative behaviour in horses: A note on the relationship between mugging behaviour, hand-feeding titbits and clicker training. Applied Animal Behaviour Science, 2010; 127: 104–107.

Hockenhulla J, Creighton E. Training horses: Positive reinforcement, positive punishment, and ridden behavior problems. Journal of Veterinary Behavior, 2013; 8: 245-252.

Innes L, McBride S. Negative versus positive reinforcement: An evaluation of training strategies for rehabilitated horses. Applied Animal Behaviour Science, 2008; 112: 357–368.

Keiper R. The assateague ponies. Tidewater Publishers, Centreville, MD, 1985.

Kurland A. Clicker training for your horse. Ringpress Books, Lydney, 2001.

Langbein J, Siebert K, Nuernberg G, Manteuffel G. The impact of acoustical secondary reinforcement during shape discrimination learning of dwarf goats *(Capra*

Hircus). Applied Animal Behaviour Science, 2007; 103 (1-2): 35-44.

Le Scolan N, Hausberger M, Wolff A. Stability over situation in temperemental traits of horses as revealed by experimental and scoring approaches. Behavioural Processes, 1997; 41: 257-266.

Leidinger C, Herrmann F, Thöne-Reineke C, Baumgart N, Baumgart J. Introducing clicker training as a cognitive enrichment for laboratory mice. Journal of Visualized Experiments, 2017; 121: 1-12.

Lindsay SR. Handbook of applied dog behavior and training, adaptation and learning. Blackwell Publishing, Iowa, 2000.

Lindsay A, Wood MA. Clicker bridging stimulus efficacy. Master Thesis, Hunter College, New York, New York, 2007.

McCall CA, Burgin SE. Equine utilization of secondary reinforcement during response extinction and acquisition. Applied Animal Behaviour Science, 2002; 78: 253–262.

McDonnell SM, Haviland JCS. Agonistic ethogram of the equid bachelor band. Applied Animal Behaviour Science, 1995; 43: 147–188.

McDonnell S, Poulin A. Equid play ethogram. Applied Animal Behaviour Science, 2002; 78: 263-290.

McGreevy PD, Oddie C, Burton FL, McLean AN. The horse–human dyad: Can we align horse training and handling activities with the equid social ethogram? The Veterinary Journal, 2009; 181: 12–18.

McLean AN. Overshadowing: a silver lining to a dark cloud in horse training? Journal Applied Animal Welfare Science, 2008; 11: 236–248.

McLean AN, Christensen JW. The application of learning theory in horse training. Applied Animal Behaviour Science, 2017; 190: 18–27.

Mills DS, Marchant-Forde JN, McGreevy PD, Morton DB, Nicol C, Phillips CJC, Sandøe P, Swaisgood RR. Encyclopedia of applied animal behaviour and welfare (Eds.), CAB International, UK, 2010.

Paredes-Ramos P, Diaz-Morales J, Espinosa-Palencia M, Coria-Avila G, Carrasco-Garcia A. Clicker training accelerates learning of complex behaviors but reduces discriminative abilities of yucatan miniature pigs. Animals, 2020; 10: 1-11.

Pryor K. Don't shoot the dog! The new art of teaching and training, 2nd ed. Bantam Books, New York, New York, 1999.

Pryor K. Getting started: Clicker training for dogs, Rev. ed. Sunshine Books, Inc., Waltham, MA, 2005; pp. 3–7.

Sankey C, Richard-Yris MA, Henry S, Fureix C, Nassur F, Hausberger M. Reinforcement as a mediator of the perception of humans by horses (Equus caballus). Animal Cognition, 2010; 13: 753–764.

Seaman SC, Davidson HPB, Waran NK. How reliable is temperament assessment in the domestic horse (Equus caballus)? Applied Animal Behaviour Science, 2002; 78: 175–191

Skinner BF. The behavior of organisms. An experimental analysis. Appleton-Century Company, Inc., New York, 1938; pp. 61–74.

Skinner BF. How to teach animals. Scientific American, 1951; 185: 26-29.

Slater C, Dymond S. Using differential reinforcement to improve equine welfare: Shaping appropriate truck loading and feet handling. Behavioural Processes, 2011; 86: 329–339.

Smith SM, Davis ES. Clicker increases resistance to extinction but does not decrease training time of a simple operant task in domestic dogs (Canis familiaris). Applied Animal Behaviour Science, 2008; 110: 318–329.

Thorpe WH. Learning and instinct in animals. 2nd ed. Methuen, UK, 1963.

Waran N, McGreevy P, Casey RA. Training methods and horse welfare. In: Waran N. (Ed.), The Welfare of Horses. Kluwer Academic Publishers, Dordrecht, 2002, pp. 151–180.

Williams BA. Conditioned reinforcement: experimental and theoretical issues. Behavior Analyst, 1994; 17 (2): 261–285.

Williams JL, Friend TH, Nevill CH, Archer G. The efficacy of a secondary reinforcer (clicker) during acquisition and extinction of an operant task in horses. Applied Animal Behaviour Science, 2004; 88: 331–341.

Kocatepe Veterinary Journal

Kocatepe Vet J. (2022) 15(2):233-238

DOI: 10.30607/kvj.1012012

RESEARCH ARTICLE

Effects of *Yucca Schidigera* and Zeolite Supplementation to Diet on Blood Cytokines and Thyroid Hormones Concentrations in Sheep

Abdullah ERYAVUZ[1]*, İbrahim DURMUŞ[2], İsmail KÜÇÜKKURT[3], Sinan İNCE[4]

[1]*Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Physiology, Afyonkarahisar, Turkey*
[2]*Afyon Kocatepe University, Şuhut Vocational High School, Laboratory and Veterinary Health, 03100, Afyonkarahisar, Turkey*
[3]*Afyon Kocatepe University, Veterinary Faculty, Department of Biochemistry, TR-03200 Afyonkarahisar, Turkey*
[4] *Afyon Kocatepe University, Veterinary Faculty, Department of Pharmacology and Toxicology, TR-03200 Afyonkarahisar, Turkey*

ABSTRACT

This study is aimed to compare the effects of combined supplementation of Yucca schidigera (YS) and clinoptilolite as a zeolite source to diet on the blood IL-1β, IL-6 and TNF-α and thyroid hormones concentrations in sheep. A total of 24 sheep were divided four groups as the Control, YS group, Zeolite and YS + Zeolite group and each group was contained 6 animals. The basal diet was given to animals in control group, while the animals in the Zeolite, YS and YS + Zeolite groups were fed with the experimental diet contained the basal diet plus 3% clinoptilolite, 1500 ppm YS and 1500 ppm YS + 3% clinoptilolite, respectively. The blood samples were taken from the animals in the end of experimental period lasted 30 days. The plasma IL-1β, IL-6, TNF-α, T3 and T4 concentrations were determined by using the commercial ELISA. The supplementation of YS decreased the plasma IL-1β concentration and the combined supplementation of Zeolite and YS lowered the plasma TNF-α concentration. The plasma IL-6, T3 and T4 concentrations were not affected by the treatments. As a result, it was concluded that the combined supplementation of zeolite and YS, which are two natural feed additives, instead of adding them separately to the feed, may affect the proinflammatory cytokines production in sheep blood.

Keywords: Cytokines, saponins, sheep, thyroid hormones, zeolite.

***

Koyunlarda Yeme *Yucca Schidigera* ve Zeolit Katılmasının Kan Sitokin ve Tiroid Hormon Düzeylerine Etkileri

ÖZ

Bu çalışmanın amacı, koyunlarda yeme steroidal saponin içeren Yucca schidigera (YS) ve zeolit kaynağı olarak klinoptilolitin birlikte ilavesinin kan IL-1β, IL-6, TNF-α ve tiroid hormon düzeylerine etkilerini karşılaştırmaktır. Çalışmada, toplam 24 koyun kullanıldı ve koyunlar her biri 6 hayvan içeren Kontrol, YS, Zeolit ve YS + Zeolit olmak üzere dört gruba ayrıldı.Kontrol grubundaki hayvanlar temel yemle beslenirlerken, YS gruptaki hayvanlar yeme 1500 ppm düzeyinde YS ilave edilmiş yemle, Zeolit gruptaki hayvanlar %3 düzeyinde klinoptilolit ilave edilmiş yemle ve YS+Zeolit gruptakiler ise 1500 ppm YS ile %3 klinoptilolit ilave edilmiş yemle beslendiler. Toplam 30 gün süren deneme döneminin sonunda, hayvanlardan kan örnekleri alındı. IL-1β, IL-6, TNF-α, T3 ve T4'ün plazma düzeyleri, ticari kitler kullanılarak ELİSA cihazında belirlendi. Yeme YS ilavesi plazma IL-1β düzeiyini azalttırken, YS+Zeolitin birlikte yeme ilavesi ise plazma TNF-α düzeyini düşürdü. Buna karşın, çalışmada, uygulamaların IL-6, T3 ve T4 düzeylerine etkisinin olmadığı tespit edildi. Sonuç olarak, iki doğal yem katkı maddesi olan zeolit ve YS'nın yeme ayrı ayrı katılması yerine birlikte ilavesinin koyun kanında proinflamatuar sitokinlerin üretimini etkileyebileceği kanaatine varıldı.

Anahtar kelimeler: Koyun, saponinler, sitokinler, tiroid hormonları, zeolit.

To cite this article: Eryavuz A., Durmuş İ., Küçükkurt İ., İnce S. Effects of Yucca Schidigera and Zeolite Supplementation to Diet on Blood Cytokines and Thyroid Hormones Concentrations in Sheep. Kocatepe Vet J. (2022) 15(2):233-238

Submission: 19.10.2021 **Accepted**: 29.05.2022 **Published Online**: 01.06.2022

ORCID ID; *AE: 0000-0001-8602-2400, İD: 0000-0003-1360-8843, İK; 0000-0003-0198-629X, Sİ; 0000-0002-1915-9797.*
*Corresponding author e-mail: eryavuz@aku.edu.tr

GİRİŞ

İnsanların beslenmesinde önemli bir hayvansal protein kaynağını oluşturan sığır, koyun ve keçi gibi ruminant hayvanların yetiştiriciliği; yoğun üretim teknikleri, kapsamlı ve dengeli beslenme, otomasyon ekipmanı ve diğer yeni teknolojiler ile gün geçtikçe gelişme göstermektedir. Rumene yerleşmiş olan mikroorganizmalar sayesinde bitki ve kaba yemleri enerji kaynağı olarak kullanabilmeleri ruminant hayvanların sindirim fizyolojisi bakımından çok önemli bir özelliktir. Bu nedenle, ruminant hayvanların tek mideli hayvanların kullanamadıkları düşük kaliteli besinleri değerlendirmeleri ve bunları et ve süt gibi insanların büyük gereksinimi olan proteinli maddelere dönüştürmeleri hem yetiştiricilere hem de ülke ekonomisine önemli katkı sağlamaktadır (Cammack ve ark., 2018). Bununla birlikte, ruminant hayvan yetiştiriciliğinin daha ekonomik olması ve karlılığını önemli ölçüde artırmak için tükettikleri yemin, çok sayıda mikroorganizmaları barındıran rumende optimum bir mikrobiyel fermentasyona maruz kalmaları ve bu fermentasyonun geliştirilmesi gerekmektedir (Kutlu ve Serbester, 2014). Rumende fermentasyon devam ettikçe kana metabolit geçişi sürmekte ve hayvanların tükettikleri yemin içeriği ve rumen fermentasyonunun düzenliliği kan metabolit düzeyindeki değişiklikler üzerine önemli etkenlerden birini oluşturmaktadır (Durmuş ve ark., 2017). Bu nedenle, rumen fermentasyonunun manipülasyonu, ruminant hayvanların verimlerini iyileştirmenin en etkili yollarından biridir.

Çevre kirliliği, bulaşıcı hastalıkların patlaması ve gıda güvenliği endişeleri günümüz modern hayvan yetiştiriciliğini rahatsız eden üç ciddi endişeyi oluşturmaktadır. Bu nedenle, hayvanların beslenmesinde kullanılan yem ham maddeleri ile yem katkı maddelerinin hayvan yetiştiriciliğini etkileyen bu endişelere yol açmaması gerekmektedir. Hayvanlarda verimi artırma, kalitesi yüksek besin madde gereksinimlerini karşılama ve yemlerden daha iyi yararlanma hedefi yem katkı maddeleri adı verilen bir sektörün oluşmasına yol açmıştır (Kutlu ve Serbester, 2014). Enzimler, antibesinsel faktörler ve antibiyotikler, yem katkı maddeleri olarak yemden yararlanmayı geliştirmek ve hayvan verimini artırmak amacıyla kullanılmaktadır. Bununla birlikte, insan tüketimine yönelik hayvansal ürünlerin güvenliğini sağlamak ve çevresel endişeleri gidermek için hayvan yemlerine antibiyotiklerin yem katkı maddesi olarak kullanılması üzerinde sosyal bir baskı vardır. Son yıllarda, antibiyotiklerin yem katkı maddesi olarak kullanımından kaynaklanan mikroorganizmaların geliştirdiği direnç ve çevre kirliliğinin azaltılmasına katkı sağlayacak potansiyele sahip doğal yem katkı maddelerinin, hayvansal üretim sektöründe büyük önem kazandığı gözlenmektedir (Chattopadhyay, 2014, Shaaban ve ark., 2021). Bu yem katkı maddeleri arasında üzerinde en yoğun çalışılanlar arasında zeolitler (Çolpan, 2013) ve saponin içeriği yüksek bitkiler (Bodas ve ark., 2012, De Sousa ve ark., 2019, Chen ve ark., 2021) de yer almış olmasına rağmen hala biyolojik etkilerinin tam olarak ortaya konması sağlanamamıştır.

Yem katkı maddelerinin hayvansal üretim ve hayvan sağlığı açısından risk oluşturmadan, hem teknolojik hem maliyet açısından dünya ile rekabet edilebilir ve sürdürülebilir şekilde üretimi gerekmektedir. Bu nedenle, ruminant hayvan yetiştiriciliğinde, yem katkı maddesi olarak antibiyotiklerin yerine kullanılacak güvenli ve etkili yem katkı maddelerinin bağışıklık sistemine olan etkilerinin tespit edilmesi; patojenleri kontrol etme, hayvan vücut savunma sistemlerini destekleme, rumen ve bağırsak sağlığını teşvik etme ve yemden yararlanmayı iyileştirmedeki etkinliklerini göstermesi bakımından önem arz etmektedir. Bu beklentiye yönelik artan ilgiye bağlı son yıllardaki yapılan araştırmalarda, zeolit ve saponinlerin söz konusu alanda ümit verici özellikler taşıdığı ortaya konmuştur (Eryavuz ve ark., 2015, De Sousa ve ark., 2019, Chen ve ark., 2021, El-Nile ve ark., 2021). Zeolitlerin ve saponin içeriği yüksek Yucca schidigera (YS)'nın yalnızca ağırlık artışı, yem verimliliği ve sağlığı dahil olmak üzere çiftlik hayvanları ve kümes hayvanlarının performansını iyileştirmekle kalmayıp, aynı zamanda hayvan gübresinden kaynaklanan amonyak emisyonlarını azalttığı ve sonuç olarak hayvan tesislerinde kokuyu kontrol ettiği gösterilmiştir (Chen ve ark., 2021, El-Nile ve ark., 2021). Bununla birlikte, yem katkı maddesi olarak hem zeolit hem de YS'nın birlikte kullanılmasının özellikle ruminant hayvanlarda oluşturdukları biyolojik etkilerin karşılaştırılmasına yönelik çalışmalar oldukça yetersizdir. Yem katkı maddesi olarak zeolit ve YS'nin ruminant hayvanların yemine birlikte katılmasının sitokinlere etkilerini ortaya koyan bir çalışma bulunmamaktadır. Bu çalışmanın hipotezi; ruminant hayvanların gastrointestinal kanal içerisinde etkisini gösterdiği bilinen zeolit ve YS'nin yeme birlikte takviyesinin proinflamatuar sitokinlerin üretimini ayrı ayrı katılmalarına göre düşürebileceği şeklindedir. Bu nedenle araştırma; iki doğal yem katkı maddesi olan zeolit ve YS'nın yeme katılmasının koyunların kanında pro-enflamatuar sitokinler ve tiroid hormonları düzeylerine etkilerini karşılaştırmak ve ikisinin birlikte kullanılmasıyla oluşacak etkileşimi belirlemek amacıyla planlanmıştır.

MATERYAL ve METOT

Hayvan Materyali

Afyon Kocatepe Üniversitesi Hayvan Deneyleri Etik Kurulu'ndan (AKÜHAYDEK – 281-17 referans nolu 49533702/160 sayılı araştırma) onay alındıktan sonra çalışma başlatılmıştır. Çalışmada, Afyon Kocatepe Üniversitesi Veteriner Fakültesi çiftliğinde var olan 24 adet merinos ırkı erkek koyun kullanılmıştır.

Çalışmada koyunlar; Kontrol, Zeolit, YS ve Zeolit+YS grupları olmak üzere 4 gruba ayrıldı ve her grupta 6 baş koyun birbiriyle karışmayacak şekilde ayrı bölmelere konuldu. Çiftlikte uygulanan bakım ve besleme şartlarına göre barındırılan koyunlara; kaba yem olarak buğday samanı ve yonca kuru otu verilirken, karma yem olarak kimyasal bileşeni Tablo 1'de verilen ticari fabrika yemi verildi. Hayvanlar; yeni doğan kuzularda yeme %1.5 ve %3 düzeyinde klinoptilolit ilavesinin hematolojik değerler üzerine olumsuz etkisinin olmadığı bildiriminden (Norouzian ve ark., 2010) hareketle %3 klinoptilit ilave edilmiş yem ve 1500 ppm YS ilavesinin kuzularda rumen amonyak düzeyini düşürdüğü yönündeki bildiriminden (Eryavuz ve ark., 2015) hareketle de 1500 ppm ilave edilmiş yem ya da hem %3 klinoptiloit hem de 1500 ppm YS birlikte ilave edilmiş ve mikserde karıştırılmış yemlerle beslendiler. Çalışmada kullanılan klinoptilolit Gordes Zeolit Madencilik Sanayi ve Ticaret Anonim Şirketi'den, YS ise Ekol Gıda Tarım Hayvancılık Sanayi Ticaret Anonim Şirketi 'den temin edildi.

Tablo 1. Ticari Fabrika Yeminin Kimyasal İçeriği (%)

Ham Protein (HP)	16
Ham selüloz (HS)	11
Ham Yağ (HY)	3.5
Ham Kül (HK)	9
Sodyum	0.3

Tüm gruplardaki hayvanlar 15 gün boyunca yeme alıştırma dönemi ve sonrasında 30 gün araştırma dönemi olmak üzere toplam 45 gün süreyle kendileri için hazırlanmış olan yemle beslendiler. Önlerinde sürekli temiz içme suyu bulundurulan hayvanlara, günde sabah ve akşam olmak üzere iki öğün yem verildi, aynı öğün içerisinde de kaba ve karma yemler farklı verildi.

Vena jugularisden heparin içeren tüplere yeterli miktarda kan örnekleri, çalışmanın son gününde sabah yemlemesinden önce, usulüne uygun olarak alındı. Bu kan örneklerinin plazmaları 10 dakika boyunca 3000 rpm'de santrifüj yapılarak elde edildi. Plazma örnekleri 1.5 ml'lik ependorf tüplere konuldu ve analizler yapılıncaya kadar -20°C' derin dondurucuda tutuldu. Analiz için plazmaların çözdürülmesinden sonra Interlökin-1β (IL-1β), tumor nekrozis faktör alfa (TNF-α), interlökin-6 (IL-6) ile T3 ve T4 düzeyleri ticari kitler kullanılarak (SUNRED, Shanghai Sunred Biological Technology Co., Ltd., Çin) ELİSA cihazında, üretici firmanın katoloğunda önerdiği gibi ölçümleri yapılarak belirlendi.

Çalışmada elde edilen verilerin istatistiksel analizi SPSS 16.0 istatistiksel paket programı kullanılarak değerlendirildi. Değişkenler ortalama ± standart hata olarak ifade edildi. Verilerin normallik testleri ile gruplar arasındaki istatistiksel farkları saptamak için ANOVA testi, post-hoc test olarak Duncan testi uygulandı. İstatistiksel anlamlılık $p<0.05$ olarak alındı.

BULGULAR

Denemenin son gününde sabah yemlemesinden önce alınan kan örneklerinde yapılan ölçümlerle elde edilen plazma IL-1β, IL-6 ve TNF-α ile tiroid hormon düzeylerine yönelik veriler Tablo 2'de gösterilmiştir. Yeme % 3 düzeyinde zeolit katılmasının plazma IL-1β, IL-6 ve TNF-α ile tiroid hormon düzeylerinin Kontrol grubu hayvanlarınkinden farklılık göstermediği tespit edildi. 1500 ppm YS ilave edilmiş yemle beslenen koyunların plazma IL-1β düzeylerinin Kontrol grubundaki hayvanlarınkinden önemli oranda ($P<0.05$) düşük olduğu, buna karşın incelenen diğer parametreler bakımından önemli bir değişim göstermediği gözlendi. Hem zeolit hem de YS ilave edilmiş yemle beslenen koyunların plazma TNF-α düzeylerinin; Kontrol grubuna göre istatistiksel anlamda önemsiz, buna karşın, Zeolit ve YS grubuna göre önemli oranda ($P<0.05$) düşük olduğu bulundu (Tablo2).

Tablo 2: Koyunlarda yeme zeolit, *Yucca schidigera* (YS) ve zeolit + YS ilavesinin plazma IL-1β, IL-6 ve TNF-α ile tiroid hormon düzeylerine etkisi (n=6, Ort.±SH).

Parametreler	**Kontrol**	**Zeolit**	**YS**	**Zeolit+YS**
IL-1β (pg/ml)	2.06 ± 0.164^{a}	1.71 ± 0.284^{ab}	1.37 ± 0.142^{b}	1.85 ± 0.143^{ab}
TNF- α (pg/ml)	1.18 ± 0.067^{ab}	1.36 ± 0.124^{a}	1.42 ± 0.063^{a}	1.04 ± 0.034^{b}
IL-6 (pg/ml)	1.30±0.167	0.99±0.223	0.88±0.236	1.38±0.268
T_3 (µg/L)	1.98±0.121	2.00±0.154	1.80±0.215	2.11±0.216
T_4 (µg/L)	35.5±5.54	32.4±3.79	30.9±4.59	39.1±5.16

[a,b]: Aynı satırda farklı harf taşıyan değerler farklıdır ($P< 0.05$). IL-1β: Interleukin 1 Beta, IL-6: Interleukin 6, TNF-α: Tümör Nekrozis Faktör.

TARTIŞMA

Hayvanların sağlık ve hastalık durumlarında kandaki metabolit düzeylerindeki değişimlerin belirlenmesi, veteriner hekimler ile yetiştiriciler için önemli verileri oluşturmakta ve hayvanın sağlığı ve beslenme durumu hakkında bilgiler vermektedir. Metabolitlerin kan düzeyleri; hayvanların ırk, fizyolojik durum, yaş ve beslenmeleri ile mevsime bağlı olarak değişmektedir. Bu çalışmada; koyunlar, antibiyotiklere alternatif doğal yem katkı maddelerinden olan, zeolit % 3, YS 1500 ppm ve zeolit %3 + YS 1500 ppm düzeyinde ilave edilmiş yemlerle beslendiler. Çalışmada; zeolit kaynağı olarak, sertliği ve küçük parçacıklara ince bir şekilde öğütülme kabiliyeti nedeniyle çok sayıda uygulama için en uygun tipi olduğu bildirilen (Wu ve ark., 2013) klinoptilolit kullanılmıştır. Çalışmada koyunların yemine ilave edilen klinoptilolit düzeyi (30000 mg/kg), hayvanların yeminde toksik olmayan düzey olarak bildirilen (EFSA, 2013) düzeyin (10000 mg/kg) oldukça üstündeydi. Bununla birlikte, Bartko ve ark. (1983), bu çalışmada kullanılan düzeyinden çok daha fazla düzeyde (her bir kg canlı ağırlık için 0.15 g) koyunların yemine klinoptilolit ilave ettikleri çalışmada, koyunların sağlık durumlarında ve genel davranışlarında bir farklılık gözlemediklerini bildirmektedirler.

Bağışıklık sisteminin önemli bileşenleri olan sitokinlerden oluşan interlökinlerin, hayvanların yemine katılan besin bileşikleri tarafından plazma düzeylerinde değişiklikler olduğu gözlenmiştir (Su ve ark., 2016). Yapılan çalışmalarda, YS'nin biyolojik etkilerinin içerdiği siteroidal saponinlerden kaynaklandığı ifade edilmektedir (Eryavuz ve ark., 2015, De Sausa ve ark., 2019). Nitekim, koksidiozlu etçi piliçlerin yemine YS saponinleri ilave edildiğinde duodenum ve çekal tonsillerde IL-1β gen ekspirasyonunun azaldığı gözlenmiştir (Oelschlager ve ark., 2019). Bununla birlikte, YS'nin biyolojik fonksiyonlarının sadece saponin bileşenlerinin varlığı nedeniyle olmayabileceği de ifade edilmektedir (Cheek ve ark., 2006, Küçükkurt ve ark., 2016). Nitekim, YS'nın, resveratrol gibi polifenollerden de zengin bir kaynak olduğu bildirilmektedir (Piacente ve ark., 2005). Resveratrolün, splenositlerde, artan resveratrol seviyesi ile birlikte IL-1β ve TNF-α gen ekspresyonunun da düştüğü ve IL-1β ve TNF-α gibi proinflamatuar sitokinlerin transkripsiyonunun baskılanmasına neden olabildiği ifade edilmektedir (Zhang ve ark., 2014). Resveratrolün, immünosüpresif farelerde IL-1α / β, IL-2 ve TNF-α seviyeleri gibi bağışıklık fonksiyonuna bağlı serum sitokin konsantrasyonlarını doza bağlı olarak yukarı yönde regüle edebileceği bildirilmektedir (Lai ve ark., 2016). Bu çalışmada; yeme YS ilavesinin IL-1β'nın plazma düzeyini azalttığı ancak zeolitlerle birlikte katılmasının bu etkiyi ortadan kaldırdığı gözlendi. Elde edilen bu bulgu; splenositlerde, artan resveratrol seviyesi ile birlikte IL-1β gen ekspirasyonunun azaldığı (Zhang ve ark., 2014) ile koksidiyozlu etlik piliçlerde YS saponinlerin duedonumda IL-1β gen ekspirasyonunu azalttığı yönündeki bildirimleri (Oelschlager ve ark., 2019) desteklemektedir. Bununla birlikte, çalışmada; yeme YS ilavesinin, plazma IL-6 ve TNF-α düzeylerine etkisinin olmadığı tespit edildi. YS'nin yeme ilave miktarlarının da sitokinler üzerine etkisinin olduğu, yüksek düzeylerinin immünolojik durumu bozabileceği ve hastalıklara karşı duyarlılıkları artırabileceği ileri sürülmektedir (Su ve ark., 2016). Nitekim, kanatlı yemine 100 ppm'den daha yüksek dozlarda YS takviyesinin, IL-6 üretiminin azalması ile sonuçlandığı gözlenmiştir (Su ve ark., 2016). Bu çalışmada, yeme YS ilavesinin plazma IL-6 ve TNF-α düzeylerine etkisinin olmaması, yeme katılan düzeyi ve hayvan türlerinin farklılığından kaynaklanabilir (Su ve ark., 2016). Kırmızı kemik iliğinden polimorfonükleer lökositlerin geçiş süresini kısaltarak dolaşıma geçişlerini hızlandırdığı bildirilen (Suwa ve ark., 2000) IL-6'nın, bu çalışmadaki uygulamalardan etkilenmemesi, dolaşımdaki polimorfonükleer lökosit sayısına da etkilerinin olmayacağını göstermektedir. Nitekim, koksidiyozlu etlik piliçlerin yemine YS saponinleri ilave edildiğinde, kanda lökosit sayısının etkilenmediği gözlenmiştir (Oelschlager ve ark., 2019). Çalışmada, yeme zeolit takviyesinin plazma proinflamatuar sitokinlere etkisinin olmaması; Tirtaatmadja ve ark (2015)'ın in vitro insan kanı kullanarak yaptıkları çalışmada, zeolitlerin sitokin düzeylerini etkilemediği yönündeki gözlemlerini desteklemektedir.

Dolaşımdaki proinflamatuar sitokinlerin seviyesi ölçülerek immunolojik etkileri değerlendirebilmenin mümkün olduğu bildirimi (Elsabahy ve Wooley, 2013) dikkate alınırsa, çalışmada elde edilen bulguların; yeme zeolit ve YS ilavelerinin bu çalışmada kullanılan düzeylerinin ruminant hayvanların immünolojik durumlarını bozmayacağına işaret etmektedir. Aynı zamanda, doğal yem katkı maddesi olarak gerek zeolitlerin gerekse YS'nin ayrı ayrı ya da birlikte ruminant yemine ilave edilmesinin IL-1β ve TNF-α'nın plazma düzeylerini düşürmesi nedeniyle, bağışıklık homeostazını koruyabildiğini ve bağışıklık sisteminin daha fazla aktivasyonunu önleyebileceğine işaret etmektedir. Nitekim, IL-1β'nın, bir bağışıklık tepkisinin başlatılmasında çok önemli olan metabolik, hormonal ve hücresel değişikliklere yol açabileceği ileri sürülmektedir (Klasing, 2007). Bu çalışmada elde edilen bulguların, ruminant yemlerine YS ve YS+Zeolit ilavelerinin antiinflamatuar özelliklere sahip olabileceğini ve enflamatuar yanıtın hafifletilmesi için etkili bir strateji olarak hizmet edebileceğini düşündürmektedir. Ruminant hayvanlardaki bağışıklık durumlarına etkilerinin daha fazla aydınlatılması için hem pro- hem de antiinflamatuar sitokinlerin birlikte değerlendirildiği yeni çalışmalara ihtiyaç bulunmaktadır.

Hücresel fonksiyonların düzenlenmesinde önemli rolleri bulunan (Huszenicza ve ark., 2002) tiroid hormonlarının plazma düzeylerindeki değişimlerin bağışıklık sistemlerini de etkileyebileceği bildirilmektedir (Marchiori ve ark., 2015, Kandır ve Keskin, 2016). Nitekim, hipotiroidili hastalarda TNF-α, IL-6 ve C-reaktif protein (CRP) seviyelerinin yükseldiği bulunmuştur (Tayde ve ark. (2017). Hipotiroidizmli hastalara levotiroksin verildiğinde, verilmeyenlere göre; plazma IL-1, IL-6 ve TNF-α düzeylerinin önemli oranda daha düşük olduğu ve anti-enflamatuar sitokin olan IL-10 düzeylerinin ise önemli oranda daha yüksek olduğunun gözlemlendiği bildirilmektedir (Marchiori ve ark., 2015). Bu çalışmada, koyunların yemine zeolit ve YS ilavelerinin tiroid hormonlarının plazma düzeylerine etkisinin olmadığı gözlendi. Bu bulgu; ruminant hayvanlarda, yem katkı maddesi olarak katılan zeolit ve YS'nin tiroid hormonları ile proinflamatuar sitokinlerin değerlendirildiği ilk bulgular olması nedeniyle, çalışmada kullanılan düzeyde koyunların yemine ilave edilecek zeolit ve YS'nin hem ayrı ayrı hem de birlikte katılmasının sitokin üretiminde etkisi olduğu bildirilen (Marchiori ve ark., 2015) tiroid hormonlarına etkisinin olmadığını göstermektedir.

SONUÇ

Koyunların yemine zeolit, YS ve zeolit+YS ilavesi, proinflamatuar sitokinlerden IL-6'nın plazma düzeylerine etkisi olmamış ancak YS ilavesi IL-1β, ve YS+zeolit ilavesi ise TNF-α'nın plazma düzeylerini azaltmıştır. Bu durum; YS ve zeolit'in ruminant hayvanların yemine birlikte ilave edildiklerinde, hayvanların immünolojik ve enflamatuar mekanizmalarını bozmayacağı gibi inflamatuar yanıtlarının hafifletilmesine de katkıda bulunabileceğini düşündürmektedir. Bu çalışmada elde edilen bulgular; hayvan beslenmesinde katkı maddesi olarak yemde kullanılan YS ve zeolitlerin hem ayrı ayrı hem de birlikte kullanılmasına bağlı ruminant hayvanların bağışıklık sisteminin çalışmasında önemli aracı moleküllerden proinflamatuar sitokinlere olan etkilerine yönelik ilk bulguları oluşturmakta ve yeni perspektifler sunmaktadır. Bununla birlikte, immun sistemin kompleksliği, diğer sistemlerle olan etkileşimi ve ölçülen sitokinlerin multifonksiyonel olmaları dikkate alındığında, YS ve YS+zeolit takviyesinin proinflamatuar sitokinlerin ekspresyonunu veya fonksiyonunu nasıl düzenlediği bilinmemektedir. Bu nedenle, YS ve YS+zeolit takviyesinin ruminant hayvanlarda enflamatuar yanıt üzerindeki yararlı etkisinin altında yatan mekanizmalar ile bunların fizyolojik etkilerinin aydınlatılması için daha fazla araştırma yapılması gerekmektedir.

Çıkar Çatışması: Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

Etik İzin: Bu çalışma Afyon Kocatepe Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu (AKUHADYEK) 281-17 referans numaralı 49533702/160 nolu kararı ile Etik İzni alınmıştır. Ayrıca yazarlar Araştırma ve Yayın Etiğine uyulduğunu beyan etmişlerdir.

Finansal Destek: Bu çalışma Afyon Kocatepe Üniversitesi Bilimsel Araştırmaları Koordinasyon Birimi tarafından desteklenmiştir (Proje kodu: 17 Kariyer 250).

Açıklama: Bu çalışmanın bir kısım verileri, "2nd International Eurasian Conference on Science, Engineering and Technology (EurasianSciEnTech 2020) October 07-09, 2020 / Gaziantep, Turkey" yapılan kongrede Özet Bildiri olarak sunulmuştur.

KAYNAKLAR

Bartko, P., Vrzgula, L., Prosbova, M., Blazovsky, J. 1983. The effect of feeding zeolite (clinoptilolite) on the health status of sheep. Vet.Med. (Praha). 1983; 28: 481-492.

Bodas, R., Prieto, N., Garcia-Gonzalez, R., Andres, S., Giraldez, F.J., Lopez, S. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim Feed Sci Technol., 2012; 176:78-93.

Cammack, KM., Austin, K J., Lamberson, WR., Conant, GC., Cunningham, HC. RUMINNAT NUTRITION SYMPOSIUM: Tiny but mighty: the role of the rumen microbes in livestock production. J. Anim. Sci., 2018; 96:752–770. doi:10.1093/jas/skx053

Chattopadhyay, M.K. Use of antibiotics as feed additives: a burning question. Front. Microbiol., 2014; doi.org/10.3389/fmicb.2014.00334

Chen, F., Lv Y., Zhu, P., Cui, C., Wu, C., Chen, J., Zhang, S., Guan, W. Dietary Yucca schidigera extract supplementation during late gestating and lactating sows improves animal performance, nutrient digestibility, and manure ammonia emission. Frontiers Vet.Sci., 2021; 8: 676324. DOI:10.3389/fvets.2021.676324

Cheeke, PR., Piacente, S., Oleszek, W. Anti-inflammatory and anti-arthritic effects of Yucca schidigera: A review. J. Inflam., 2006; 3: 6.

Çolpan, İ. Kanatlı beslenmesinde zeolitin etkileri. Vet.Tavuk.Dern.Derg.Mektup, Ankara, 2013; 11:12-22.

De Sousa, O.A., Cooke, R.F., Brandão, A.P., Schubach, M.K., Schumaher, T.F., Bohnert, D.W., Marques, R.S. Productive and physiological responses of feeder cattle supplemented with Yucca schidigera extract during feedlot receiving. J. Anim. Sci., 2019; 97:208–219.

Durmuş, İ., Evcimen, M., Salim, M.N., Küçükkurt, İ., İnce, S., Eryavuz, A. Koyunlarda yemlemeden sonra kan oksidan-antioksidan denge ile bazı biyokimyasal parametrelerdeki değişimlerin belirlenmesi. Kocatepe Vet.J., 2017; 10:1-6.

EFSA Panel on Additives and Products or Substances used in Animal Feed. Scientific Opinion on the safety and efficacy of clinoptilolite of sedimentary origin for all animal species. EFSA J., 2013; 11: 1–14.

El-Nile, A., Elazab, M., El-Zaiat, H., El-Azrak, K., Elkomy, A., Sallam, S., Soltan, Y. In vitro and in vivo assessment of dietary supplementation of both natural or nano-zeolite in goat diets: Effects on ruminal fermentation and

nutrients digestibility. Animals, 2021; 11: 2215. https://doi.org/10.3390/ani11082215

Elsabahy, M., Wooley, K.L. Cytokines as biomarkers of nanoparticle immunotoxicity, Chem. Soc. Rev., 2013; 42: 5552–5576.

Eryavuz A., Küçükkurt İ., İnce S., Fidan AF., Avcı G., Bülbül T. Kuzularda Rasyona Yucca Schidigera Tozu Katılması ve Günlük Dozunun Rumen Fermentasyonu ile Verime Etkilerinin Araştırılması. Kocatepe V.J., 2015; 8: 1-10.

Huszenicza, GY., Kulcsar, M., Rudas, P. Clinical endocrinology of thyroid gland function in ruminants. Vet. Med. (Czech), 2002; 47: 199–210.

Kandır, S., Keskin, E. Serum IL-1β, IL-6, IL-10 and TNF-α Levels in Thyroidectomized Rats. Kafkas Univ Vet Fak Derg., 2016; 22: 297-300.

Klasing, K. C. Nutrition and the immune system. Br. Poult.Sci., 2007; 48:525–537.

Kucukkurt I, Akkol EK, Karabag F, Ince S, Suntar I, Eryavuz A, Sozbilir NB. Determination of the regulatory properties of Yucca schidigera extracts on the biochemical parameters and plasma hormone levels associated with obesity. Rev. Bras. Farmacogn., 2016; 26: 246–250.

Kutlu HR, Serbester U. Ruminant beslemede son gelişmeler. Türk Tarım – Gıda Bil.Tek, Derg., 2014; 2(1): 18-37.

Lai X, Pei QS, Song X, Zhou X, Yin ZQ, Jia RY, Zou YF, Li LX, Yue GZ, Liang XX, et al. The enhancement of immune function and activation of NF-jB by resveratrol-treatment in immunosuppressive mice. Int Immunopharmacol., 2016; 33:42–47.

Marchiori, R.C., Pereira, L.A., Naujorks, A.A., Rovaris, D.L., Meinerz, D.F., Duarte, M.M., Rocha, J.B. Improvement of blood inflammatory marker levels in patients with hypothyroidism under levothyroxine treatment. BMC Endocr. Disord., 2015; 15:32 DOI 10.1186/s12902-015-0032-3.

Norouzian M.A, Valizadeh R, Khadem AA, Afzalzadeh A, Nabipour A. The effects of feeding clinoptilolite on hematology, performance, and health of newborn lambs. Biol Trace Elem Res., 2010; 137:168--176.

Oelschlager, M.L., Rasheed, M.S.A., Smith, B.N., Rincker, M.J., Dilger, R.N. Effects of Yucca schidigera-derived saponin supplementation during a mixed Eimeria challenge in broilers. Poultry Science, 2019; 98: 3212–3222.

Piacente S, Pizza C, Oleszek W. Saponins and phenolics of Yucca schidigera Roezl: chemistry and bioactivity. Phytochem Rev., 2005; 4:177–190.

Shaaban, M.M., Kholif, E.A., El Tawab, A.M., Radwan, M. Thyme and celery as potential alternatives to ionophores use in livestock production: their effects on feed utilization, growth performance and meat quality of Barki lambs.Small Rum.Res., 2021; 200: 106400. DOI:10.1016/j.smallrumres.2021.106400

Su JL, Shi BL, Zhang PF, Sun DS, Li TY, Yan SM. Effects of yucca extract on feed efficiency, immune and antioxidative functions in broilers. Braz Arch Biol Technol., 2016; 59:e16150035.

Suwa T, Hogg JC, English D, van Eeden SF. Interleukin-6 induces demargination of intravascular neutrophils and shortens their transit in marrow. Am. J. Physiol. Heart Circ. Physiol., 2000; 279: H2954–60.

Tayde, P., Bhagwat, N., Sharma, P., Sharma, B., Dalwadi, P., Sonawane, A., Subramanyam, A., Chadha, M., Varthakavi, P. Hypothyroidism and depression: are cytokines the link? Indian J. Endocrinol. Metab., 2017; 21:886-892.

Tirtaatmadja, N.; Mortimer, G.; Ng, E.-P.; Ahmad, H.; Mintova, S.; Serpooshan, V.; Minchin, R.; Mahmoudi, M. Nanoparticles-induced inflammatory cytokines in human plasma concentration manner: An ignored factor at the nanobio-interface. J. Iran. Chem. Soc. 2015; 12: 317-323.

Wu Y., Wu Q., Zhou Y., Ahmad H., Wang T. Effects of Clinoptilolite on Growth Performance and Antioxidant Status in Broilers. Biol. Trace Elem. Res., 2013; 155: 228-235.

Zhang CY, Tian YD, Yan FB, Kang XT, Han RL, Sun GR, Zhang HR. Modulation of growth and immunity by dietary supplementation with resveratrol in young chickens receiving conventional vaccinations. Am. J. Vet. Res., 2014; 75:752–759.

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.