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Phone: +90 474 2426807-2426836/5228
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RESEARCH ARTICLE

Use of Immunoperoxidase Technique in Smears Prepared from Vaginal Secretions in Early Diagnosis of Listerial Abortions in Cattle [1]

Serpil DAĞ * Doğan AKÇA ** Musa KARAMAN * Özgür ÇELEBİ ***
Hasan ÖZEN * Fatih BÜYÜK *** Mitat ŞAHİN ***

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* Department of Pathology, Faculty of Veterinary Medicine, Kafkas University, TR-36100 - TURKEY
** School of Health, Kafkas University, TR-36100 - TURKEY
*** Department of Microbiology, Faculty of Veterinary Medicine, Kafkas University, TR-36100 - TURKEY

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Summary

Listeriosis is a sporadic disease of ruminants that causes meningoencephalitis, septicemia and abortion. In this study, the usefulness of immunoperoxidase technique was investigated in early diagnosis of cattle listerial abortions. For this purpose, 96 smears prepared from vaginal swab samples that were collected from aborting cattle were stained for *L. monocytogenes* by immunoperoxidase technique. Presence of the agent in vaginal swab samples were investigated by bacteriological culture technique and the results were compared to that of immunoperoxidase technique. A total of 7 samples, 4 out 5 bacteriological culture positive smear samples and 3 out of 91 bacteriological culture negative smear samples, were detected to be positive by immunoperoxidase technique. Compared to bacteriological culture technique, sensitivity and specificity of immunoperoxidase technique was calculated as 80% and 96.7%, respectively. In conclusion, immunoperoxidase technique in smears prepared from vaginal swabs can be used in early diagnosis of listerial abortions since it can give results the same day samples collected, however the technique must be supported by bacteriological culture technique which is performed for bacterial isolation and identification of the agent.

Keywords: *Listeriosis, Abortion, Cattle, Immunoperoxidase*

Sığır Listerial Abortlarının Ön Tanısında Vajinal Akıntılardan Hazırlanan Sürme Preparatlarda İmmunoperoksidaz Yönteminin Kullanılması

Özet

Listeriozis; ruminantlarda meningoensefalitis, septisemi ve abortusa neden olan, genellikle sporadik seyirli bir enfeksiyondur. Bu çalışmada sığır listerial abortlarında erken ön tanı amacıyla immunoperoksidaz boyama yönteminin kullanılabilirliği araştırıldı. Bu amaçla atık yapan 96 sığırdan alınan vajinal sıvap örneklerinden hazırlanan sürme preparatlar *L. monocytogenes* antikoru ile immunoperoksidaz boyama yöntemi kullanılarak boyandı. Bakteriyolojik kültür metodu ile vajinal sıvap örneklerinde etkenin varlığı araştırılarak immunoperoksidaz yönteminin sonuçları ile karşılaştırıldı. Bakteriyolojik kültürü pozitif olan 5 adet vajinal sıvap örneğinden hazırlanan sürme preparatların 4'ünde ve negatif sonuçlanan 91 adet örneğin 3'ünde olmak üzere toplam 7 örnekte immunoperoksidaz ile pozitif reaksiyon saptandı. İmmunoperoksidaz metodu kültür metodu ile karşılaştırıldığında duyarlılığı ve özgüllüğü sırasıyla %80 ve %96.7 olarak saptandı. Sonuç olarak vajinal akıntılardan hazırlanan sürme preparatlarda immunoperoksidaz tekniğinin aynı gün içerisinde sonuç alınması nedeni ile listerial abortların ön tanısı amacıyla kullanılabileceği ancak bu yöntemin etken izolasyon ve identifikasyonuna yönelik olarak yürütülen bakteriyolojik kültür yöntemi ile desteklenmesi gerektiği sonucuna varıldı.

Anahtar sözcükler: *Listeriozis, Abort, Sığır, İmmunoperoksidaz*

İletişim (Correspondence)
+90 474 2426807/5086
serpildag06@hotmail.com

INTRODUCTION

Listeriosis is a sporadic, occasionally seen as an enzootic disease of ruminants that causes meningoencephalitis, septicemia and abortion [1]. It can manifest some other prenatal diseases in all ruminants, resulting important economic losses. Being a zoonosis, it might cause drastic problems in humans. Among the known *Listeria* species, namely; *L. monocytogenes*, *L. ivanovii, L. innocula, L. seeligeri, L. welshimeri, L. grayi,* and *L. murrayi*, only *L. monocytogenes* can cause clinical listeriosis [2]. However, there are few studies reporting that *L. ivanovii* might be an etiological agent in some cases [3,4].

Listeria species are Gram positive, non-capsulated, non-spore forming, aerobic to facultative anaerobic, short round edged, rod-shaped bacteria measuring 0.5x0.5x2.0 µm. The bacteria can be observed as single, chained, V- shaped or grouped in parallel forms under microscope [5]. They grow best at 30-37°C, and can replicate well in silage that are not prepared properly. Moreover, the bacteria can survive at 1-45°C, which gives them a wider range of survival chance [6]. *Listeria* species are very common in environment and can be isolated from the infected animals of several species [7]. Soil is a good reservoir for the agent. Since the agent is found in the micro flora of digestive tract of healthy and diseased animals, feces is the natural reservoir [8]. In addition, infected animals spread the bacteria to the environment via milk, urine, aborted fetus, and uteral and vaginal secretions. Contaminated silage and feedings for ruminants, and raw meat, fish, and vegetables, non-pasteurized milk and milk products for humans are the major sources of the agent [9,10].

Definitive diagnosis of listeriosis is made by the agent's isolation and identification by bacteriological culture techniques [11]. Serological means such as agglutination test and ELISA, and PCR as a molecular test are also in use for determination of the bacteria [12,13]. However, all these techniques possess some drawbacks. A simple, reliable and cheap detection technique is therefore still needed. Immunoperoxidase technique was previously shown to be successful in diagnosis of listerial infections [14,15]. However, the technique has not been tried before on fetal tissues or vaginal smears in cattle with Listerial abortion.

In this study, immunoperoxidase technique, as a potentially early detection technique for *L. monocytogenes* was investigated in smears prepared from vaginal swab samples of cattle that had abortion. The technique was also compared to the standard bacteriological culture technique for its sensitivity and specificity.

MATERIAL and METHODS

Material

Vaginal swab samples were collected between February-May 2008 in Kars province from 96 cattle that had abortion in their history. Three vaginal swab samples were obtained from each animal, and placed in Tryptose Broth containing screw capped-tubes, and then sent to Kafkas University, Faculty of Veterinary Medicine, and Department of Microbiology under cold-chain in the same day.

Methods

Microbiological Investigations: Bacteriological isolation of *Listeria* from vaginal swab samples was performed according to the technique suggested by FDA [16]. Vaginal swab samples in Trpytose Broth were transferred to selective supplement (Oxoid, SR141) containing Listeria Enrichment Broth (Oxoid, CM862) and incubated at 30°C for 48 h. Then, they were inoculated onto selective supplement (Oxoid, SR140 containing Listeria Selective Agar (LSA) (Oxoid CM856), and incubated at 30°C for 24 h. Smooth, round, grey black centered colonies were inoculated onto Tryptic Soy Agar-Yeast Extract (TSA-YE), and incubated at 30°C for 24 h. Identification of the bacteria as *L. monocytogenes* were succeeded by Gram staining, catalase and oxidase tests, motility, umbrella-like growth in semi-solid agar, CAMP test, growth in D-mannitol, D-xylose, L-rhamnose, α-methyl-D-mannoside and D-glucose. Negative and positive controls for identification were also performed.

Immunoperoxidase Technique: Smears prepared from total of 96 vaginal swab samples were used in immunoperoxidase staining. The technique was performed broadly as follows; all smears were fixed in alcohol and then dehydrated. Following blocking the endogenous peroxidase activity by treating the smears with 3% H_2O_2 for 30 min, antigen retrieval was performed by microwaving in citrate solution (pH 6.0) for 25 min. Non-specific antibody binding was blocked by a blocking solution for 30 min. Smear samples were then incubated for 60 min at room temperature by anti-Listeria polyclonal antibody (LSBio, LS C122061) diluted 1:1000. Biotinylated secondary antibody and Streptavidin peroxidase complex (Zymed Histostain Plus Bulk Kit, Cat No: 85-9043) were consecutively applied for 30 min each with three times phosphate buffer saline, pH 7.4 (PBS) washings between. With the use of substrate, 3,3-diaminobenzidine H_2O_2, antibody binding was visualized. Background staining was provided by Harris hematoxylin. Finally, immunmount was applied and smears were observed under a microscope. Negative controls, at which primer antibody was not applied, and positive controls of smears prepared from *L. monocytogenes* standard strains and smears prepared from 5 isolates of swab samples that were determined to be *L. monocytoges* positive by microbiological means were also performed.

Quantitative Assessment of Immunoperoxidase Technique: Sensitivity and specificity of the immunoperoxidase technique was evaluated as described by Moore et al.[17].

RESULTS

In bacteriological culture technique, colonies grown in LSA were characterized as *Listeria* spp. based on Gram positivity, non-capsulation, non-spore forming, motility at 20-25°C, and umbrella-shaped growth at 20°C with short rod-like morphology. By microbiological investigation of 96 vaginal swab samples, 5 (5.2%) were determined to be positive for Listeria *(Table 1)*. All *Listeria* spp. were also identified as *L. monocytogenes* by β-hemolysis, positive reactivity with *Staphylococcus aureus* at CAMP test, glucose, methyl-D-mannoside and rhamnose positivity and xylose, mannitol and nitrat reduction negativity.

The results of the immunoperoxidase technique were summarized in *Table 1*. Total of 7 samples were detected positive for listeria by immunoperoxidase technique. Out of 5 samples that were positive by bacteriological culture technique 4 were determined to be positive, and out of 91 samples that were negative by bacteriological culture technique 3 were found to be positive by immunoperoxidase technique. The positive stained bacteria were observed as bacillus shaped and mostly single or in groups of two or forming V-shapes *(Fig. 1a-b)*. Smears prepared from the isolates of cultures were all shown positive immunoreactivity *(Fig. 2)*. While all negative controls in which primer antibody was switched by PBS gave negative results, all positive controls that were prepared from *L. monocytogenes* standard strain (ATTC-7644) showed positive immunoreactivity. According to these results, sensitivity and specificity of the immunoperoxidase technique compared to the bacteriological culture technique were calculated as 80% and 96.7%, respectively.

DISCUSSION

Cases of abortion negatively affect cattle breeding not only in Kars province but also nationwide. Most of the abortions are known to be caused by bacterial agents, and *Listeria* is a significant bacterium in the list [18,19]. Stillbirth, infertility and drops in milk production and quality are the major reasons of economic losses due to Listeriosis. The disease is also a zoonosis, and therefore poses a risk for human health [20].

Listerial abortions in cattle are often sporadic and take place mostly within the last trimester [21]. The agent could be easily detected in fetal tissues as well as in uteral and

Table 1. *Time of abortion, time lapse after abortion until sample collection, and results of the bacteriological culture and immunoperoxidase techniques for Listeria (Total of 88 cases that were negative by both techniques were not shown)*

Tablo 1. *Abort zamanı, abortu takiben örnek toplamaya kadar geçen süre ve Listeria yönünden bakteriyolojik kültür ile immunoperoksidaz yöntemlerinin sonuçları (Her iki yöntemle de negatif sonuçlanan toplam 88 vaka tabloda gösterilmemiştir)*

Case No	Time of Abortion (Months)	Sample Collection (Days)	Bacteriological Culture	Immunoperoxidase
1	7-8	30	+	+
2	6-7	45	+	-
3	6-7	45	+	+
4	8-9	30	+	+
5	8-9	45	+	+
6	7-8	45	-	+
7	7-8	45	-	+
8	8-9	45	-	+

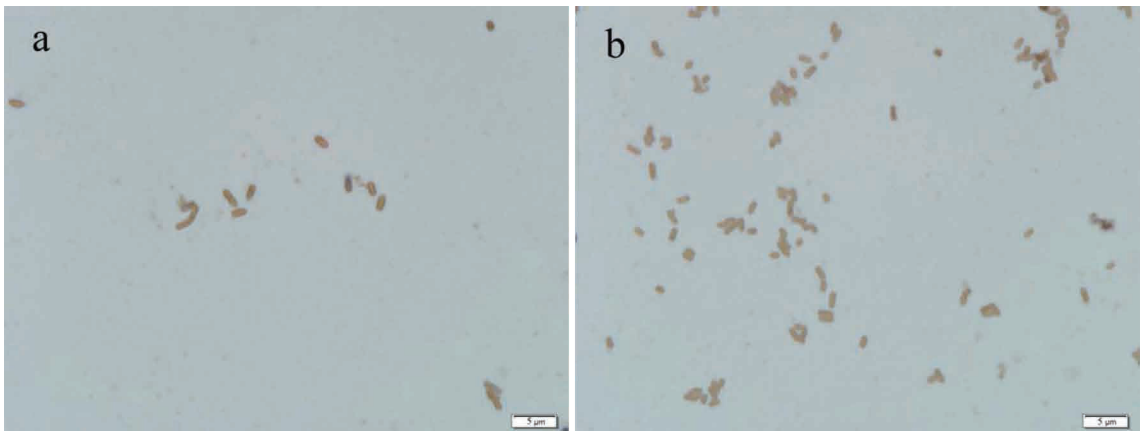

Fig 1. a-b. Listeria agents observed in smears prepared from vaginal swab samples by immunoperoxidase technique

Şekil 1. a-b. Vajinal sıvap örneklerinden hazırlanan sürme frotilerde immunoperoksidaz yöntemi ile gözlenen Listeria etkenleri

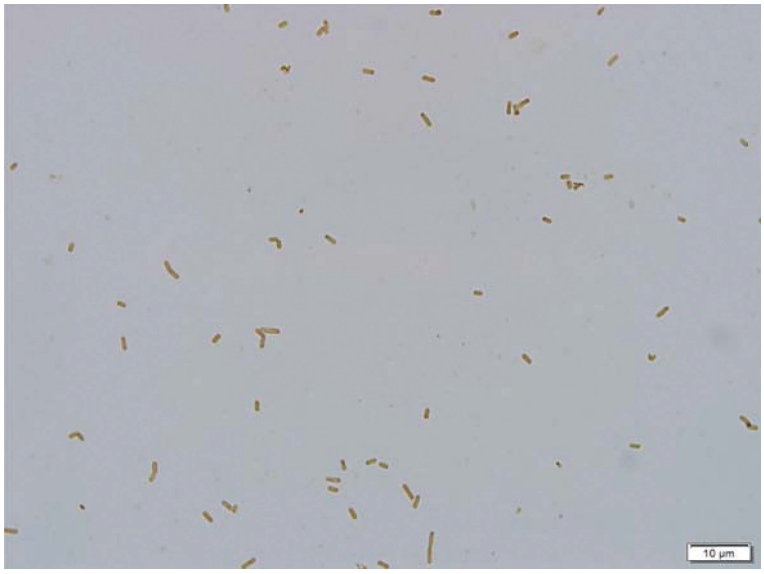

Fig 2. Listeria agents showing positive immunoreactivity in smears prepared from culture isolates

Şekil 2. Kültür izolatlarından hazırlanan sürme frotilerde pozitif immunoreaksiyon gösteren Listeria etkenleri

vaginal secretions after abortion [18,19]. Although it is not known exactly how long the bacteria shade in uteral and vaginal secretions it is thought to last for several months after abortion [22]. Studies regarding to bacteriological isolation of the agent in vaginal secretions are however, quite limited [23-25]. In a study conducted in servico-vaginal swab samples, *L. monocytogenes* was detected at 10% of cattle with genital system diseases [25]. In another study, *L. ivanovi* was isolated in vaginal and fecal swab samples collected from sheep with abortion [24]. Similarly, *L. monocytoges* and *L. ivanovi* were detected in the vaginal swab samples of buffalos at 2.4% and 0.8%, respectively [23]. In Turkey, most studies of listeriosis in animals have aimed to seroprevalence detection [13,26,27]. However, there are limited studies on the bacteriological isolation and identification for *Listeria*. In a study conducted by Erdogan et al.[13] sero-prevalence of listeriosis was determined 78.9% in Kars province. A bacteriological study for isolation and identification of the agent was also performed in Kars [4]. In the current study, *L. monocytogenes* was isolated and identified in 5 vaginal swab samples of 96 cattle that had abortion or stillbirth in their history. In spite of the time lapse and delayed sampling after the abortion the isolation rate seems to be similar to previous studies in the literature [23,25].

Listerial agents are known to spread by aborted fetuses, fetal membranes, vaginal secretions, milk, urine and feces. The agent was also reported to be present in digestive tract of healthy animals as well as humans [28]. Therefore, soil, water, and foodstuffs could be easily contaminated with the bacteria and become a treat for spread to humans. Hence, rapid and reliable detection of the agent is important in taking precautions in epidemics. The definitive detection of listeriosis can only be possible with isolation and identification of the agent by bacteriological means. Although bacteriological culture technique is the reliable and standard method it takes time and require equipped laboratory environment and personnel. The technique might also suffer from inadequacies in samplings and low bacterial presence in samples. Possible laboratory spread to laboratory technician is the main drawback of bacteriological culture technique. Moreover, isolation of the bacteria in clinical samples might not be reliable since the agent can be found in brain and feces [28,29]. Therefore, a rapid, simple, and reliable technique is still needed.

Complement fixation, agglutination and ELISA are the commonly used serological techniques to determine listeriosis [13]. They are based on the detection of antibodies that are reactive against somatic (O) and flagella (H) antigens. Although these techniques are simple and sensitive they might be insufficient in diagnosis. The tests might also suffer from cross reactions with other Gram negative bacteria such as *Streptococcus*, *Staphylococcus*, and *Enterococcus* [30].

Polymerase chain reaction and DNA hybridization techniques are some molecular methods used in detection *Listeria* especially in foodstuffs. Virulence factors such as haemolysin and p60 extracellular protein are mostly used for detection. PCR technique has been successfully used in determination of *Listeria* species in milk and vaginal secretions [31]. However, it is comparably expensive and requires equipped laboratory equipment and personal. Optimization problems and longer time requirement to obtain results renders the use of molecular techniques [12].

Pathological findings in aborted fetuses are not specific for *Listeria*, and therefore definitive cause in abortions could not be easily determined in listerial abortions. Rapid and reliable determination of the causative agent however could be succeeded by peroxidase-antiperoxidase technique [32,33]. The technique has also been shown to provide better detection results compared to the bacteriological culture techniques in certain studies, though it was stated that the technique must be supported by other laboratory detection means [33].

In the current investigation, 4 out of 5 samples that were determined to be positive for *Listeria* by bacteriological culture technique showed positive immunoreactivity by immunoperoxidase technique. Therefore, 20% false negativity in comparison to the bacteriological culture technique was observed. Low number of bacteria in the collected samples might be the cause of the false negativity in one sample. Use of enriched broth agar, therefore would provide better results for isolation of the agent, and hence might be superior to immunoperoxidase technique in such cases. In addition, presence of low number of *Listeria* cases studied in the current study might be the cause of high false reactivity for immunoperoxidase technique. Moreover, 3 out of 91 negative samples by bacteriological culture technique showed positive immunoreactivity for *Listeria*. Therefore, immunoperoxidase technique yielded 3.3% false positivity. This false positivity might be caused by cross reactivity with other bacterial agents and/or possible contamination during the sample staining. According to the results obtained, sensitivity and specificity of the immunoperoxidase technique was calculated as 80% and 96.7%, respectively. In a previous study for *Brucella*, similar results were also obtained [34].

In conclusion, immunoperoxidase technique was shown to be a useful tool in rapid, easy, relatively inexpensive and fairly reliable detection of *Listeria* in smears prepared from vaginal secretions of cattle which have abortion. However, the results of the technique should be evaluated carefully keeping in mind that the listerial agents could be found in environment and feces of healthy animals and possible contamination could occur during sampling. Finally, it was concluded that the immunoperoxidase technique might provide some help in early determination of *Listeria* cases; however the results should be supported by some other detection techniques.

REFERENCES

1. Hird DW, Genigeorgis C: Listeriosis in food animals: Clinical signs and livestock as a potential source of direct (non food-borne) infection for humans. **In,** Miller AJ, Smith JL, Somkutti GA (Eds): Foodborne Listeriosis. Society for Industrial Microbiology. Elsevier Science Publishing, Inc. New York. pp. 31-39, 1990.

2. McLauchlin J: *Listeria monocytogenes*, recent advances in the taxonomy and epidemiology of listeriosis in humans. *J Appl Bacteriol,* 63 (1): 1-11, 1987.

3. Elischerova K, Cupkava E, Urgeova E, Lysy J, Sesevikova A: Isolation of *Listeria ivanovii* in Slovakia. *Cesk Epidemiol Microbiol Imunol,* 39 (4): 228-236, 1990.

4. Şahin M, Beytut E: Abortions in sheep due to *Listeria ivanovii* in the Kars region. *Turk J Vet Anim Sci,* 30, 503-506, 2006.

5. Seeliger HPR, Jones D: Genus Listeria Pierie 1940. **In,** Sneath PHA, Mair NS, Sharpe ME, Holt JG (Eds): Bergey's Manual of Systematic Bacteriology. pp. 1235-1245, Williams and Wilkins Co., Baltimore, 1986.

6. Low JC, Donachie W: A rewiew of *Listeria monocytogenes* and listeriosis. *Vet J,* 153 (1): 9-29, 1997.

7. Müller HE: Listeriosis in animals. *İnfeksiyon Dergisi*, 2 (4): 505-519, 1988.

8. Vazquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Doninguez-Bernal G, Goebel W, Gonzalez-Zorn B, Wehland J, Kreft J: *Listeria* pathogenesis and molecular virulence determinants. *Clin Microbiol Rev,* 14 (3): 584-640, 2001.

9. Güven A, Patır B: Elazığ ilinde tüketime sunulan et ve bazı et ürünlerinde *Listeria* türlerinin araştırılması. *Tr J Vet Anim Sci,* 22, 205-212, 1998.

10. Kalender H: Detection of *Listeria monocytogenes* in faeces from chickens, sheep and cattle in Elazığ province. *Tr J Vet Anim Sci,* 27, 449-451, 2003.

11. Gasanov U, Hughes D, Hansbro PM: Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: A review. *FEMS Microbiol Rev,* 29 (5): 851-875, 2005.

12. Peters M, Pohlenz J, Jaton K, Ninet B, Bille J: Studies of the detection of *Listeria monocytogenes* by culture and PCR in cerebrospinal fluid samples from ruminants with listeric encephalitis. *Zentralbl Veterinarmed B,* 42 (2): 84-88, 1995.

13. Erdoğan HM, Gökçe G, Gökçe Hİ, Kırmızıgül AH, Güneş V, Sural E, Yılmaz K: Kars yöresindeki sığırlarda *Listeria monocytogenes* enfeksiyonlarının ELISA yöntemi ile araştırılması. *Kafkas Univ Vet Fak Derg,* 5 (1): 43-46, 1999.

14. Karademir N, Güvenç T, Yarım M, Esendal ÖM, Gümüşsoy S: Gebe tavsanlar ile fötüslerinde deneysel listeriozisin histopatolojik, immunopatolojik ve mikrobiyolojik yöntemlerle tanısı. *Ankara Üniv Vet Fak Derg*, 43, 311-319, 1996.

15. Kabakçı N, Yarım M: The expression of CD14 antigen in experimental encephalitic Listeriosis in rabbits. *Revue Méd Vét,* 155 (3): 151-155, 2004.

16. Lovett J, Hitchins AD: *Listeria* Isolation. FDA Bacteriological Analytical Manual (BAM), 6th ed., Supp. Sept. (2nd Print): 29.01.1989.

17. Moore FM, Petrak ML, McMillian MC: Diagnosis of chlamydial infection in pet birds: Comparison of cloacal-swab culture and peroxidase-antiperoxidase methods. *Avian Dis*, 32 (1): 157-162, 1988.

18. Kirkbride CA: Bacterial agents detected in a 10-year study of bovine abortions and stillbirths. *J Vet Diagn Invest*, 5, 64-68, 1993.

19. Anderson ML: Infectious causes of bovine abortion during mid-to late-gestation. *Theriogenology*, 68 (3): 474-486, 2007.

20. Sutherland PS, Porritt RJ: *Listeria monocytogenes*. **In,** Hocking AD, Arnold G, Jensen I, Newton K, Sutherland PS (Eds): Foodborne Microorganisms of Public Health Significance. 5th ed., pp. 333-378, AIFST (NSW Branch) Food Microbiology Group, 1997.

21. Radostits OM, Blood DC, Gay CC: Veterinary Medicine. A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses, 8th ed., pp. 660-666, Bailliare Tindall, Philadelphia, London. 1994.

22. The Merck Veterinary Manual: Listeriosis. 2011. http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/51400.htm, *Accessed*: 21.04.2012.

23. Chaudhari SP, Malik SVS, Chatlod LR, Barbuddhe SB: Isolation of pathogenic *Listeria monocytogenes* and detection of antibodies against phosphatidylinositol-specific phospholipase C in buffaloes. *Comp Immunol Microbiol Infect Dis,* 27 (2): 141-148, 2004.

24. Kaur S, Malik SVS, Vaidya VM, Barbuddhe SB: Phenotypic and genotypic characterization of *Listeria* species isolated from spontaneous cases of abortion in humans and animals. *The 16th International Symposium on Problems of Listeriosis (ISOPOL XVI)*. 20-23 March, Savannah, Georgia, USA, 2007.

25. Parihar VS, Barbuddhe SB, Chakurkar EB, Danielsson-Tham ML, Tham W: Isolation of *Listeria* species from farm bulk milk at the receiving dairy plant and cervico-vaginal swabs from dairy cows in Goa. *Ind J Comp Microbiol, Immun Infect Dis,* 28 (1): 53-55, 2007.

26. Kennerman E, Babür C, Kılıç S: Determination of seroprevelance of *Listeria monocytogenese* antibodies in cattle in Bursa Province of Turkey. *Uludağ Univ Vet Fak Derg,* 24, 95-98, 2005.

27. Öcal N, Babür C, Yağcı BB, Macun HC, Çelebi B, Kılıç S, Yağcı İP: Kırıkkale yöresinde süt sığırlarında Brusellozis, Listeriozis ve Toksoplazmozis' in seroprevelansı ve birlikte görülme sıklığı. *Kafkas Univ Vet Fak Derg,* 14 (1): 75-81, 2008.

28. Abay S, Aydın F: Sağlıklı sığırların dışkılarından *Listeria* spp. izolasyon ve identifikasyonu. *Erciyes Üniv Sağlık Bil Derg,* 14 (3): 191-197, 2005.

29. Gronstol H: Listeriosis in sheep. Isolation of *Listeria monocytogenes*

from organs of slaughtered animals and dead animals submitted for post-mortem examination. *Acta Vet Scand,* 21 (1): 11-17, 1980.

30. Bennett RW, Weaver RE: Serodiagnosis of *Listeria monocytogenes.* US Food and Drug Administration Bacteriological Analytical Manual, 8th ed., Revision A, Chapter 11, 1998.

31. Akça D, Şahin M: Investigation of *Listeria* species isolated from milk and vaginal swab samples of cows in the province of Kars, Turkey. *Kafkas Univ Vet Fak Derg,* 17 (6): 987-993, 2011.

32. Marco A, Ramos JA, Dominguez L, Domingo M, Gonzalez L: Immunocytochemical detection of *Listeria monocytogenes* in tissue with the peroxidase-antiperoxidase technique. *Vet Pathol,* 25 (5): 385-387, 1988.

33. Johnson GC, Fales WH, Maddox CW, Ramosvara JA: Evaluation of laboratory tests for confirming the diagnosis of encephalitic listeriosis in ruminants. *J Vet Diagn Invest,* 7 (2): 223-228, 1995.

34. Dağ S, Büyük F, Özen H, Çelebi Ö, Karaman M, Akça D, Şahin M: Detection of *Brucella* spp. in vaginal swab samples of aborting cattle: Comparison of immunoperoxidase to bacteriological culture technique. *Kafkas Univ Vet Fak Derg,* 18 (4): 617-622, 2012.

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RESEARCH ARTICLE

Farelerde 3-Metilkolantren ile İndüklenen Fibrosarkoma Üzerine Sisteaminin Etkileri

Abdullah DOĞAN * Pınar AKSU * Dinçer ERDAĞ *
Murat BAYEZIT * Ertan DOĞAN ** Kadir ÖZCAN ***

* Kafkas Üniversitesi, Veteriner Fakültesi, Farmakoloji ve Toksikoloji Anabilim Dalı, TR-36100 Kars - TÜRKİYE
** Göle İlçe Tarım Müdürlüğü, TR-75700 Ardahan - TÜRKİYE
*** Uşak Üniversitesi Sağlık Yüksekokulu, Patoloji Anabilim Dalı, TR-64100 Uşak - TÜRKİYE

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Özet

Tümörlerin biyolojilerinin anlaşılması, korunma ve tedavi yöntemlerinin geliştirilmesi büyük önem arz etmektedir. Bu çalışmada farelerde 3-metilkolantrenle (3-MC) indüklenen fibrosarkoma üzerinde sisteaminin koruyucu etkileri araştırıldı. Deneyde yaklaşık 20±2.0 g ağırlığında beyaz erkek fareler *(Mus musculus albino)* kullanıldı. Fareler her grupta 15 adet olacak şekilde beş gruba ayrıldı, standart diyet ve su ile *ad libitum* olarak beslendi. Birinci gruptaki hayvanlara hiçbir ilaç uygulaması yapılmadı. İkinci gruptaki farelere susam yağı (0.2 ml, deri altı), üçüncü gruba sisteamin (%0.1 suda oral), dördüncü gruptaki farelere 3-metilkolantren çözeltisi (1 mg/0.2 ml susam yağı) 0.2 ml hacimde deri altı yolla enjekte edildi. Beşinci gruba 3-metilkolantren çözeltisi (1 mg/0.2 ml susam yağı) 0.2 ml deri altı ve %0.1 oranında suda çözdürülmüş sisteamin oral yolla *ad libitum* olarak verildi. Hayvanlar 4 ay süreyle takip edildi. Süre sonunda servical dislokasyonla ötanazi edilen farelerin otopsileri yapıldı. Organlardaki morfolojik değişiklikler ve alınan doku örneklerindeki tümöral oluşumlar histopatolojik yöntemlerle araştırıldı. Araştırma sonucunda sisteaminin farelerde 3-metilkolantrenle indüklenen fibrosarkoma karşı koruyucu etki gösterdiği belirlendi.

Anahtar sözcükler: *Fibrosarkoma, 3-Metilkolantren, Sisteamin*

Effects of Cysteamine on 3-Methylcholanthrene - Induced Fibrosarcoma in Mice

Summary

Understanding the biology, developing methods for prevention and treatment of tumors is of great importance. The present study was investigated protective effects of the cysteamine on *3*-methylcholantrene-induced fibrosarcoma in mice. In the experiment, white male mice *(Mus musculus albino)* were used approximately 20±2.0 g in weight. Divided into five groups, per group of 15 mice and were fed *ad libitum* with a standard diet and water. No drug was performed in the first group of animals. Sesame oil (0.2 ml volume) for the second group of mice were injected subcutaneously. The third group was given in drinking water 0.1% solution of cysteamine *(ad libitum)*. 3-methylcholantrene solution (1 mg/0.2ml sesame oil) were injected for the fourth group of mice subcutaneously with 0.2 ml volume. The five group was performed 0.2 ml volume of *3*-methylcholantrene solution (1 mg/0.2 ml sesame oil) subcutaneously and 0.1% dissolved in water was *ad libitum* disintegrating orally. Animals were observed for 4 months. The mice that euthanasiaded cervical dislocation were autopsied at the end. Morphological changes in organs and tissue samples taken from tumor formation was investigated with histopathological methods. In conclusion ıt is suggested that cysteamine has a protective effect on 3-methylcholantrene-induced fibrosarcoma in mice.

Keywords: *Fibrosarcoma, 3-Methylcholanthrene, Cysteamine*

GİRİŞ

Kanser hücrelerin kontrolsüz ve hızlı üremesiyle karakterize ölümcül bir hastalıktır [1]. Özellikle kolon ve akciğer kanserleri yüksek oranda malignite ve ölümle seyretmektedir [2]. Bu nedenle dünyada kanserin korunma ve tedavi-

İletişim (Correspondence)
+90 474 2251150/5128
adogankars@hotmail.com

sinde kullanılabilecek ilaçların keşfi için çok sayıda araştırmaların yapıldığı görülmektedir. Tümörlerin nedenini %70-80 oranında kimyasal maddeler oluşturmakta olup, bunlar arasında doymamış aromatik hidrokarbonlar (antresen, benzantresen, fenantren vb), 1-naftilamin gibi aromatik aminler, benzidin, dimetilaminobenzen, aflatoksin, sterigmatosistin, safrol, pirazolidon alkaloidleri, nitrozo bileşikleri (dialkilnitrozamin, nitrozopiperidin vb), azerenler, aromatik nitrobileşikleri (nitrofuranlar vb), 1,2-dibromometan, halojenli hidrokarbonlar (vinil klorür vb), azobileşikleri (azometan, 4-dimetilamino-azobenzen), dialkilhidrazinler, aldehidler, tioüre, asbest, kadmiyum, krom gibi maddeler sayılabilir [3-6]. Kimyasal kanserojenler içerisinde bulunan ve deneysel çalışmalarda kullanılan 3-metilkolantren (3-MC)'in farelere deri altı, periton içi ve oral yolla uygulanmasıyla yaklaşık bir ay içerisinde fibrosarkoma oluşturulabilmektedir [7-9].

Kimyasal maddeler doğrudan ya da etkinleştikten sonra genetik materyal üzerine etki ederek tümör hücresi oluştururlar (başlama basamağı). Oluşan bu tümör hücresi immun sistem tarafından ortamdan uzaklaştırılamazsa, çoğalarak kendine benzeyen hücre grubuna dönüşür (gelişme safhası). Tümör giderek büyür, sonra metastaz yaparak uzak organlarda yeni tümörlerin oluşmasına neden olur (ilerleme safhası) [1-6,10]. Kimyasal karsinojenler hücrelerin nükleofillerine karşı affinite gösterip, onları bağlayarak genetik yapının bozulmasına neden olurlar (sistinine aromatik aminler, alkile edici ajanlar, adenine polisiklik ajanlar, guanine nitrozoaminler vb) [9,10]. Doymamış aromatik hidrokarbonlardan olan 3-metilkolantren yağda çözünen kuvvetli bir karsinojen maddedir. Araştırmalarda kanserojen amaçla kullanılmaktadır [2,9,11-14]. Arilhidrokarbon reseptörünü uyarır ve DNA'ya bağlanır. Karaciğer başta olmak üzere organlarda 1,2-hidroksilasyon, cis ya da trans dihidroksilasyona uğrar, ayrıca keto türevlerine dönüştürülerek vücuttan atılır [10,13].

Çevre sağlığı açısından 3-MC büyük önem arz etmektedir. Alındığında mikrozomal P-450 enzim sistemini stimüle ederek fizyolojik madde (steroid hormonlar gibi) ve ilaçların metabolizma ve toksisitesini değiştirmektedir [4,9,11,12]. Vücutta N-oksidasyon ve hidroksilasyon reaksiyonlarında artışa neden olmaktadır [9,11,15]. İndüklenen sitokrom P-4501A çok sayıda maddenin biyoaktivasyonunda rol oynamaktadır. Bu nedenle 3-MC hem doğrudan hem de diğer maddeleri aktive ederek mutasyona neden olmaktadır. Genotoksik etkiler sonucu teratojenite, lösemi, özellikle akciğer ve serviks kanserleri görülür [9,11,14]. Ratlara 40 mg/kg dozda enjekte edilen 3-MC teşhisde kullanılabilen onkojen proteinlerin sentezine neden olmaktadır [9,11].

Sisteamin kimyasal olarak trietanolamin (beta-merkaptoetilamin) yapısında olup, vücutta sisteinin dekarboksilasyonuyla oluşmaktadır. Koenzim A'nın yapısına girdiğinden enerji üretimi için gerekli bir maddedir. İlaç ve zehirlerin detoksifikasyonunda rol oynayan ve sistein, glutamin ve glisinden oluşan bir tirpeptit olan glutatiyonun yapısında dolayılı yoldan da olsa yer almaktadır [4,16,17]. Genetik bir hastalık olan sistinozisin tedavisinde orfans ilaç olarak kullanılır. Sistein vücutta sistin halinde depo edilir (İki sülfür atomu disülfid bağı ile birleşmiştir). Sistin redüktaz enzimiyle indirgenerek protein sentezinde kullanılmaktadır. Mutasyon sonucu sistini indirgeyen enzim bazı bireylerde bulunmaz. Özellikle böbrek, göz, kas ve beyin başta olmak üzere organların hücre içlerinde fazla miktarda çökerek sistinozise neden olur. Sisteamin sistin molekülündeki disülfid bağını koparark sisteamin-sistin ve sistein açığa çıkarır. Sisteamin hipotaurine dönüşerek idrarla atılmaktadır [18-21]. Sisteamin dopamin beta-hidroksilaz enzimini inhibe ederek somatostatin sentezini bloke eder. Büyümeyi ve insülin sekresyonunu artırır [18,21-23]. Ayrıca ACTH-releasing hormon düzeyini düşürür ve bakırla şelat yapar [23].

Sisteamin hücre metabolizmasında görev alır. Ayrıca sisteine dönüşerek glutatiyonun yapısına girer. Bu nedenle sisteaminin hücre savunma sistemlerinde bir rol oynayabileceği düşünülmektedir. Bu araştırmada sisteaminin farelerde 3-metilkolantrenle indüklenen tümör üzerine koruyucu etkisinin olup, olmadığı araştırılmıştır.

MATERYAL ve METOT

Bu araştırmada deney hayvanları kullanıldığından Kafkas Üniversitesi Hayvan Deneyleri Yerel Etik Kurulundan izin alınmıştır (KAÜ-HADYEK 26.11.2010/49). Araştırmada ağırlıkları 18-22 g (20±2.0) g olan, 8-12 haftalık erkek, Mus musculus albino fareler kullanıldı. Fareler standart diyet ve çeşme suyuyla *ad libitum* olarak 4 ay süreyle beslendi. Yem Erzurum Bayramoğlu Yem Fabrikasından, sisteamin (CAS: 156-57-0) Fluka ve 3-MC (CAS: 56-49-5) Supelco firmalarından temin edildi. Toplam 75 adet fare her grupta 15 adet olacak şekilde 5 gruba ayrıldı. Birinci grup, kontrol grubu olarak tutuldu. İkinci gruptaki hayvanlara skapula hizasından sırt bölgesi derisi altına 0.2 ml susam yağı bir kez enjekte edildi. Üçüncü gruba *ad libitum* olarak içme suyuyla %0.1 oranında sisteamin 4 ay süreyle verildi (Sisteamin çözeltileri üç günlük hazırlandı). Dördüncü gruptaki hayvanlara sırt bölgesi derisi altına 1 mg 3-MC/0.2 ml susam yağı solüyonundan 0.2 ml bir kez enjekte edildi (bu gruba sisteamin içme suyuyla verilmedi). Beşinci gruptaki farelere sırt bölgesi derisi altına 1 mg 3-MC/0.2 ml susam yağı solüsyonundan 0.2 ml deri altı yolla bir kez enjekte edildi. Bu gruptaki hayvanlara enjeksiyonu takiben içme suyuyla %0.1 oranında sisteamin 4 ay süreyle *ad libitum* olarak verildi (farelerin günde yaklaşık 2-3 ml su içtikleri görüldü). Her gün makroskobik olarak muayene edilerek tümör gelişimi izlendi. Süre sonunda (4 ay) fareler tartıldıktan sonra servical dislokasyonla ötanazi edilerek, otopsileri yapıldı. Tümöral oluşumların boyutları ve ağırlıkları ölçüldü. Dokulardan alınan tümör örnekleri formol-alkol solüsyonunda tespit edildi. Parafin bloklardan yaklaşık 6 mikrometre kalınlığında kesitler alındı. Örnekler dereceli ksilol, alkol, hemotoksilen ve eozinden geçirilerek boyandı [24]. Mikroskop altında histo-

patolojik olarak incelendi. Gruplardaki tümörlü hayvan sayıları istatiksel yöntemle karşılaştırılarak (Minitab Realese 12.1) aralarındaki farkın önemli olup, olmadığı belirlendi.

BULGULAR

Birinci, ikinci ve üçüncü gruptaki farelerde deney süresince (4 ay süre ile) ölüm görülmedi. Dördüncü grupta bulunan farelerde enjeksiyondan sonraki üçüncü, sekizinci ve on sekizinci günde birer adet olmak üzere toplam 3 ölüm olayına rastlandı. Beşinci gruptaki farelerde enjeksiyondan sonraki dokuzuncu günde bir ölüm olayı görüldü. Ölen hayvanlarda tümör tespit edilmedi. İkinci grupta bir, üçüncü grupta üç ve dördüncü grupta dört adet farede enjeksiyon yerlerinde kıl dökülmeleri ve ülsere rastlandı.

Deney sonunda grup 1, 2 ve 3'deki farelerde tümör görülmedi. Dördüncü grupta (3-MC) 8 hayvanda tümöre rastlandı. Farelerden birinin karaciğerinde yaklaşık 1.5 g ağırlığında ve "1x1x1 cm" boyutlarında metastaz tespit edildi. Grup 4'de 3-MCin deri altı enjeksiyonu ile yaklaşık %66.6 oranında tümöre rastlandı. Beşinci gruptaki farelerde beş adet tümör oluşumu tespit edildi. Bu grupta otopside metastaza rastlanmadı. Sisteamin ve 3-MC verilen grup 5'deki hayvanların yaklaşık %35.7'sinde tümör oluştuğu belirlendi. Belirtilen oranlar erken ölen hayvanlar dahil edilmeden elde edilmiştir. Dahil edildiğinde oranlar sırasıyla %53.3 ve %33.3 olarak hesaplandı. Tümör oluşumu açısından kontrol grubu ile deney grupları arasındaki fark istatitiksel açıdan önemli ($P \leq 0.05$) bulunmasına rağmen, grup 4 ve 5 arasındaki farklılık önemsiz bulunmuştur. Tümörlerin büyük oranda sırt bölgesi, boyun, sağ-sol ön ve arka bacakların üst bölümlerine lokalize olduğu tespit edilmiştir. *Tablo 1*'de dördüncü ve beşinci gruplardaki hayvan sayısı, ağırlıkları, hayvanlarda belirlenen fibrosarkomaların ağırlık ve çapları gösterilmiştir.

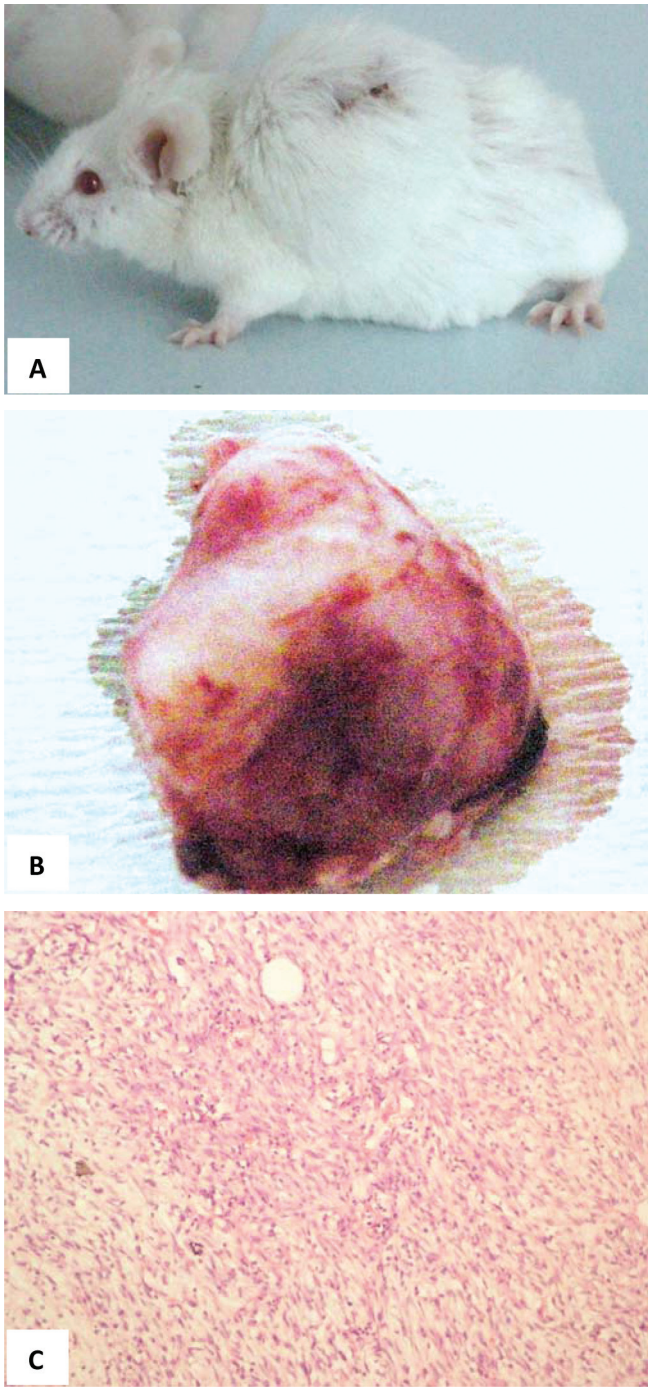
Histopatolojik muayene ile oluşumların tümör olduğu tespit edildi. Grup 4'te görülen tümörlü fare, rezerke fibrosarkoma ve bu tümörden elde edilen kesitlerin resimleri sunulmuştur *(Şekil 1)*. Belirtilen olgunun mikroskopik muayenesinde bağ doku kesitlerinde yaygın fibroblastlara rastlandı. Fibroblastlar arasında lenfositler ve bundan çok daha az sayıda plazma hücreleri tespit edildi. Aynı grupta bulunan farelerden hazırlanan karaciğer kesitlerinin mikroskopik muayenesinde ise kanama odaklarına rastlandı. Ancak bu grupta böbrekten alınan örneklerin mikroskopik muayenesinde patolojik bir bulguya rastlanmadı.

TARTIŞMA ve SONUÇ

Organik yapıların tam yanmaması sonucu ortaya çıkan kimyasal maddelerden olan arilhidrokarbonlar, çevre kirliliği, mutasyon, kanser, teratojenite ve immun sistem bozuklukları gibi çok sayıda olumsuz etkilere neden olurlar [4,5,6,9,11,25]. Danovan et al.[26] 3-MC verilmiş fare ve ratlarda fototoksisite gözlemiş ve mutasyonu mikronükleus testi ile tespit etmiştir. Balıklara 3-MC'in 15 mg/kg dozda verilmesiyle T-lenfositlerinde sitotoksisite, mutasyon, immunodepresyon gözlenmiş ve intrasellülar kalsiyum düzeyinin arttığı belirlenmiştir [25]. Lutz et al.[27] yaptıkları çalışmada 200 mg/kg dozda verilen 3-MC'in bir haftada timus bezinde atrofiye neden olduğu, T ve B lenfositopeni ve kanser yaptığı tespit edilmiştir. Hidroksile 3-MC (dihidroksile metaboliti)

Tablo 1. *Grup 4 ve 5'de tespit edilen tümörlerin ağırlık ve boyutları*
Table 1. *The weights and the sizes of the detected tumors in the 4th and 5th groups*

No	Vücut Ağırlığı (g)		Tümör Ağırlığı (g)		Tümör Boyutu (cm)	
	Grup 4	Grup 5	Grup 4	Grup 5	Grup 4	Grup 5
1	**28.6**	29.1	-	-	-	-
2	**41**	37.4	**6**	5	**2x2.5x2**	2x2x2
3	44.3	32.6	4.4	-	2x2x1.5	-
4	48.2	-	8.5	-	3x3x2.5	-
5	-	34.3	-	-	-	-
6	25.7	32.8	-	-	-	-
7	27.1	45.3	-	1.4	-	1x1x0.5
8	-	33	-	-	-	-
9	48	36.2	2.1	-	1x1x0.5	-
10	38.5	45.4	3.5	8.9	1x1x1	3.5x3x2
11	41.4	42.1	2.2	4.2	1x1x0.5	2x2x1.5
12	-	46.1	-	-	-	-
13	39.5	48.3	-	12.1	-	4x3x3
14	49.2	34.5	8	-	3x3x2.5	-
15	36.7	46.5	6.4	-	2x3x1.5	-



Şekil 1. A- Tümörlü fare, **B**- Rezekte edilmiş fibrosarkoma, **C**- Fibrosarkomadan elde edilen kesitlerin mikrokopik görünümü x 200

Fig 1. A- Tumourous mause, **B**- Resected fibrosarcoma, **C**- Microscobic scene of sections derived from fibrosarcoma x 200

hücrelerin nükleofilik makromolekülleri ile birleşerek genetik hasar yaptığı bilinmektedir [25,28]. Genotoksik etkili olan 3-MC oksidatif stresi de artırmaktadır [15]. Bu araştırmalardan 3-MC'in doğrudan mutasyon ve dolaylı olarak immun sistem depresyonu yapmak suretiyle kanser biyolojisinde rol oynadığı söylenebilir.

Vücuda verilen 3-MC biyoaktivasyona uğrar ve genetik materyale bağlanarak mutasyon yapar. Tümör gelişiminin kolay izlenebilmesi nedeniyle deneysel çalışmalarda deri altı yolla 1 mg dozda uygulanmaktadır [7-9]. Periton içi yolla da benzer doz (40 mg/kg) uygulanabilmektedir. Bu çalışmada indüktörün uygulanmasından yaklaşık bir ay sonra tümör makroskobik olarak görülmeye başlanmıştır. Oysa Keshava [29] aynı dozda ilaç ile 2-4 hafta sonra farelerde fibrosarkoma gözlemiş ve üç ay sonra bunları mikroskobik olarak hemotoksilen-eozin boyama yöntemiyle mitotik indeksi yüksek, nükleus oranı artmış hücreler olarak tespit etmiştir. Keshava akciğer alveollerinde metastaza rastlamıştır.

Bu araştırmada 3-MC farelerde %66.6 oranında tümöre neden olduğu belirlenmiştir. Bu oran hayvanın türü, ırkı, çevre şartları (beslenme vb), immun sistemin durumu, doz ve süre gibi faktörlerle değişebilir. Grup 5'te ise 5 farede (yaklaşık %35.7 oranında) fibrosarkoma tespit edilmiştir. Gruplardaki tümörlü hayvan sayıları kontrol grubuna göre istatistiksel açıdan önemli bulunmuştur ($P \leq 0.05$). Ancak grup 4 ve 5 arasındaki tümör sayısı farkı önemsiz bulunmuştur ($P \leq 0.05$). Hayvan sayılarının artırılarak tekrarlanması araştırmanın güvenirliğini artırabileceği söylenebilir.

Gruplarda erken ölen farelerin tümörden öldükleri gösterilememiştir. Sisteaminin şelatör etkisinin ölümde rolü olduğu düşünülebir. Ölüm akut toksisite ya da bireysel duyarlılık gibi başka nedenlerden de kaynaklanabilir. Sisteamin verilen grupta tümör oranının düşük bulunması, ilacın antioksidan, antimutajenik etkilerine; ayrıca hücre içi tampon görevi görme ve enerji metabolizmayla olan ilişkilerine bağlanabilir. Bütile edilmiş hidroksile toluen gibi antioksidanlar 3-metilkolantrenin genotoksik etkilerini azaltmaktadır. Bu nedenle besinlerle alınan antioksidanların tümör olaylarını düşüreceği ileri sürülmektedir [11]. Polifenol ve diğer antioksidanlar oksidatif stresi azaltıp, genotoksisite ve kanser riskinin düşmesine neden olmaktadır [30]. Sisteamin, sistein ve diğer sistein bileşiklerinin antiklastojenik etkileri gösterilmiştir. Bu konuda sistein ile sisteamin arasında aynı yönde etkileşme bulunduğu bildirilmektedir [31]. Bu etkilerin ortaya çıkmasında doğrudan klastojenlerle reaksiyona girmenin bir rolü olduğu düşünülmektedir. Antimutajenlerin kanser riskini düşürebileceği belirlenmiştir [32]. Sisteaminin antimutajenik etkilerinin olduğu bilinmektedir. Sisteamin vücutta enerji üretimi için gerekli olan Coenzim A'nın yapısına girmektedir (sisteamin, beta-alanin, pantonoik asit ve 3'-fosforileadenodindifosfat) [16]. Pantotenik asit ile sisteamin pantetein olarak adlandırılmaktadır. Pitari et al.[33] yaptıkları çalışmada farelerde timik hücreler tarafından sentezlenen hücre membranına bağlı vanilin I molekülünün panteteinaz aktivitesine sahip olduğu gösterilmiştir. Memeli ve kuşların karaciğer ve böbreklerinde de bu enzimin aktivitesi belirlenmiştir. Panteteinaz enzimi CoA'da bunan panteteini antioksidan ve antiklastojen etkili siteamine hidrolize etmektedir. Benzer bir araştırma Kaskow et al.[34] tarafından yapılmıştır. Vanin molekülünün Panteteinaz (ya da Pantetein hidrolaz) gibi görev yaptığı belirlenmiştir. Vaninin farelerde sisteamin düzeyini yükseltiği ve yangısel beyin hasarlarını önlediği bildirilmektedir.

Kükürtlü bileşiklerin koruyucu etkileri diğer araştırıcılar tarafından da çalışılmıştır. Juan et al.[35] yaptıkları çalışmada 3-metilkolantrenin endotel lezyonlar sonucu arterosklerotik değişikliklere ve koroner vaskülojenezde azalmaya neden olduğu, verilen N-asetilsisteinin hücre proliferasyonunu inhibe ettiği, ancak anjiogenezi etkilemediğini belirlemiştir.

Sisteaminin 3-MC genotoksisitesinden koruyucu etkisi sistein üzerinden glutationa dönüşmesinden kaynaklanabileceği gibi doğrudan taşıdığı SH gruplarıyla da ilişkili olabileceği düşünülmektedir. Grachev et al.[36] yaptıkları çalışmada siteaminin hidroksile pirimidin bazları ile birleştiğini ortaya koymuştur (timin, urasil). Bu durumun genetik materyalde hidroksile radikallerin toksisitesinde bir azalmaya neden olabileceği söylenebilir. Alkile edici ajanlardan olan iodoasetat ve iodoasetamidin SH grupları ile birleşerek panteteinaz aktivitesini irreversibil olarak inhibe ettiği bildirilmektedir [33]. Bu durum doğal olarak sisteamin, sistein ve glutatiyon düzeyinde bir azalmaya neden olmaktadır. Kanser hücreleri normal hücrelere göre daha fazla aminoasit, DNA yapısına giren bazlara ve enerjiye ihtiyaç duymaktadır. Daha çok metiyonin kullanılacaktır. Bazı kanser türlerinde tiamin yetersizliği söz konusudur. Tiamin piruvat dehidrojenaz enziminin ko faktörüdür ve pirüvik asitin asetilCoA üzerinden TCA siklusuna girmesinde rol oynamaktadır. Adenin, guanin ve pentoz şekerlerin artması bir adenozin bileşiği olan CoA sentezinin çoğalmasına neden olmaktadır. Bu durumda daha fazla sisteamin bağlanacağından sistein ve glutatiyon düzeyleri düşecektir. Sonuçta kanser hastaları toksik ajanlara karşı daha savunmasız kalacaktır. Metiyonin esansiyel bir aminoasittir. Metil grubunu tetrahidrofolata aktararak L-hemosistein üzerinden serinle birleşip sistetione ve sonra sisteine dönüşmektedir. Sisteaminin apoptozisde de rolü olabileceği ileri sürülmektedir. Sanina et al.[37] yaptıkları bir çalışmada, nitrik oksitin hemoglobin, deoksihemoglobin ve sitokrom oksidazlardaki bağlantısının sisteamin ile kompleks yaptığı ve bu bağlantının insanlarda eritroblastik lösemide apoptozisi indüklediği bildirilmektedir.

Sonuç olarak, sisteaminin 3-MC ile indüklenen fibrosarkoma karşı fareleri istatiksel açıdan önemsiz derecede de olsa koruyabildiği dikkati çekmektedir. Elde edilen bu sonucun daha geniş araştırmalarla doğrulanması gerektiği önerilmektedir.

KAYNAKLAR

1. Traş B: Genetik Toksikoloji. **In,** Kaya S, Pirinçci İ, Bilgili A (Eds): Veteriner Hekimliğinde Toksikoloji. 2. Baskı, s. 647-670. Medisan Yayınları No: 53, Ankara, 2002.

2. Baijal PK, Fitzpatrick DW, Bird RP: Phenobarbital and 3-methylcholanthrene treatment alters phase I and phase II enzymes and sensitivity of the rat colon to the carsinogenic activity of Azoxymethane. *Food and Chem Toxicol,* 35, 789-798, 1997.

3. Doğan A: Toksikoloji. Ders Notları. Kafkas Üniversitesi Veteriner Fakültesi Farmakoloji ve Toksikoloji Anabilim Dalı, Kars, 2012.

4. Kaya S, Ünsal A: Zehirlerin etki şekilleri ve koruyucu makanizmalar. **In,** Kaya S, Pirinçci İ, Bilgili A (Eds): Veteriner Hekimliğinde Toksikoloji. 2. Baskı, s. 149-179, Medisan Yayınları No: 53, Ankara, 2002.

5. Pirinçci İ: Zehirlenme tipleri ve zehirlerin etkileri. **In,** Kaya S, Pirinçci İ, Bilgili A (Eds): Veteriner Hekimliğinde Toksikoloji. 2. Baskı, s. 9-14, Medisan Yayınları No: 53, Ankara, 2002.

6. Ünsal A, Kaya S: İlaçların istenmeyen etkileri. **In,** Kaya S, Pirinçci İ, Bilgili A (Eds): Veteriner Uygulamalı Farmakoloj. Cilt 1, 2. Baskı, s. 163-173, Medisan Yayınları No: 41, Ankara, 2000.

7. Sacu D, Bildik A: Deneysel olarak fibrasarkom oluşturulan ratların İnterlökin 6 (IL-6) ve Tümör Nekrozis Faktör-α (TNF-α) düzeylerinin belirlenmesi. *Kafkas Univ Vet Fak Derg,* 15 (5): 681-686, 2009.

8. Sozmen M, Tunca R, Erginsoy SD: Cyclin a expression in associated whit apoptosis and mitosis in murine 3-methylcholanthrene-induced fibrosarcomas. *Exp and Toxicol Pathol,* 61, 41-49, 2009.

9. Polat F, Turaçlar N, Gül E, Özdemir Ö, Bingöl G: Mutation analysis of the proto-oncogenes ki-rasand c-myc in the soft tissue tumors of the rats that were format by 3-methylcholanthrene *in vivo*. *DÜ Fen Bil Enst Derg,* 17, 11-18, 2008.

10. Forth W, Henschler D, Rummel W: Pharmakologie und Toxikologie. 4. Völlig neu bearbeitete auflage, Bibliographisches Institut, Mannheim, 1983.

11. Polat F, Özdemir Ö, Elagöz Ş: Analysis of ki-ras exon 2 gene mutations in 3-methylcholanthrene and butylated hydroxytoluene-induced rat lung tissues. *T J Biol,* 32, 277-282, 2008.

12. Murphy SE, Nunes MG, Hatala MA: Effects of phenobarbitale and 3-methylcholanthrene ınduction on the formation of tree glucronide metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNK. *Chem-Biol Interact,* 103, 153-166, 1997.

13. Celius T, Pansoy A, Mathews J, Okey AB, Henderson MC, Krueger HS, Williams DE: Flawin-containing monooxygenase-3: Induction by 3-methylcholanthrene and complex regulation by xenobiotic chemicals in hepatome cells and mause liver. *Toxicol and Appl Pharmacol,* 247, 60-69 2010.

14. Kuroda M, Oikawa K, Yoshida K, Takeuchi A, Takeuchi M, Usui M, Umezawa A, Mukai K: Effects of 3-methylcholanthrene on the transcriptional activity and mRNA accumulation of the oncogene hWAPL. *Cancer Let,* 221, 21-28, 2005.

15. Lemaire B, Beck M, Jaspart M, Debier C, Calderon PB, Thome JB, Rees JF: Precision-cut liver slices salmo salar as a tool to investigate the oxidative of CYP 1A-mediated PCP 126 and 3-methylcholanrthrene metabolism. *Toxicol in Vitro*, 25, 335-342, 2011.

16. Bentley R: From reaktive C2 unitsto acetyl coenzyme A: A long trail with an acetyl phoshate detour. *Trends Biochem Sci,* 25 (6): 302-304, 2000.

17. Miller SL, Schlesinger G: Prebiotic syntheses of vitamin coenzymes: I. Cysteamine and 2-mercaptoethanesulphonic acid (Coenzym M). *J Mol Evol,* 36, 302-307, 1993.

18. Wilmer, M J, Kluijtmans ,AJ, Van Der Welden TJ, Willems PH, Scheffer PG, Masereeuv M, Monnens LA, Van Den Hauvel LP, Leftchenko EN: Cysteamine restores glutatione redox status in cultured cystinotic proximal tubular epithelial cells. *Biochim Biophysi Acta,* 1812, 643-651, 2011.

19. Basouw M, Levtchenko E: Pharmacokinetics of cysteamine in cystinosis patient treated with hemodialysis. *Pediatr Nephrol*, 26, 639-640, 2011.

20. Wenner WJ, Murphy JL: The effects of cysteamine on the upfer gastrointestinal tract of children with cystinosis. *Pediatr Nephrol,* 11, 600-603, 1997.

21. Broyer M, Tete MJ, Guest G, Bertheleme JP, Labrousse F, Paisson, M: Clinical polymorphysim of cystinosis encephalopathy. Results of treatment with cysteamine. *J Inher Methab Dis,* 19, 65-75, 1996.

22. Ahren B, Böttcher G, Ekman R, Sundler F: Cysteamine and the endocrine pancreas: Immunocytochemical, immunochemical and fonctional aspects. *Cell Tissues*, 256, 159-166, 1998.

23. Vecsei L, Ekman R, Alling C, Widerlöv E: Influence of cysteamine and cysteine on open-field behaviour, and on brain concentration of catecholamine, somatostatin, neuropeptide Y and corticotropin releasing hormone in the heart. *J Neuoral Transm,* 78, 209-220, 1989.

24. Luna LG: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. Third ed., Mc Graw-Hill Book Comp, 1968.

25. Reynaud S, Duchiron C, Deschaux P: 3-Methylcholantrene inhibits lymphocyte proliferation and increases intracellular calcium levels in common carp (*C. caprio* L). *Aqua Toxicol,* 63, 319-331, 2003.

26. Donovan PJ, Smith GT, Nardone R: The mutagenic effect of 7,12-dimethylbenz (a)anthacene, 3-methylcholanthrene and benzo(a) pyrene to the developing syrian hamster fetus measured by an *in vivo/in vitro* mutation assay. *Mut Res,* 554, 111-117, 2004.

27. Lutz CT, Browne G, Petzold CR: Methycholantrene causes increased thymocyte apoptosis. *Toxicology,* 128, 151-167, 1998.

28. Jacobus JA, Wang B, Maddox C, Esch H, Lehmann L, Robertson LW, Wang K, Kirby B, Ludewig G: 3-Methylcholantrene (3-MC) and 4-chlorobiphenyle (PCB3) genotoxicity is gender-related in fischer 344 transgenic rats. *Environ Int,* 36, 970-979, 2010.

29. Keshava N: Tumorigenicity of morphologically distinct transformed foci induced by 3-methylcholantrene in BALB/c 3-T3 cells. *Mut Res*, 447, 281-286, 2000.

30. Rastogi S, Shukla Y, Paul BN, Chowdhuri K, Khanna SK, Das M: Protective effect of ocimum sanctum on 3-methylcholantrene, 7,12-dimethylbenz(a)anthrachene and aflatoxin B1 induced skin tumorigenesis in mice. *Toxicol Appl Pharmacol,* 224, 228-240, 2009.

31. Gebhard H. The Anticlastogenic effect of various combinations of cysteamine, AET, HCT and amino acids on choromosome damage by trenimon and bleomycin in human lymphocytes *in vitro. Hum Genet*, 43, 185-203, 1978.

32. Hoffman GR, Shorter RA, Quaranta RL, McMaster PD: Two mechanisms of antimutagenicity the aminothiols cysteamine and WR 1065 in *Saccharomyces sereviciae. Toxicol in Vitro,* 13, 1-9, 1999.

33. Pitari G, Malergue F, Martin F, Philippe JM, Massucci MT, Chabret C, Maras B, Dupre S, Naquet P, Galland F: Panteteinase activity of membrane-bound vanilin 1: Lack of free cysteamine in tissues of vanilin 1 deficient mice. *FEBS Letters*, 483, 149-154, 2000.

34. Kaskow BJ, Proffit JM, Blangero J, Moses EK, Abraham LJ: Diverse biological activities of the vasculer non-inflammatory molecules. The vanin pantetheinases. *Biochem Biopys Res Com,* 2011 (article in press).

35. Juan S.H, Lee JL, Ho PY, Lee YH, Lee WS: Antiproliferative and antiangiogenic effects of 3-methylcholantrene an aryl-hydrocarbon reseptor agonist in human umbilical vascular endothelial cells. *Euro J Pharmacol,* 530, 1-8, 2006.

36. Grachev S A, Kropachev EV, Litvyakova GI: Synthesis of 5-s-cysteamine-6-hydroxytymine and evidence of its formation in the gama radiolysis of aqueous solutions of tymine and cysteamine. *UDC,* 541 (15): 1595-1599, 1984.

37. Sanina NA, Syrtsova LA, Psikha BL, Shkondina NI, Rudneva TV, Kotelnikov AI, Aldoshim SM: Ferrocytochrome C and deoxyhemoglobin in the reaktion with the iron cysteamine nitrosyl complex. *Russian Chem Bull Int Ed,* 59 (10): 1944-1948, 2010.

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RESEARCH ARTICLE

Unbiased Estimate of the Epithelial Cell Number and Epithelial Cell Nuclear Volume in the Bulbourethral (Cowper) Glands of Holstein Bulls - A Stereological Study

Murat Sırrı AKOSMAN * Vural ÖZDEMİR * Hasan Hüseyin DEMİREL **
Johnnie Bremholm ANDERSEN ***

* Department of Anatomy, Faculty of Veterinary, University of Afyon Kocatepe, ANS Kampüsü, TR-03200 Afyonkarahisar - TURKEY
** Department of Pathology, Faculty of Veterinary, University of Afyon Kocatepe, ANS Kampüsü, TR-03200 Afyonkarahisar - TURKEY
*** Stereology and Electron Microscopy Laboratory and Centre for Stochastic Geometry and Advance Bioimaging, Aarhus University Hospital, Aarhus - DENMARK

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Summary

The bulbourethral gland (or Cowper's gland) plays an important role for fertility. It is, therefore, essential to have quantitative data about the morphological and histomorphological structure of this gland in nonpathological conditions. In the present study, Holstein bull's bulbourethral glands were collected, and the volume of the glands, total epithelial cell number and epithelial cell's nuclear volume was estimated for the first time by stereological methods. The smooth fractionator technique for epithelial cell counting was used. Epithelial cell's nuclear volume was estimated on vertical sections. The mean number of the epithelial cells (coefficient of error; CE) and the mean epithelial cell's nuclear volume were found $322x10^7$ (0.1) and 59.1 μm^3 (*SD*=3.7) respectively. Finally, the present studies stereological findings are in the acceptable range.

Keywords: *Bulbourethral gland, Cell number, Nuclear volume, Smooth fractionator, Stereology*

Holştayn Boğalarda Bulbourethral (Cowper) Bezin Epitel Hücre Sayısı ve Epitel Hücre Çekirdeği Hacminin Hesaplanması - Stereolojik Çalışma

Özet

Glandula bulbourethralis'in fertilitedeki önemli rolünden dolayı bu eklenti bezinin sağlıklı hayvanlarda morfolojik ve histomorfolojik yapısının sayısal değerleri önemlidir. Bu çalışmada, Holştayn ırkı boğalardan alınan gl. bulbourethralis'lerin hacim, toplam epitel hücre sayısı ve epitel hücre çekirdeklerinin hacimleri ilk kez stereolojik metotlar kullanılarak hesaplandı. Epitel hücre sayımı için smooth fractionator tekniği kullanıldı. Epitel hücrelerin çekirdek hacimleri vertikal kesitler ile saptandı. Epitel hücrelerinin sayısı $322x10^7$,(CE:0,1) ve epitel hücre çekirdek hacmi 59.1 μm^3 (*SD*=3.7) olarak hesaplandı. Elde edilen bulguların CE ve SD değerleri normal sınırlar içinde olduğu görüldü.

Anahtar sözcükler: *Çekirdek hacmi, Gl bulbourethralis, Hücre sayısı, Smooth fractionator, Stereoloji*

INTRODUCTION

Sex accessory tissues play a crucial role in the reproductive process [1]. The reproduction process of the ruminants is of considerable economic and biological interest [2]. Therefore, a better understanding of the morphology and function of the reproductive organs in ruminant species is highly desirable. The bulbourethral (cowper's) glands are male accessory sex glands which are present in most mammals but absent in aquatic mammals and a few carnivores [3].

İletişim (Correspondence)
+90 272 2281312/165
vozdemir@aku.edu.tr

The two main bulbourethral glands are situated in the bulbospongial tissue which's found at a dorsolateral location at the caudal end of the urethra masculine [4,5]. The gland has a mucoid secretion that produces by the epithelial cells in the gland and this secretion lubricates the urethra for the semen consistence [4-6]. There is another physiological importance of the cowper's gland which is promoting the semen coagulation. In the absence of the coagulant, spermatozoa are not transported through the cervix to the site of fertilization [3,4,7].

Most of studies on bulbourethral glands have dealt with ultrastructural or histological aspects; only a few provided relative or total glandular cell numbers which must be correlated with the amount of secretion the tissue can produce. Thus, it seems important not only to establish methodological guidelines for accessing the total number and nuclear volume of glandular cells, but also to obtain baseline values in non-pathological conditions. In order to reach these values, stereological methods must be preferred for the researchers. Stereological tools are unbiased, precise and in this case independent of shrinkage and can estimate the total number of cells in an organ [8].

Even though the prominent changes in cell number tumours of the bulbourethral gland rarely occur as opposed to the prostate gland. This is hard to explain because both glands are from the same developmental anlagen, the urogenital sinus, and are driven by dihydrotestosterone [1]. It is unknown whether the difference in the pathology between these two glands resides in intrinsic factors within the gland or with extrinsic environmental or pathological factors. The answers to these questions may provide important insight into the carcinogenesis and/or prevention of prostate cancer [1].

The purpose of the present study was to determine the number and the nuclear volume of the glandular epithelial cells in the bulbourethral glands by means of design-based stereological methods.

MATERIAL and METHODS

Animals

This study was performed using 7 healthy Holstein bull's paired (2.5 - 3.0 years, 650-700 kg) bulbourethral glands. All glands were removed immediately from their carcass after slaughter in abattoir.

Tissue Preparation and Sampling Protocol

The organs total volume measurement was performed on seven paired bulbourethral glands, their epithelial cell's number estimated on the five (3 left and 2 right) bulbo-urethral glands secretory epithelium cells and their epithelial cell's nuclear volume estimation performed on the five (2 left and 3 right) bulbourethral glands secretory epithelium cells. Beside these measurements, glands residual parts and two paired remained glands were embedded in paraffin and cut into 4 µm thicknesses by rotary microtome (Leica RM 2155) and stained with hematoxylin-eosin for pathological evolution.

All glands were weighed (Kern, Balingen-Germany) and the lengths and the diameters measured by vernier caliper (Labomar, 304B-01- Turkey). The volumes of the glands were estimated by water displacement method. After this, all glands were immersion fixed in neutral buffered 10% formalin for two weeks.

For cell counting; 3 left and 2 right glands were sliced into 3 mm intervals (the first cut at a random distance between the edge and 3 mm) and every second slab was chosen giving rise to a fraction of ½ *Fig. 1 A-B*. These slabs were placed according to the smooth fractionator design [9], smaller slabs were placed peripherally; larger ones were placed centrally and they were totally embedded in paraffin. The whole length of the tissue in the paraffin block was measured and was divided into 9 equal pieces by cutting in 8 equidistant intervals. First cut on the paraffin block was performed randomly into first interval length and then continue cutting same intervals to obtain 9 equal pieces. Every piece was turned 90° to the same side and all pieces were re-embedded paraffin in a metal block holder. This paraffin block was exhaustively sliced in 40 µm sections by rotary microtome (Leica RM2155) and slices were taken onto glass slide which pass through the center of the tissues in the paraffin block and were stained by Giemsa *(Fig. 1)*.

To estimate the epithelial cell's nuclear volume on vertical sections, 2 left and 3 right glands were sliced into 3 mm intervals (again the first cut at a random distance between the edge and 3 mm) and every second slab was chosen with a random start [10]. The sampled slabs were cut into 3 mm bars with a random start and every 5th bar was chosen. The vertical axis of the bars was determined, and then the bars were rotated freely around this axis and embedded in paraffin. A 40 µm thick through the center of the tissue in the paraffin block was held for the volume estimation of the nuclei [11].

Stereological Analysis

The counting was performed with epithelial cell nucleus as counting units. The number of the epithelial cells were estimated using the computer software loaded Shtereom I [12], Olympus BH2 light microscope with motorized stage (Lang MS 316) (for the step lengths on the X, Y axis) and 3.2 MP Cmex camera (Euromex, Holland), under x100 oil-immersion lens objective. The thickness of the tissue measured and the movements in the Z axis was controlled using a microcator (Heidenhain, Germany). For counting the nearly 100-200 nucleus per gland according to disector principle [13], area of the counting frame was 91 µm^2 and the step lengths for the X and Y axis was 1250 µm. The height of the disector was 10 µm.

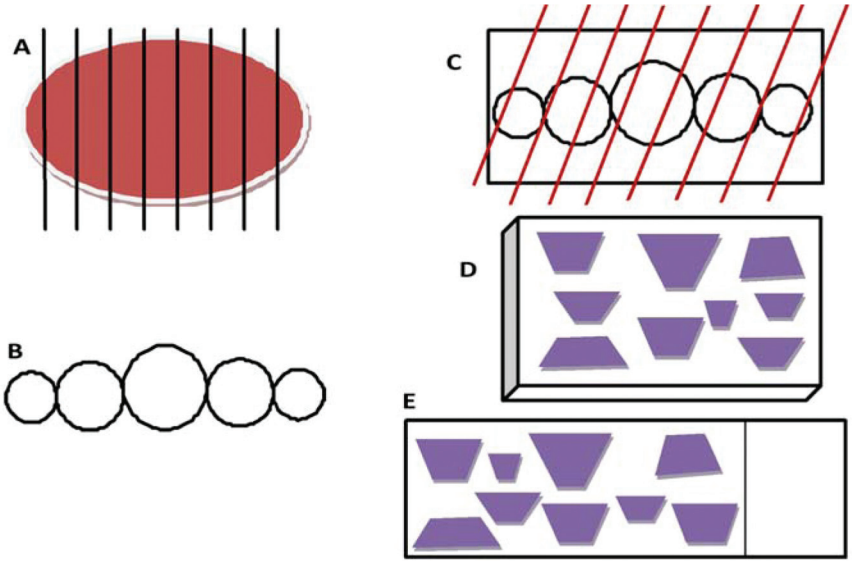

Fig 1. Diagram of the baseline sampling scheme: **A-** The gland was sectioned and every second slab was systematically sampled, **B-** The slabs were arranged according the smooth fractionator principle and totally embedded in paraffin, **C-** The length of the tissue was measured, divided by eight, started to cut with a random start **D-** Nine piece were totally embedded in paraffin at metal holder, **E-** The counting procedure was performed on the tissues which pass through the centre

Şekil 1. Örnekleme prosedürünün şeması: **A-** Bez eşit aralıklarla dilimlenerek sistematik olarak her ikinci dilim seçildi, **B-** Dilimler smooth parçalama prensibi gereğince sıralanarak hep birlikte parafine gömüldü, **C-** Parafin blok içerisindeki dokunun uzunluğu ölçülerek 8'e bölündü ve eşit bırakılan aralıkta rastgele bir başlangıç yapılarak kesilmeye başlandı, **D-** Kesim işlemi sonucunda elde edilen 9 parça birlikte parafine gömüldü, **E-** Dokuların merkezlerinden geçen kesitler değerlendirilerek sayım yapıldı

The nuclear volume estimation was performed on epithelial cell nuclei which came into focus within the counting frame and in the disector height. The orientation frame and the transparent test probe were used for managed the ruler. Area of the counting frame, the step lengths for the X and Y axis, the height of the disector, the magnification of the Cmex, the monitor's final magnification and the total final magnification was; 150 µm^2, 1250 µm, 10 µm, x0.4, x49, x1956 respectively.

The total cell number was estimated according to following formula [14].

$N= 1/F_{ssf} \times 1/F_{slab} \times 1/F_{asf} \times 1/F_{hsf} \times \Sigma Q^-$

F_{ssf}; is the section sampling fraction (ssf =1/2)

F_{hsf}; is the height sampling fraction; the mean section thickness divided to the height of the disector (~20 µm /10 µm)

F_{asf}; is the area sampling fraction; multiplying of the x-y step lengths together and divided to the frame area ((1250 µm x 1250 µm)/91 µm),

F_{slab}; is the obtained value of the whole tissue length in the paraffin block when divided by 8, and this value converted to micrometer and again divided to the height of the slicing section (40 µm).

ΣQ^-; is the number of the counted epithelial cell nucleus

The coefficient of error for the number of counted nucleus and the section thickness was estimated altogether according to formulas below [13]:

$$CE= \frac{\sqrt{Var(noise)+Var(SRS)}}{\sum Q^-}$$

$Var(noise)= \Sigma Q^-$

$$Var\ (SURS)= \frac{3(A-Var(noise))-4B+C}{240}$$

$$CE(N)= \sqrt{CE^2(Di\,sec\,tor)+CE^2(t)}$$

The statistical significance between the left and right glands for the mean weight and mean length were investigated with the Wilcoxon test.

RESULTS

Qualitative findings

All examined bulbourethral gland tissues displayed a normal histology and the epithelial cells were clearly observed at every depth in all the sections throughout the glandular tissue. Almost all of epithelial cells had clear cytoplasmic spots (*Fig. 2* and *3*).

The nuclei of the epithelial cells exhibited their typical morphology, prominent membrane because the chromatin and heterochromatin areas on the nucleus.

Quantitative Findings

The average length and the average weight of the glands were 3.1 cm (*SD = 0.4*) and 7.7 g (*SD = 1.7*) respectively. The mean front width (1.0 cm, *SD = 0.1*) and the mean front height (1.6 cm, *SD = 0.3*) were smaller than the mean back width (1.8 cm, *SD = 0.2*) and mean back height (2.2 cm, *SD = 0.2*). These calculations were performed with seven coupled gland and represented in *Table 1* and *Fig. 4*.

There were no significant statistical difference between the mean weight and mean length of the left and right glands.

The number of epithelial cells from each bull is presented in table 2 -the mean number of the epithelial cells was found 3.224.738.050, which also includes the estimates of the CE (N) per animal. The mean coefficient of error for the cell number and tissue's final thickness were 0.1 altogether.

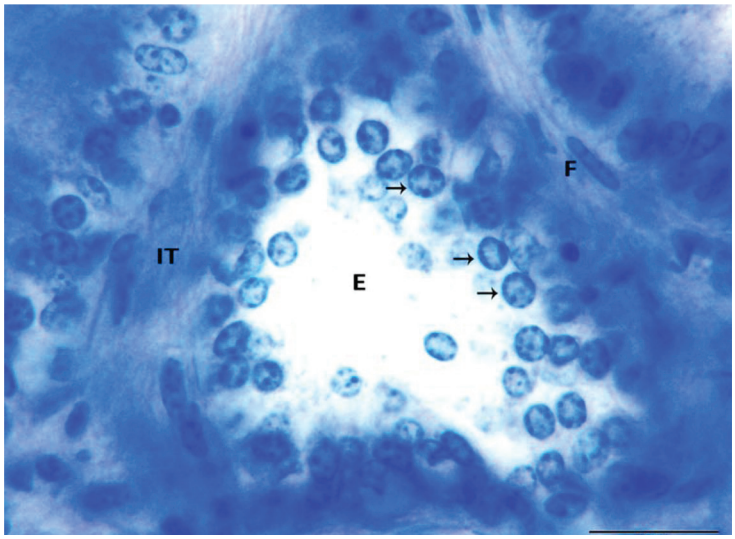

Fig 2. Image from a 40 µm thick section (x100). Arrows indicate the epithelial cells, **(E)** Endpiece, **(F)** Fibroblast, **(IT)** Intestitiel tissue

Şekil 2. 40 µm kalınlığında bir örneğin resmi (x100): Oklar epitel hücreleri göstermektedir, **(E)** Endpiece, **(F)** Fibroblast, **(IT)** Intestitiel tissue

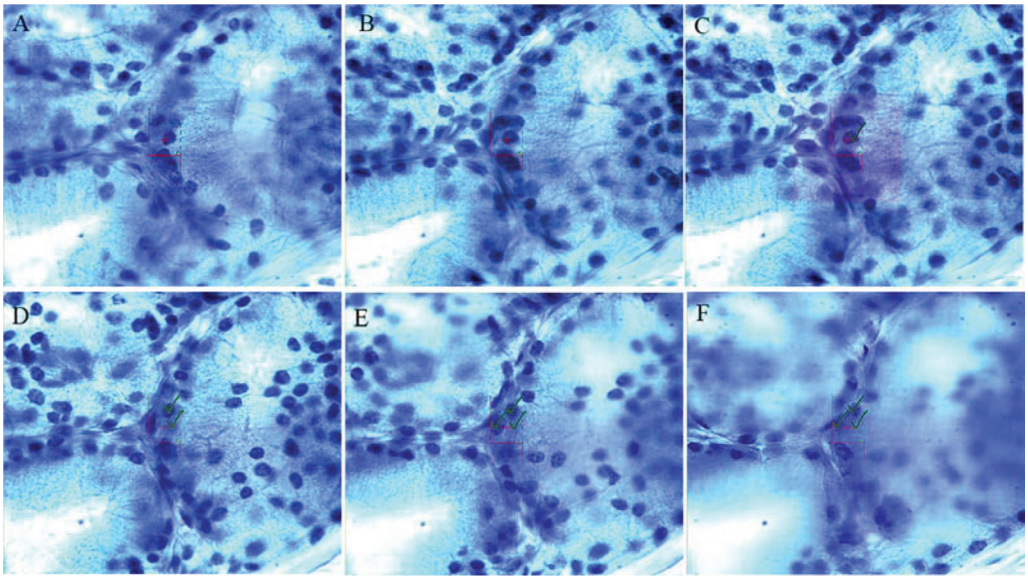

Fig 3. Cell counting was performed in optical disectors placed with a fixed sampling period along the centre line counting Giemsa stained nuclei's: **A-** (0 µm), **B-** (4 µm), **C-** (6 µm), **D-** (10 µm), **E-** (13 µm), **F-** (20 µm) The area of the counting frame is 91 μm^2

Şekil 3. Optik disektör kullanılarak güven aralığı içerisinde yapılan hücre sayım işlemi: **A-** (0 µm), **B-** (4 µm), **C-** (6 µm), **D-** (10 µm), **E-** (13 µm), **F-** (20 µm) Sayım çerçevesinin alanı 91 μm^2dir

Fig 4. The bulbourethral gland with a scale bar

Şekil 4. Bulbourethral bezin ölçekli görünümü

Total volumes, measured by water displacement method, of the glands were 7.12 cm^3 (*SD=1.5*).

The mean volume of the epithelial cell nuclei was 59.1 μm^3 (*SD=3.7*).

The total shrinkage in the z axis was calculated with the microcator for the thickness of the sliced tissue on the slide by making depth measurements on the counting areas and was found 44.5 % and the mean final thickness of the sliced tissue's was found 22.2 µm (*SD=0.97*).

Table 1. *The morphometric information of bulbourethral glands.*
Tablo 1. *Bulbourethral bezlerin morfometrik değerleri*

No	Side	Weight (g)	Length (cm)	Front width	Back width	Front height	Back height	Side	Weight (g)	Length (cm)	Front width	Back width	Front height	Back height
1	left	6.6	3.0	0.8	1.6	1.7	2.1	right	6.2	2.8	0.9	1.6	2.0	2.2
2	left	9.8	2.6	1.0	2.0	1.5	2.5	right	10.0	2.8	1.0	2.0	1.6	2.4
3	left	9.6	3.4	1.0	1.9	1.4	2.5	right	9.3	3.4	1.1	2.1	1.4	2.2
4	left	6.5	3.2	1.0	1.7	1.5	2.1	right	6.1	3.1	1.1	1.6	1.7	2.1
5	left	5.9	2.8	0.9	1.7	1.6	2.2	right	5.1	2.7	0.8	1.6	1.1	1.8
6	left	8.3	3.5	1.2	1.7	2.0	2.3	right	8.7	3.6	1.1	1.7	1.5	2.3
7	left	7.1	3.2	1.1	1.7	1.6	2.0	right	7.5	3.5	0.8	1.8	1.3	2.2
mean		7.7	3.1	1.0	1.8	1.6	2.2		7.6	3.1	1.0	1.8	1.5	2.2
SD		1.6	0.3	0.1	0.1	0.2	0.2		1.8	0.4	0.1	0.2	0.3	0.2

Table 2. *The mean epithelial cell number of the bulbourethral glands.*
Tablo 2. *Bulbourethral bezlerin epitel hücre sayıları*

Gland No	Epithelial Cell Number	CE	CV
1	$3.86.10^9$	0.095	
2	$2.39.10^9$	0.105	
3	$3.49.10^9$	0.102	
4	$2.80.10^9$	0.102	
5	$3.55.10^9$	0.098	
Mean	$3.22.10^9$	0.100	0.19

DISCUSSION

At the histological level in goats, boars and buffalo, Type I and Type II cells are observed in the entire glandular complex, though Type I cells dominate within the cranial disseminate glands, and Type II cells dominate within the bulbourethral glands [15-17]. However, this study is not related with the types of the cells, just performed on the secretory portion of the bulbourethral gland. The number or abundance of the cells in the tissue is the important fundamental information in non-pathological conditions to recognize tumours.

From the morphometrical aspect, Abdel-Razek and Ali [18] were measured the length of the 2 years old bull's bulbourethral glands and they found 1.2-2.1 cm long, however we found 3.1 cm long on the 2.5-3 years old bulls bulbourethral glands.

In this study, we showed the epithelial cell number and the epithelial cell's nuclear volume in the bull's bulbourethral gland. However we detect that there is a lack of any other studies releated with the number and nuclear volume on this gland for to compare our results. We applied design based stereological methods for the cell counting and nuclear volume estimation. The optical fractionator is a precise, unbiased and modern stereological method which combines the optical disector with the fractionator principle [14]. The optical disector is a 3D probe for particle counting within a thick section [13,14,19,20]. The fractionator is a sampling design that samples the organ systematic and in known fractions randomly [14,21]. The smooth fractionator technique [9], which we performed on certain parts of the present study, is even more efficient than the original method [22]. In order to increase efficiency, the items arrange (slab, sections, etc.) in a symmetric design with one peak and minimal jumps [9]. In the present study, we applied general and smooth fractionator techniques modification. The application performed as sliced the whole gland into known distance and arranged these slices side by side [9] and totally embedded them into the paraffin and sliced that paraffin block into 9 equal distance pieces and these pieces embedded in a metal block holder and at the final step slices hold on the glass slide which pass through these pieces center. Thus, in order to save time there is no need to slice all selected tissues exhaustively.

Modern design based stereological methods produced unbiased estimates of the mean nuclear volume of arbitrarily shaped particles [11]. Evaluation of the mean cell nuclear volume of an organ is especially important for objective histopathologic malignancy grading. Prognostic significance of the nuclear volume has been reported by Sørensen [23]. The method produces efficient and precise results with highly statistical efficiency [11,23]. This method was here performed with the combination of the vertical sectioning procedure

which is simple and consumed less time than isotropic uniform sectioning [11,24] and enabled us to perform unbiased and very efficient estimations [25] of the cell nucleus. However, the paraffin embedding makes almost 50% shrinkage in the volume [26]. In our study, we estimate that the mean shrinkage for the tissue on the slide was 44.5%.

In general, the error estimation of the method squared divided by the coefficient of variance squared should follow the following relationship: $0.25 < CE^2/CV^2 < 0.5$. If it is below 0.25, you might be working too much, whereas if it is above 0.5 you might be working too little. In our study, we obtained a ratio of 0.29, which is in the suitable range [14,27].

Further studies, currently in progress, are oriented towards supplying a detailed functional interpretation of the secretory epithelium as well as the secretion of the gland.

REFERENCES

1. Chughtai B, Sawas A, O'Malley RL, Naik RR, Khan SA, Pentyala S: A Neglected gland: A review of the cowper's gland. *Int J Androl*, 28, 74-77, 2005.

2. Abou-Elmagd A, Wrobel KH: The periurethral glandular complex in the Water buffalo: An ultrastructural, histological and lectin-histochemical study. *Arch Histol Cytol*, 52 (5): 501-512, 1989.

3. Dyce KM, Sack WO, Wensing CJG: Anatomia Veterinaria. 2nd ed., pp. 193, 567, McGraw-Hill Interamericana, Mexico, 1999.

4. Badia E, Briz MD, Pinart E, Sancho S, Garcia N, Bassols J, Pruneda A, Bussalleu E, Yeste M, Casas I, Bonet S: Structural and ultrastructural features of boar bulbourethral gland. *Tissue Cell,* 38 (1): 7-18, 2006.

5. Campero CM, Ladds PW, Thomas AD: Pathological findings in the bulbourethral glands of bulls. *Aust Vet J,* 65 (8): 241-244, 1988

6. Dursun N: Veteriner Anatomi II. 6th Baskı, s. 152, Medisan Yayınevi, Ankara, Türkiye, 1998.

7. Hart RG, Greenstein JS: A newly discovered role for cowper's gland secretion in rodent semen coagulation. *J Reprod Fertil*, 17 (1): 87-94, 1968.

8. Santos M, Marcos R, Santos N, Malhao F, Monteiro RA, Rocha E: An unbiased stereological study on subpopulations of rat liver macrophages and on their numerical relation with the hepatocytes and stellate cells. *J Anat*, 214 (5): 744-751, 2009.

9. Gundersen HJG: The smooth fractionator. *J Microsc*, 207 (3): 191-210, 2002.

10. Møller A, Strange P, Gundersen HJG: Efficient estimation of cell volume and number using the nucleator and the disector. *J Microsc*, 159 (1): 61-71, 1990.

11. Sørensen FB: Stereological estimation of the mean and variance of nuclear volume from vertical sections. *J microsc*, 162(2): 203-229, 1991.

12. Oguz EO, Conkur ES, Sarı M: Shtereom I simple windows based software for stereology. volume and number estimations. *Image Anal Stereol,* 26, 45-50, 2007.

13. Gundersen HJG, Jensen EBV, Kieu K, Nielsen J: The efficiency of systematic sampling in stereology- reconsidered. *J Microsc*, 193 (3): 199-211, 1999.

14. West MJ, Slomianka L, Gundersen HJG: Unbiased stereological estimation of the total Number of neurons in the subdivisions of the rat hippocampus using the Optical fractionator. *Anat Rec*, 231 (4): 482-97, 1991.

15. Wrobel KH: Untersuchungen zur feinstruktur und histochemie der glandula bulbourethralis der Ziege. *Cell Tissue Res,* 108 (4): 582-596, 1970.

16. Wrobel KH: Histochemische untersuchungen am prostataparanchym der Ziege. *Anat Histol Embryol,* 1 (1): 64-72, 1972.

17. Wrobel KH, Sinowatz F: Vergieichende studien an den anhangsdrüsen der mannlichen Urethra. *Acta Histochem Suppl*, 31, 193-200, 1985.

18. Abdel-Razek KH, Ali A: Developmental changes of bull (Bos taurus) Genitalia as evaluated by caliper and ultrasonography. *Reprod Domest Anim,* 40, 23-27, 2005.

19. Braendgaard H, Evans SM, Howard CV, Gundersen HJG: The total number of neurons in the human neocortex unbiasedly estimated using optical disectors. *J Microsc*, 157(3): 285-304, 1990.

20. Evans S, Janson AM, Nyengaard JR: Quantitative Methods in Neuroscience: A Neuroanatomical Approach. 1st ed., 82, Oxford University Press, USA 2004.

21. Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sørensen FB, Vesterby A, West MJ: Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *Apmis,* 96, 379-394, 1988.

22. Gundersen HJG: Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. *J. Microsc,* 143, 3-45, 1986.

23. Sørensen FB: Objective histopathologic grading of cutaneous malignant melanomas by stereologic estimation of nuclear volume. *Cancer*, 63, 1784-1798, 1989.

24. Baddeley AJ, Gundersen HJG, Cruz-Orive LM: Estimation of surface area from vertical sections. *J Microsc,* 142 (3): 259-276, 1986.

25. Michel RP, Cruz-Orive LM: Application of the cavalieri principle and vertical sections method to lung: Estimation of volume and pleural surface area. *J Microsc,* 150 (2): 117-136, 1988.

26. Dorph-Petersen KA, Nyengaard JR, Gundersen HJG: Tissue shrinkage and unbiased stereological estimation of particle number and size. *J Microsc,* 204 (3): 232-246, 2001.

27. Lodrup AB, Karstoft K, Dissing TH, Pedersen M, Nyengaard JR: Kidney biopsies can be used for estimations of glomerular number and volume: A pig study. *Virchows Arch,* 452, 393-403, 2008.

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RESEARCH ARTICLE

Borrelia burgdorferi s.l. and *Rickettsia* spp. in Ticks Collected from European Part of Turkey

Sirri KAR * Nadim YILMAZER * Kenan MIDILLI ** Sevgi ERGIN ** Aysen GARGILI ***&****

* Namik Kemal University, Department of Biology, 59030 Tekirdag, TÜRKİYE
** Istanbul University, Cerrahpasa Medical Faculty, Department of Microbiology and Clinical Microbiology, TR-34303 Istanbul - TÜRKİYE
*** University of Texas Medical Branch, Department of Microbiology & Immunology, Galveston, Texas - USA
**** Marmara University, Infectious Diseases Epidemiology Research Center, TR-34660 Istanbul - TURKEY

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Summary

This study was performed in ticks collected with the flagging method from localities situated along Anatolian side of Istanbul to the Bulgarian border of Turkey which is under the effect of Black Sea climate. All ticks which were collected seasonally were screened for the presence of *B. burgdorferi* s.l. and *Rickettsia* spp. in pools. As a result, indicated agents were revealed to be common in ticks of studied localities, *Ixodes ricinus* being the predominant tick which was active throughout the year.

Keywords: *Tick, Borrelia burgdorferi s.l., Rickettsia, Turkey*

Türkiye'nin Avrupa Yakasından Toplanan Kenelerde *Borrelia burgdorferi* s.l. ve *Rickettsia* spp. Varlığı

Özet

Bu çalışma, İstanbul'un Anadolu yakasından, Bulgaristan sınırına kadar devam eden, Trakya'nın karadeniz iklimi etkisi altında bulunan alanlardan bayraklama yöntemiyle toplanmış kenelerde yürütülmüştür. Mevsimsel düzende toplanmış olan kene havuzlarında *B. burgdorferi* s.l. ve *Rickettsia* spp. varlığı araştırılmıştır. Sonuç olarak, çalışılan alanlarda ilgili etkenlerin yaygın olarak görüldüğü ve yıl boyu aktivite gösterebilen *Ixodes ricinus*'un en baskın kene olduğu anlaşılmıştır.

Anahtar sözcükler: *Kene, Borrelia burgdorferi s.l., Rickettsia, Türkiye*

INTRODUCTION

Rickettsiosis is a zoonotic disease caused by obligate intracellular gram-negative alphaprotobacteria in the genus *Rickettsia* included in the order Rickettsiales and the family Rickettsiaceae. Belonging to the genus *Rickettsia*, 25 species the pathogenicity of which is certain, and some 20 species with pathogenicity which remains unascertained were reported from the vertebrates. Most of them are included in spotted fever group, and transmitted by ticks. A great number of tick species of different genera are responsible for transmission of one or more rickettsial species [1]. Vector ticks are reservoir for the disease at the same time, but every tick species is not reservoir for every agent [2].

Lyme borreliosis which is caused by *Borrelia burgdorferi* sensu lato (s.l.) complex in the genus *Borrelia*, family Spirochaetaceae, and seen in temperate zone of the northern hemisphere is the most common arthropod-borne disease. *Borrelia burgdorferi* s.l. complex is reported to comprise some 18 genospecies, of which the pathogenities, resulting clinical pictures, and species of vertebrate host and vector ticks are different. In general, vector ticks for transmission of Lyme borreliosis fall within the genus *Ixodes* [3,4].

Ticks are biological or mechanical vectors of many species specific or zoonotic agents in humans and animals. Depending on species and developmental stages, ticks exhibit varying degrees of host selection. However, when they could not quest for their own appropriate host, they

İletişim (Correspondence)
+90 282 2502636
sirrikar@yahoo.com

can use different animals as host. There is not a known tick species which tends to complete its life cycle on humans. On the other hand, from the reported cases to date, we know that 222 tick species, especially 33 of them feed on humans [5,6], and especially some of those display high interest in humans [7,8]. This factor plays an important role in epidemiological characters of tick-transmitted diseases. The data on the tick abundance, frequency of tick-bite cases, and infection prevalence of these ticks in a given region is very significant to determine the related risk for human. This study aims to investigate the presence of *Rickettsia* spp. and *Borrelia burgdorferi* s.l. in ticks collected from some spesific localities in the Thrace region and Istanbul, Turkey seasonally over one year period.

MATERIAL and METHODS

Study Area and Material

The study covered five localities situated along Anatolian side of Istanbul to the Bulgarian border. These areas were selected as a subsample of the natural biotopes of Black Sea climatic effects *(Fig. 1)*. Deciduous oak forest patches with scattered natural or artificial grasslands, shrub and scarce vegetation which are known to be suitable for *Ixodes ricinus* especially were sampled. Although sampling areas differed based on habitat and structure composition, they had common features. Ticks were collected from two foci (Beykoz and Belgrat Forest) monthly and from other foci twice per season in the year 2009.

The flagging method, slightly modified from Ginsberg and Ewing [9], was used to collect ticks. A white, cotton flannel (1x1 m) was dragged through vegetation for 100 m, by checking every 10 to 20 m. Dragging was conducted five to ten times per sampling site in the morning (8.00 am-11.00 am) and in the evening (5.00 pm-8.00 pm) in order to weaken the effects of changing weather conditions in collection days. All ticks were removed from the flag, preserved in 70% ethanol and transported to the laboratory for identification. Adult ticks were identified to species level, while larval and nymphal stages to genus level with the exception of *Hyalomma aegyptium* nymphs under a stereomicroscope [10-12]. The collected ticks were sorted according to the species, site and season of collection, and stored in 70% ethanol at 4°C until further studies.

Pooling of Ticks and DNA Extraction

Of 194 tick groups, 77 groups were selected based on collection sites, species and developmental stages for DNA extraction. Those groups were pooled as follows: two groups of *D. marginatus* adult (one individual in each group), one group of *H. aegyptium* adult (one individual), eight groups of *H. aegyptium* nymphs (1-4 individuals), six groups of *Haemaphysalis* nymphs (1-5 individuals), 32 groups of *Ixodes* spp. nymphs (1-27 individuals), 23 groups of *I. ricinus* adult (1-25 individuals), one group of *R. bursa* (one individual), four groups of *R. sanguineus* group (1-13 individuals).

Ticks of each pool were decontaminated in 70% ethanol, washed with sterile double distilled water, air-dried for a maximum of 10 min, and then homogenized in liquid nitrogen. DNA was extracted with QIAamp DNA Mini Kit (10) [13], and the obtained samples were stored at -80°C until use.

Fig 1. Topographic map of the study area. Stars point out the studied localities (The map was modified from *http://www.arcgis.com*)

Şekil 1. Çalışma alanının topografik haritası. Yıldızlar, çalışılan lokaliteleri işaret etmektedir (Harita *http://www.arcgis.com*'dan uyarlanmıştır)

Semi Nested PCR

OspA gene of *Borrelia burgdorferi* s.l. (Primers; OSPAFw1: ttgggaataggtctaatattagc, BorR: actaatgttttvccatcttc, OSPAFw2: atttcctggaagcttaatgc, PCR conditions; first step: OSPAFw1-BorR, second step: OSPAFw2 BorR, 94°C/2 min (94°C/1 min, 50°C/1 min, 72°C/2 min) 45 cycles, 72°C/10 min) and citrate synthase (gltA) gene of *Rickettsia* spp. (Primers; RickF1: gggttttggtcatcgtgtat, RickR1: cccgaataaaaatcaacatt, RickR2:tctctcaataaaatattcatc tttaag, PCR conditions; first step: Rick F1-RickR1, second step: Rick F1-RickR2, 95°C/2 min (95°C/30 s, 50°C/1 min, 72°C/1 min) 44 cycles, 72°C/5 min) were targeted [14].

RESULTS

A total of 2624 ticks were collected within the scope of the study. Given the percentage and developmental stages, the numbers and species of those ticks were as follows: 2343 (89.29%) *Ixodes* spp. (*I. ricinus* 16.35%, nymph 22.70%, larva 60.95%), 126 (4.80%) *Hyalomma* spp. (*H. aegyptium* 3.97%, nymph 23.02%, larva 73.01%), 106 (4.03%) *Rhipicephalus* spp. (*R. sanguineus* gr. 66.98%, *R. bursa* 2.83%, nymph 11.32%, larva 18.87%), 35 (1.34%) *Haemaphysalis* spp. (*H. inermis* 14.29%, nymph 60.0%, larva 25.71%) and 14 (0.54%) *Dermacentor* spp. (*D. marginatus* 100%). Most of the ticks were obtained over the summer (978 ticks, 37.27%), whereas 223 ticks (8.50%), all *Ixodes* spp., were collected during the winter. It was possible to find *Ixodes* spp. at all developmental stages in both summer and winter seasons; however nymphs were more abundant in spring (44.55%) and winter (27.07%), while larvae in summer (48.25%) and fall (45.17%). The data are summerized in *Table 1*.

We found that 60 of 77 pools yielded positive PCR results for *Rickettsia* spp. (%77.92 positive), and 12 pools for *Borrelia burgdorferi* s.l. (15.58% positive). *Table 2* indicates PCR results.

DISCUSSION

Distribution, density and seasonal activities of tick species depend on vegetation, seasonal parameters and host availability [15]. Forestlands with high rate of precipitation and high humidity (80% at least) are reported to be more suitable for *I. ricinus* especially [6,16]. The preferred study areas which are covered by oak forests are in fact convenient for this tick species; however mean areal values of precipitation (79.0 mm, 75.6 mm, 68.1 mm and 79.2 mm in winter, spring, summer and fall, respectively, as found out from records of Turkish State Meteorological Service) were lower than the averages in the year the study was performed. On the

Table 1. *Tick numbers according to seasonal distribution*
Tablo 1. *Toplanan kene sayılarının mevsimsel dağılımı*

Ticks	Number of Ticks Collected by Seasons				Total
	Spring	Summer	Fall	Winter	
Ixodes spp.					
Larva	40	689	645	54	1428
Nymph	237	34	117	144	532
I. ricinus	219	123	16	25	383
Hyalomma spp.					
Larva	57	35	-	-	92
Nymph	-	28	1	-	29
H. aegyptium	4	1	-	-	5
Rhipicephalus spp.					
Larva	-	20	-	-	20
Nymph	6	6	-	-	12
R. bursa	2	1	-	-	3
R. sanguineus gr.	54	17	-	-	71
Haemaphysalis spp.					
Larva	-	9	-	-	9
Nymph	8	13	-	-	21
H. inermis	5	-	-	-	5
Dermacentor spp.					
D. marginatus	9	2	3	-	14
Total	**641**	**978**	**782**	**223**	**2624**

Table 2. *Semi nested PCR results of tick pools*
Tablo 2. *Kene havuzlarında uygulanan semi nested PCR sonuçları*

Tick Species	Counts of Pools	Tick Numbers in the Pools	Number of *Rickettsia* spp. Positive Pools (%)	Number of *B. burgdorferi* sl. Positive Pools (%)
D. marginatus	2	1	1 (50)	-
H. aegyptium	1	1	1 (100)	-
H. aegyptium nymph	8	1-4	5 (62.2)	1 (12.5)
Haemapysalis spp. nymph	6	1-5	4 (66.7)	-
Ixodes spp. nymph	32	1-27	28 (87.5)	4 (12.5)
I. ricinus	23	1-25	19 (82.61)	7 (30.43)
R. bursa	1	1	1 (100)	-
R. sanguineus gr.	4	1-13	1 (25)	-
Total	**77**	**1-27**	**60 (77.92)**	**12 (15.58)**

other hand, the preferred study areas are under the effect of Black Sea climate, a proxy for high humidity.

Studies show that *I. ricinus* share humid forested places with species of *Haemaphysalis*, another forest tick. It was reported that *Hyalomma* spp. is a typical open field tick, whereas *Dermacentor* spp. and *Rhipicephalus* spp. have characteristics of both tick genera [17]. Except for *H. inermis*, some *Haemaphysalis* species live in more arid regions [18], and *R. bursa* is found in habitat and has period of activity similar to that of *Hyalomma* spp.[7,19]. Throughout the same region, ratio of adults of human biting *H. marginatum*, *H. aegyptium*, *R. bursa*, *R. sanguineus* gr., *I. ricinus*, *H. inermis*, *H. parva* and *D. marginatus* to each other was 4.8:7.7:6.2:1 2.8:65.3:0.1:1.9:1.2 [7,8]. However, that ratio is 0.0:1.0:0.6:14.8: 79.6:1.0:0.0:2.9 in this study. Considering that the previous studies [7,8] have been conducted on human biting ticks, differences between the ratios may have arisen from the human contact opportunity and other environmental determinants in the collection sites.

Ixodes ricinus is active at all times of the year in many regions in Europe. Its adults show peak in spring especially, and the major peak for nymphs occurs in the end of spring, starting up with the beginning of the season. However, emergence of larvae takes place a couple of months after nymphs, although it may change according to region [4,20]. Adults and nymphs were seen in spring in our study as well, and larvae showed a second peak in fall, as confirmed by the study of Randolph et al.[21]. Yet, a 10°C temperature in the environment is reported to be threshold for larvae, and other developmental stages are affected by cold weather, the degree of affection being lower [20]. Seasonal temperature average were 8.2°C, 12.2°C, 23.8°C and 16.8°C in winter, spring, summer and fall, respectively in the study area during the year 2009, and ticks of three developmental stages were found in every season. We could not find any ticks in areas of grassland and scarce vegetation at temperatures below 10°C, but it was possible to collect them from the places covered by oak leaves. Therefore, it was obvious that fallen oak leaves protected ticks against the unfavourable weather conditions at certain level.

Many tick species are known to play role in transmission of the rickettsial agents in the spotted fever group [22,23]. Prevalence of this agent ranges from 1.6% to 67% [24-27] in European populations of *I. ricinus*, and 1% to 84.57% in other tick species [2,28]. In Turkey, almost %50 *Rickettsia* spp. positivity was detected in ticks other than *Ixodes* spp. collected from domesticated animals in different regions [29]. In two serological surveys of people in risk groups from Black Sea [30] and Mediterranean regions [31] of the country, seropositivity was found as 11.7% and 13.7%, respectively. However, rickettsial infections are rarely seen (3-5 annual cases per million), and cases are mostly from coastal provinces and Thrace region, which was sampled in the present study, being in the lead. In addition, *Rickettsia conorii* subsp. *conorii* was detected in clinical cases [32] from Thrace region, which is the only reported species from human cases in the country.

Overall, 77.92% of tick pools tested was found to be positive for *Rickettsia* spp. in the present study, and *Ixodes* spp. nymphs and *I. ricinus* adults showing higher percentages (87.5% and 82.61%, respectively) than the other tick species. Even though the primers used were specific to infectious *Rickettsia* spp.[14], molecular techniques employed in analysis of restricted regions of relevant gene are known to be conflictual, and thus examining of more than one gene region is suggested, especially in case of facultative endo-symbiont *Rickettsia* spp.[27]. On the other hand, importance of spotted fever group rickettsiae in vertebrates is a little known fact [33], although some endosymbiont species are vertically transmitted among the ticks with no involvement of a vertebrate [34]. Detailed molecular analyses indicated that *Rickettsia* spp. is a more complex group than expected [1]. Moreover, although many *Rickettsia* species isolated from ticks have not yet been implicated in human pathology, it was reported that these ricketsiae should be considered as potential pathogens [35,36].

As to studies on the prevalence of *Borrelia burgorferi* s.l. in *I. ricinus* in Europe, positivity was found to be 0-2.9%,

8.9-10.1% and 2-56.3% in larvae, nymphs and adults, respectively [37-40]. From the studies carried out with different methods 4-38.7% positivity was reported in field-collected *I. ricinus* in different regions in Turkey [41-43]. In fact, the relevant agent was detected in *Ixodes spp.* nymphs and adults as 12.5% and 30.4%, respectively and in *H. aegyptium* nymphs as 12.5% in our study. Comparatively, *H. aegyptium* nymphs collected from tortoises in the same study area had a positivity of 14.3% [44]. Nevertheless, Guner et al.[45] reported that agents isolated from tortoise ticks differ from common *B. burgdorferi* s.l. species genetically, and therefore suggested that those agents should be named as *B. turcica*.

Approximately 65.500 Lyme patients are seen annually in Europe [4]. Lyme disease was reported from Turkey for the first time in 1990. While a seropositivity of 44% was detected in risky areas, recorded cases do not pass a couple of dozen [46-48]. Moreover, the disease shows different clinical pictures depending on the agent and the patient, and its symptoms can be confused with a variety of systemic diseases [49].

In conclusion, 1) Contrary to the common belief, some tick species can cause health problems even in winter months in Turkey. 2) Higher *Rickettsia* spp. and *B. burgdorferi* s.l. positivity and tick-attachment rates [7,8] indicate that status of tick-borne diseases in humans can be far worse than previously known. 3) Further studies involving surveillance of tick-borne disease agents are necessary to reveal transmission of pathogens of public health importance.

REFERENCES

1. Merhej V, Raoult D: Rickettsial evolution in the light of comparative genomics. *Biol Rev*, 86, 379-405, 2011.

2. Parola P, Paddock CD, Raoult D: Tick-borne Rickettsioses around the World: Emerging diseases challenging old concepts. *Clin Microbiol Rev*, 18 (4): 719-756, 2005.

3. Piesman J, Gern L: Lyme borreliosis in Europe and North America. *Parasitol*, 129, 191-220, 2004.

4. Rizzoli A, Hauffe HC, Carpi G, Vourc'h GI, Neteler M, Rosà R: Lyme borreliosis in Europe. *Euro Surveill*, 16 (27): 1-8, 2011.

5. Estrada-Peña A, Jongejan F: Ticks feeding on humans: A review of records on human-biting Ixodoidea with special reference to pathogen transmission. *Exp Appl Acarol*, 23, 685-715, 1999.

6. Anderson JF, Magnarelli LA: Biology of ticks. *Infect Dis Clin North Am*, 22, 195-215, 2008.

7. Gargili A, Kar S, Yilmazer N, Cerit C, Sönmez G, Sahin F, Alp H, Vatansever Z: Evaluation of ticks biting humans in Thrace province, Turkey. *Kafkas Univ Vet Fak Derg*, 16 (Suppl-A): S141-S146, 2010.

8. Gargili A, Kar S, Yilmazer N, Ergonul O, Vatansever Z: Different abundances of human-biting ticks in two neighboring provinces in Turkey. *Kafkas Univ Vet Fak Derg*, 17 (Suppl A): S93-S97, 2011.

9. Ginsberg HS, Ewing CP: Comparison of flagging, walking,trapping, and collecting ticks from hosts as sampling methods for northern deer ticks, *Ixodes dammini*, and lone star ticks, *Amblyomma americanum* (Acari: Ixodidae). *Exp App Acarol*, 7, 313-322, 1989.

10. Apanaskevich DA: To diagnostics of *Hyalomma aegyptium* (Acari: Ixodidae). *Parazitologiya*, 37, 47-59, 2003.

11. Walker AR, Bouattour A, Camicas JL, Estrada-Peña A, Horak IG, Latif AA, Pegram RG, Preston PM: Ticks of domestic animals in Africa. A guide to identification of species. Bioscience Reports, Scotland, UK, 2003.

12. Estrada-Peña A, Bouattour A, Camicas JL, Walker AR: Ticks of domestic animals in the Mediterranean region. A guide of identification of species. University of Zaragoza Press, Zaragoza, p. 131, 2004.

13. Sparagano O, Allsopp MTEP, Mank RA, Rijpkema SGT, Figuerova JV, Jongejan F: Molecular detection of pathogen DNA in ticks (Acari: Ixodidae). Exp Appl Acarol, 23, 929-960, 1999.

14. Gargili A, Midilli K, Ergin S, Sengoz G, Ozturk R, Altas K: Istanbul'da insanları tutan kenelerde *Borrelia burgdorferi* sl. ve *Rickettsia* spp. yaygınlığı. *17. Ulusal Parazitoloji Kongresi, 1-7 Kasım 2009, Adana,* 2009.

15. Randolph SE: Tick ecology: Processes and patterns behind the epidemiological risk posed by ixodid ticks as vectors. *Parasitol*, 129 (Suppl): 37-65, 2004.

16. Alekseev AN, Dubinina HV: Abiotic parameters and diel and seasonal activity of *Borrelia*-infected and uninfected *Ixodes persulcatus* (Acarina: Ixodidae). *J Med Entomol*, 37, 9-15, 2000.

17. Uspensky I: Preliminary observations on specific adaptations of exophilic ixodid ticks to forests or open country habitats. *Exp Appl Acarol,* 28, 147-154, 2002.

18. Hornok S, Farkas R: Influence of biotope on the distribution and peak activity of questing ixodid ticks in Hungary. *Med Vet Entomol*, 23, 41-46, 2009.

19. Castellà J, Estrada-Peña A, Almería S, Ferrer D, Gutiérrez J, Ortuño A: A survey of ticks (Acari: Ixodidae) on dairy cattle on the island of Menorca in Spain. *Exp Appl Acarol*, 25, 899-908, 2002.

20. Barandika JF, Hurtado A, Juste RA, García-Pérez AL: Seasonal dynamics of *Ixodes ricinus* in a 3-year period in Northern Spain: First survey on the presence of tick-borne encephalitis virus. *Vector Borne Zoonotic Dis*, 10 (10): 1027-1035, 2010.

21. Randolph SE, Green RM, Peacey MF, Rogers DJ: Seasonal synchrony: The key to tick-borne encephalitis foci identified by satellite data. *Parasitol*, 121, 15-23, 2000.

22. Blanco JR, Oteo JA: Rickettsiosis in Europe. *Ann NY Acad Sci*, 1078, 26-33, 2006.

23. Socolovschi C, Mediannikov O, Raoult D, Parola P: The relationship between spotted fever group Rickettsiae and Ixodid ticks. *Vet Res*, 40, 34-50, 2009.

24. Bertolotti L, Tomassone L, Tramuta C, Greco E, Amore G, Ambrogi C, Nebbia P, Mannelli A: *Borrelia lusitaniae* and Spotted Fever Group Rickettsiae in *Ixodes ricinus* (Acari: Ixodidae) in Tuscany, Central Italy. *J Med Entomol*, 43, 159-165, 2006.

25. Piccolin G, Benedetti G, Doglioni C, Lorenzato C, Mancuso S, Papa N, Pitton L, Ramon MC, Zasio C, Bertiato G: A Study of the Presence of *B. burgdorferi*, *Anaplasma* (Previously *Ehrlichia*) *phagocytophilum*, *Rickettsia*, and *Babesia* in *Ixodes ricinus* Collected within the Territory of Belluno, Italy. *Vector Borne Zoonotic Dis*, 6, 24-31, 2006.

26. Wölfel R, Terzioglu R, Kiessling J, Wilhelm S, Essbauer S, Pfeffer M, Dobler G: *Rickettsia* spp. in *Ixodes ricinus* Ticks in Bavaria, Germany. *Ann N Y Acad Sci*, 1078, 509-511, 2006.

27. Klasen ET, Fonville M, van Overbeek L, Reimerink JHJ, Sprong H: Exotic Rickettsiae in *Ixodes ricinus*: fact or artifact? *Parasites & Vectors*, 3, 54, 2010.

28. Márquez FJ: Spotted fever group *Rickettsia* in ticks from southeastern Spain natural parks. *Exp Appl Acarol*, 45, 185-194, 2008.

29. Christova I, van de Pol J, Yazar S, Velo E, Schouls L: Identification of *Borrelia burgdorferi* sensu lato, *Anaplasma* and *Ehrlichia* species, and Spotted Fever Group Rickettsiae in ticks from Southeastern Europe. *Eur J Clin Microbiol Infect Dis*, 22, 535-542, 2003.

30. Tekin A, Gözalan A, Çöplü N, Yılmaz G, Köksal İ, Esen B, Ertek M: The seropositivity and risk factors for rickettsial infections in selected areas from Black Sea Region of Turkey. *Dicle Med J*, 37 (3): 204-210, 2010.

31. Vural T, Ergin Ç, Kurşun AE: Antalya yöresinde *Rickettsia conorii* antikorlarının araştırılması. *Mikrobiyol Bült*, 29, 370-374, 1995.

32. Kuloglu F, Rolain JM, Aydoslu B, Akata F, Tugrul M, Raoult D: Prospective evaluation of rickettsioses in the Trakya (European) region of Turkey and atypic presentations of *Rickettsia conorii*. *Ann N Y Acad Sci*, 1078, 173-175, 2006.

33. Perlman SJ, Hunter MS, Zchori-Fein E: The emerging diversity of *Rickettsia*. Proceedings. *Proc R Soc B*, 273, 2097-2106, 2006.

34. Baldridge GD, Burkhardt NY, Simser JA, Kurtti TJ, Munderloh UG: Sequence and expression analysis of the ompA gene of *Rickettsia peacockii*, an endosymbiont of the Rocky Mountain wood tick, *Dermacentor andersoni*. *Appl Environ Microbiol*, 70, 6628-6636, 2004.

35. Renvoisé A, Mediannikov O, Raoult D: Old and new tick-borne rickettsioses. *International Health*, 1 (1): 17-25, 2009.

36. EFSA Panel on Animal Health and Welfare (AHAW): Scientific opinion on geographic distribution of tick-borne infections and their vectors in Europe and the other regions of the Mediterranean Basin. *EFSA J*, 8 (9): 1723, 2004.

37. Hubálek Z, Halouzka J: Distribution of *Borrelia burgdorferi* sensu lato genomic groups in Europe. A review. *European J Epidemiol*, 13, 951-957, 1997.

38. Rauter C, Hartung T: Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: A metaanalysis. *Appl Environ Microbiol*, 71 (11): 7203-7216, 2005.

39. Wielinga PR, Gaasenbeek C, Fonville M, de Boer A, de Vries A, Dimmers W, Akkerhusi Op Jagers G, Schouls LM, Borgsteede F, van der Giessen JW: Longitudinal analysis of tick densities and *Borrelia*, *Anaplasma*, and Ehrlichia infections of *Ixodes ricinus* ticks in different habitat areas in the Netherlands. *Appl Environ Microbiol*, 72, 7594-7601, 2006.

40. Faulde MK, Robbins RG: Tick infestation risk and *Borrelia burgdorferi* s.l. infection-induced increase in host-finding efficacy of female *Ixodes ricinus* under natural conditions. *Exp Appl Acarol*, 44, 137-145, 2008.

41. Guner ES, Hashimoto N, Takada N, Kaneda K, Imai Y, Masuzawa T: First isolation and characterization of *Borrelia burgdorferi* sensu lato strains from *Ixodes ricinus* ticks in Turkey. *J Med Microbiol*, 52, 807-813, 2003.

42. Gunes T, Kaya S, Poyraz O, Engin A: The prevalence of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks in the Sinop region of Turkey. *Turk J Vet Anim Sci*, 31 (3): 153-158, 2007.

43. Sen E, Uchishima Y, Okamoto Y, Fukui T, Kadosaka T, Ohashi N, Masuzawa T: Molecular detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in *Ixodes ricinus* ticks from Istanbul metropolitan area and rural Trakya (Thrace) region of north-western Turkey. *Ticks and Tickborne Diseases*, 2, 94-98, 2011.

44. Kar S, Yilmazer N, Midilli K, Ergin S, Alp H, Gargili A: Presence of the zoonotic *Borrelia burgdorferi* sl. and *Rickettsia* spp. in the ticks from wild tortoises and hedgehogs. *MUSBED*, 1 (3): 166-170, 2011.

45. Guner ES, Watanabe M, Hashimoto N, Kadosaka T, Kawamura Y, Ezaki T, Kawabata H, Imai Y, kenada K, Masuzawa T: *Borrelia turcica* sp. nov., isolated from the hard tick *Hyalomma aegyptium* in Turkey. *Int J Syst Evol Microbiol*, 54, 1649-1652, 2004.

46. Sen E (2006): Lyme hastalığının epidemiyolojisi. *Türk Mikrobiol Cem Derg*, 36 (1): 55-66, 2006.

47. Bayindir Y: Boreliyoz. **In,** Doganay M, Altintas N (Eds): Zoonozlar: Hayvandan Insanlara Bulaşan Enfeksiyonlar. pp. 71-83, Bilimsel Tıp Yayınevi, Izmir, 2008.

48. Kaya AD, Parlak AH, Ozturk CE, Behcet M: Seroprevalence of *Borrelia burgdorferi* infection among forestry workers and farmers in Düzce, North-western Turkey. *New Microbiol*, 31, 203-209, 2008.

49. Tilly K, Rosa PA, Stewart PE: Biology of infection with *Borrelia burgdorferi*. *Infect Dis Clin North Am*, 22, 217-234, 2008.

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RESEARCH ARTICLE

Increased Glucose Uptake and Insulin Binding Activity of *Nerium Oleander* in Hepatocytes and Adipocytes [1]

Nuray YAZIHAN * Ahmet Levent BAS ** Ezgi ERMIS ***
Sule DEMIRCI **** Kamil UNEY **

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* Department of Pathophysiology, Moleculer Biology R&D Unit, Faculty of Medicine, Ankara University, TR-06100 Ankara - TURKEY
** Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Selcuk University, TR-42003 Konya - TURKEY
*** Moleculer Biology R&D Unit, Faculty of Medicine, Ankara University, TR-06100 Ankara - TURKEY
**** Department of Physiology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, TR-15030 Burdur - TURKEY

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Summary

Type 2 diabetes mellitus (DM) affects a large population worldwide. DM is often considered as a syndrome of disordered glucose metabolism. Current conventional drug therapies for DM are usually insufficient and medicinal herbs with antihyperglycemic activities are increasingly sought by diabetic patients and health care professionals. Nerium Oleander (N.O.) is known to be effective in lowering of postprandial blood glucose in DM patients as a folk remedy. In this study we aimed to evaluate effect of N.O. distillate in glucose uptake activity of hepatocytes and adipocytes. The human hepatoma cells Hep3B and mouse adipocyte 3T3-L1 cells were cultured. Depending on the groups, different concentrations insulin (1, 10, 20 IU/ml) and N.O. (0.1, 1, 10, 50 µg/ml) were added to medium for 48 h. Cellular toxicity and proliferation were evaluated by LDH secretion levels and MTT test. A metabolizable fluorescent derivative of glucose, 2-NBDG and FITC-insulin were used for glucose uptake and insulin binding activity. Insulin increased cellular proliferation and decreased LDH leakage and apoptosis in both cell types. Lower dosages of N.O. has no significant effect on apoptosis and cell number while at the highest dosages minimal cytotoxicity was seen mainly in adipocytes. Main effect of N.O. treatment was increased glucose uptake in Hep3B and 3T3-L1 cells ($P<0.001$). Our results showed that N.O. may be offered as new approaches to treatment of type 2 diabetes by modulating cellular glucose uptake.

Keywords: *Nerium oleander, Hepatocytes, Adipocytes, Glucose uptake, Insulin binding, Type II diabetes mellitus*

Nerium Oleander'in Hepatositler ve Adipositlerde İnsulin Bağlanma ve Glukoz Alımını Arttırıcı Etkisi

Özet

Tip 2 diabet glukoz metabolizmasının bozukluğu şeklinde tanımlanan tüm dünyada oldukça yaygın bir hastalıktır. Mevcut tedavi yöntemleri yeterli gelmemekte, hastaların antihiperglisemik etkiye sahip tıbbi bitkilerle tedaviye ilgisi artmaktadır. Nerium Oleander (N.O.) diabetik hastalarda postprandial glukoz düzeyini düşürmek amacıyla yerel olarak kullanılmaktadır. Bu çalışmada N.O. ekstraktının karaciğer ve adiposit hücrelerinde glukoz alımına ve insulin bağlanmasına etkilerinin belirlenmesi amaçlanmıştır. Farklı dozlarda insulin (1-20 IU/ml) ve N.O. (0.1-50 µg/ml) 48 h uygulamasının İnsan hepatosit Hep3B ve fare adipositleri 3T3-L1 hücre dizileri üzerindeki etkileri değerlendirilmiştir. Bu amaçla hücre toksitesi LDH sekresyonu, hücre çoğalması MTT yöntemi ve hücre içine glukoz alımı/insulin bağlanması florimetrik yöntemle 2-NBDG ve FITC-insulin kullanılarak ölçülmüştür. Insulin uygulaması her iki hücrede de proliferasyonu arttırmış, LDH sekresyonunu azaltmıştır. Düşük dozlarda N.O. uygulamasının hücre sayısına etkisi görülmezken kullanılan üst dozlarda adipositlerde sitotoksik etkisi gözlenmiştir. N.O. nun adiposit ve hepatositlerde hücre içine glukoz alımını anlamlı arttırdığı gözlenmiştir ($P<0.001$). Çalışmamız sonuçları N.O. ekstraktının tip 2 diabette özellikle insulin ve glukoz kullanımını düzenleyici etkisi nedeniyle önemli yeni bir tedavi alternatifi olabileceğini göstermiştir.

Anahtar sözcükler: *Nerium oleander, Hepatositler, Adipositler, Glukoz alımı, İnsulin bağlanması, Tip II Diabetes mellitus*

İletişim (Correspondence)
+90 312 3103010/372
nurayyazihan@yahoo.com

INTRODUCTION

Type 2 diabetes mellitus (DM) affects a large population worldwide [1]. There have been many attempts to develop the safe and effective methods of curing diabetes. Although very intensive research is being conducted in this field, current protocols still have only limited applications. The general consensus on treatment of type 2 diabetes is that lifestyle management is at the forefront of therapy options. In addition to exercise, weight control, and medical nutrition therapy, oral glucose-lowering drugs and injections of insulin are the conventional therapies. Alternative treatments for diabetes have become increasingly popular the last several years, including medicinal herbs, nutritional supplementation and acupuncture. Plant derivatives with purported hypoglycemic properties have been used in folk medicine and traditional healing systems around the world. Approximately 1200 plants are used worldwide for the empirical treatment of DM. However, only about 350 of them are documented to present hypoglycemic activity although only a small number of these have received scientific and medical evaluation to assess their efficacy [2,3]. The World Health Organization Expert Committee on diabetes has recommended that traditional medicinal herbs be further investigated [1,4].

Some of the most common plants used traditionally for treatment of diabetes are *Pterocarpus marsupium* [5], *Bitter Melon* [6], *Gymnema Sylvestre* [7], *Asian Ginseng* [8], *Soybean* [9] and *Cinnamon* [10].

Nerium oleander (N.O.) is a member of Apocynaceae familia and grown in Mediterrian region. *Nerium indicum* is known to be effective in lowering of postprandial blood glucose in DM patients and both *oleander* and *indicum* subforms are now used as a folk remedy for type II diabetes in some regions of mediterrian region and Asia [11]. The hot water extract of the leaves of N.O. has been used as a remedy against Type II DM with subjective success but without corroborating laboratory data. First report related to the hypoglycemic activity of the plant is given by Bellakhdar et al.[12] and then reported by other authors [13,14].

DM is a complex group of metabolic disorders including hyperglycemia and impaired insulin secretion and/or insulin response. Current theories of DM include a defect in insulin-mediated glucose uptake in muscle and adipocytes, a dysfunction of the pancreatic β-cells, an impaired insulin action in liver and decreased peripheral (muscle) glucose utilization. Changes in glucose clearance, an index of efficiency of glucose removal from the circulation, by itself do not affect plasma glucose concentrations independent of changes in rates of glucose entry and exit [15,16]. In this study we aimed to evaluate *in vitro* direct effect of N.O. in hepatocyte and adipocyte cells especially with the aspect of glucose uptake metabolism and cellular proliferation.

MATERIAL and METHODS

Cell lines, Chemicals and Materials

Human hepatoma cell line Hep3B and mouse preadipocyte cells 3T3-L1 were obtained from the American Type Culture Collection (ATCC). Hep3B cells were cultured in Roswell Park Memorial Institute-1640 (RPMI, PAA, Austria) and 3T3-L1 cells in Dubellco's Modified Eagles Medium (DMEM, PAA, Austuria), supplemented with fetal calf serum (FCS), (PAA, Austuria), L-glutamine, streptomycin and penicillin (Sigma, MO, USA). Insulin (Sigma, MO, USA) was dissolved in water, sterilized by 0.22 m pore size cellulose acetate membrane filters, and added to cultures at the indicated time and concentrations. Cell counts were tested by 3-4,5-dimethylthiazol -2-yl-2,5 diphenyltetrazolium bromide (MTT, Sigma, MO, USA). Lactate Dehydrogenase (LDH) and glucose levels were measured with commercial kits using an automatic multi-analyzer (Roche; P800).

Obtaining Liyophilized Nerium oleander

Nerium oleander plant was collected among new shoots in March-September period from Mediterranean region of Turkey. After washing collected plant, fresh shoots were chopped, and adequate distilled water added. The mixture was heated in heat resistant container. After liquid started to evaporate, container lid was covered and vapor was separated to other clean glass container by causing it to come to contact with a surface cooled with cold water. N.O. distillate was liyophilized in small glass bottle (20 ml) by using liyophilizator. N.O. liyophilized distillate was dissolved in according to dosage in saline solution then sterilized by 0.22 m pore size cellulose acetate membrane filters, and added to cultures at the indicated time and concentrations.

Cell Culture and Experimental Protocol

The human hepatoma cell line Hep3B was cultured in RPMI-1640 medium and 3T3-L1 cells in DMEM, supplemented with 10% v/v fetal calf serum, 2 mmol/L L-glutamine, streptomycin (100 mg/mL) and penicillin (100 IU/mL) in a humidified atmosphere containing 5% CO_2 at 37°C. One day before the experiments, cells were seeded on 96-well microtitre plates (Nunc, Denmark) at $2X10^5$ cells/mL.

Depending on the groups, different concentrations insulin (1, 10, 20 IU/ml) and N.O. (0.1, 1, 10, 50 µg/ml) were added to medium for 48 h.

LDH levels were evaluated from control and treated cells at the 48th h. MTT was measured at the 2nd, 24th and 48th h. After supernatants were removed cell surface was washed with sterile phosphate buffered saline (PBS) and cells were harvested with lysis solution and caspase-3 levels of groups were measured from cell lysates. LDH measurement was done from both of the supernatant and cell lysates.

Evaluation of Cellular Proliferation or Death

MTT, a colorimetric assay based upon the ability of living cells to reduce MTT into formazan, was used for evaluation of the effects of dose and time dependent effects of glucose, insulin and N.O. on cellular death or proliferation (24^{th}, 48^{th} h). Cell number % was calculated as ratio of cell number of effected group *vs* control group 100 at the determined hours.

Biochemical Determination of Cell Death

Hep3B cells were plated in 96 multiwell cell culture plates as $3X10^5$ cells/mL. LDH is normally present in the cytosol of hepatocytes. In response to cell damage LDH is released from the cells. Therefore, to determine cell death, we measured secreted and intracellular LDH levels and calculated % released LDH at the 48^{th} h for each group. To do this, the medium was collected to measure enzyme activities. The adherent cells were lysed. Both medium and cell lysates were used for quantitative determination of LDH activity (IU/L) which was performed with an automatic multianalyzer (Roche, MN, USA) using commercial kit (Roche, MN, USA). Released enzyme fractions for each sample were calculated as the ratio of enzyme present in the medium *vs* the sum of the levels of same enzyme in the supernatant and in the cells.

Glucose Uptake and Insulin Binding Activity

A metabolizable fluorescent derivative of glucose, 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG, Molecular Probes, Oregon, USA), was used. Following incubation at 37°C for 120 min with the dye, culture medium was removed and after washing the cells with Hanks' balanced salt solution (HBSS) fluorescence was measured in Spectromax M2 fluorescence microplate reader (Perkin-Elmer Corp. Norwalk, CT, USA) set at an excitation wavelength of 470 nm and an emission wavelength of 550 nm. To determine the effect of insulin or N.O. on glucose uptake, the cell suspension was dispensed in 96-well microtiter plates at 2500 cells/well (200 ml). After 24 h of incubation (37°C/ 5% CO_2), all culture medium was removed from each well and replaced with 100 ml of culture medium with different concentrations of insulin and and 100 ml of 300 µM 2-NBDG diluted in the same medium to give a final concentration of 150 µM /well. In this case, cells were incubated simultaneously with both insulin/N.O. and 2-NBDG for 120 min, and then the plates were centrifuged, the culture medium was removed and the cells were washed once with HBSS. Fluorescence was measured as previously described and results of triplicate experiments expressed as 2-NBDG concentrations [17].

Same procedure was repeated with 0.3 mM Fluorescein isothiocyanate (FITC) labeled Insulin. Fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 520 nm [18].

Statistical Analysis

Results of the experiments were analyzed by One Way ANOVA, followed by a multiple comparison test using SPSS 13.0. $P<0.05$ was accepted as statistically significant. Results were given as mean± SEM.

RESULTS

We characterized the concentration-dependent effect of N.O. and insulin on human hepatocyte cell line (Hep3B) and mouse adipocyte cell line (3T3-L1) as a function of time. N.O. treatment decreased adipocyte cell number ($P<0.01$) whereas insulin increased ($P<0.01$, *Fig. 1*). Minimal toxic effect is seen at the highest dose of N.O. ($P<0.05$) in Hep3B cells *(Fig. 2)*.

Insulin treatment decreased LDH leakage, higher doses of N.O. treatment increased LDH leakage ($P<0.01$, *Fig. 3*).

Main effect of N.O. treatment was seen in glucose uptake to Hep3B and 3T3-L1 cells ($P<0.001$, *Fig. 4, 5*) and this effect is more prominent in liver cells and significantly different from control and insulin treated cells ($P<0.001$). N.O. treatment increased insulin binding capacity in both adipocytes and hepatocytes ($P<0.001$, *Fig. 6*).

DISCUSSION

Both experimental and epidemiologic studies showed that insulin resistance is not key factor only in diabetes also in cardiovascular diseases [19]. Control of blood glucose levels is a function and coordination of different organ systems such as liver, pancreas, muscle and fat. These organs and tissues

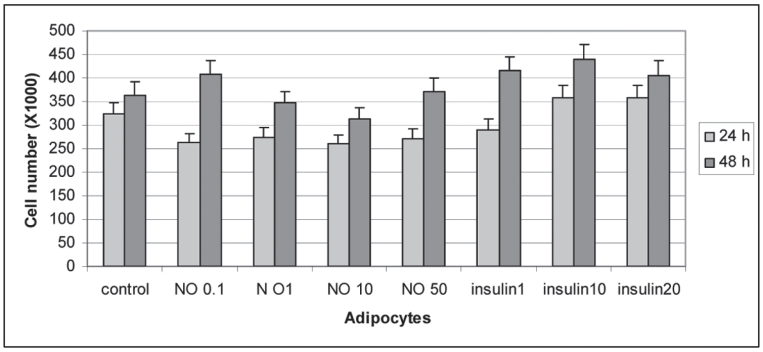


Fig 1. Cell number was determined at 24^{th} and 48^{th} h in adipocyte cell line 3T3-L1. NO treatment caused significant cytotoxicity with dose and time dependent manner ($P<0.01$). The effect is most prominent in N.O. 10 µg/ml dose and at 24 th h ($P<0.001$). Insulin treatment increased cell number at 48 th h in all concentrations ($P<0.01$). Data are presented as mean±SEM (n=6)

Şekil 1. Adiposit hücre dizisinde (3T3-L1) hücre proliferasyonunun 24 ve 48. saatlerde değerlendirilmesi. N.O. tedavisi doz ve zaman bağımlı olarak sitotoksitik etki göstermiştir ($P<0.01$). Bu etki NO 10 µg/ml dozda ve 24. saatte en belirgindir ($P<0.001$). Insulin tedavisi 48. saatte hücre proliferasyonunu tüm dozlarda arttırmıştır ($P<0.01$). Sonuçlar ortalama±SEM olarak verilmiştir (n=6)

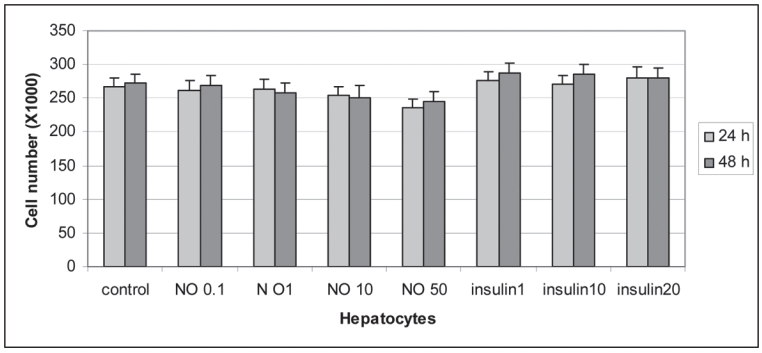
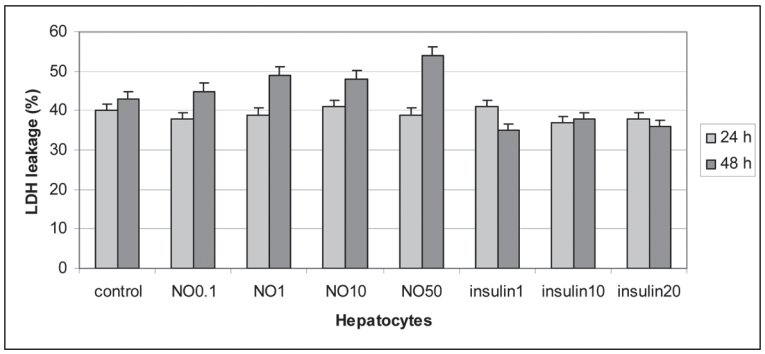


Fig 2. Cell number was determined at 24th and 48th h by the MTT assay. N.O. treatment has minimal cytotoxic effect in highest dosage in Hep3B cell line (P<0.05). Insulin treatment increased cell number in all concentrations (P<0.001). Data are presented as mean±SEM (n=6)

Şekil 2. Hepatosit hücre dizisinde 24 ve 48. saatlerde hücre sayımının MTT ile değerlendirilmesi. N.O. tedavisi en yüksek dozda hepatositlerde minimal toksik etki göstermiştir (P<0.05). Insulin tedavisi tüm dozlarda proliferative etki göstermiştir (P<0.001). Sonuçlar ortalama±SEM olarak verilmiştir (n=6)

Fig 3. N.O. treatment increased LDH released to medium at 48th h. Starting from the 1 IU/mL dosage insulin treatment decreased LDH release from hepatocytes at the 24th and 48th h (P<0.01). Data are presented as mean±SEM

Şekil 3. N.O. tedavisi hepatositlerde LDH sekresyonunu 48. saatte artırmıştır. İnsulinin 1 IU/mL dozundan itibaren hepatositlerde LDH sekresyonunu 24 ve 48. saatlerde azaltmıştır (P<0.01). Sonuçlar ortalama±SEM olarak verilmiştir (n=6)



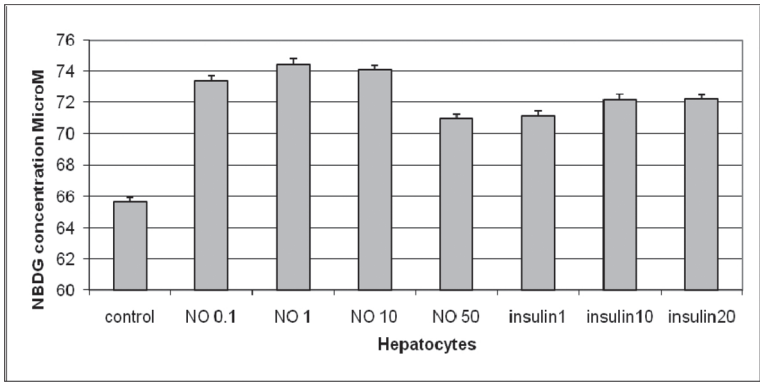
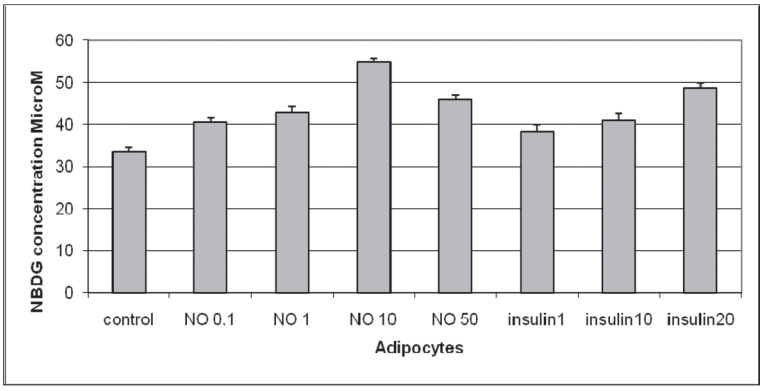


Fig 4. Basal and insulin-stimulated NBDG-glucose uptake in cultured human hepatocytes. N.O. treatment increased glucose uptake in hepatocytes compared to control and insulin treated cells (P<0.001). Data are presented as mean±SEM

Şekil 4. Hepatositlerde bazal ve insulinle uyarılmış NBDG-glukoz alımı. N.O. tedavisi hepatositlerde hücre içine glukoz alımını kontrole ve insulin gore belirgin arttırmıştır (P<0.001). Sonuçlar ortalama±SEM olarak verilmiştir (n=6)

Fig 5. Basal and insulin-stimulated NBDG-glucose uptake in cultured adipocytes N.O. treatment increased glucose uptake in adipocytes starting from lower dosages but most prominent at 10 µg/ml dosage compared to control and insulin treated cells (P<0.001). Data are presented as mean±SEM

Şekil 5. Adipositlerde bazal ve insulinle uyarılmış glukoz alımı. N.O. tedavisi adipositlerde düşük dozlardan itibaren hücre içine glukoz alımını arttırmıştır, bu etki en belirgin 10 µg/ml dozdadır (P<0.001). Sonuçlar ortalama±SEM olarak verilmiştir (n=6)



have major roles in the use and storage of nutrients in the form of glycogen and triglycerides. Type II DM is the most common disorder which is characterized by impaired insulin stimulated glucose uptake in muscle and adipose tissue [20].

It is striking that peripheral glucose levels should be well regulated by physiological control mechanisms, which, when deregulated, trigger early signs of the pathogenesis of obesity and diabetes, such as abnormal suppression of glucagon and loss of insulin secretion in the fed state and decreased peripheral tissue glucose uptake. These dysregulations become prominent before full impairment

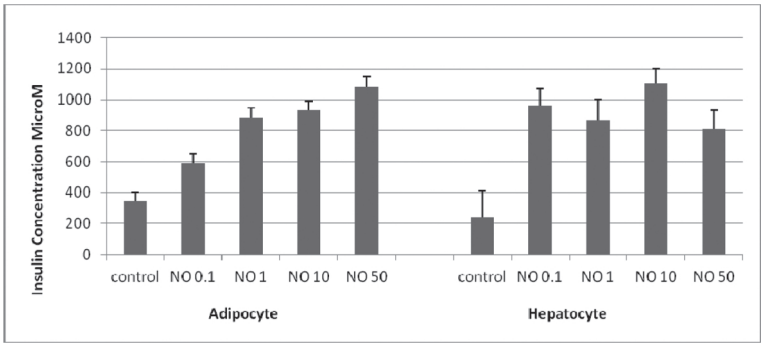


Fig 6. Basal and N.O.-stimulated FITC-Insulin binding activity in cultured adipocytes and hepatocytes. N.O. treatment increased insulin binding in adipocytes and hepatocytes as dose dependent manner (P<0.001). Data are presented as mean±SEM

Şekil 6. Hepatositlerde ve adipositlerde bazal ve N.O. ile uyarılmış insulin bağlanma kapasitesi. N.O. tedavisi adipositlerde ve hepatositlerde insulin bağlanmasını doz bağımlı olarak arttırmıştır (P<0.001). Sonuçlar ortalama±SEM olarak verilmiştir (n=6)

of beta-cell secretion and insulin resistance appears, a characteristic of established type 2 DM [20,21]. Also dysregulation of extrapancreatic glucose sensors, especially glucose uptake, may be early events in the pathogenesis of obesity and type 2 diabetes [21]. From the physiologic standpoint, activation of glucose transport and glycogen synthase is linked to the insulin-signaling mechanism in many systems. Glucose entry into the primary insulin target tissues (skeletal muscle, heart, adipose tissue, and liver) occurs by facilitated diffusion, mediated by a family of transport proteins. Glucose transporters (GLUT) mainly GLUT 1-4 mediates insulin stimulated glucose uptake by skeletal muscle, heart, and adipose tissues [21]. Adipocyte metabolism starts to take famous roles in recent studies. It is accepted now that altered adipocyte metabolism, fat storage and distribution is very important in the pathogenesis of glucose intolerance in Type 2 DM [22]. Chronically increased glucose and plasma free fatty acids FFA stimulates adipogenesis and further gluconeogenesis leading to hepatic/muscle insulin resistance. Dysfunctional fat cells produce excessive amounts of insulin resistance-inducing, inflammatory, and atherosclerotic-provoking cytokines and fail to secrete normal amounts of insulin-sensitizing adipocytokines. Fat cells start to proliferate and become enlarged and more insulin resistant. Different theraphies are used to enhance adipocyte insulin sensitivity, reduce plasma FFA, and favorably influence the production of adipocytokines [23]. Thiazolidinediones are insulin-sensitizing antidiabetic agents which are used in DM patients [24]. Researchers are seeking new alternative treatments to decrease intracellular concentrations of triglyceride metabolites in muscle, liver, and ß-cells, contributing to improvements in muscle/hepatic insulin sensitivity and pancreatic and liver function in type 2 diabetics.

Alternative therapies with anti-hyperglycemic effects are increasingly sought by patients with diabetes. This comes as no surprise since alternative treatments have been most widely used in chronic diseases, which may be only partially alleviated by conventional treatment. Herbal medications are the most commonly used alternative therapy for blood sugar control; however, their safety and efficacy need to be further evaluated by well-designed, controlled clinical studies. N.O. is well known toxic plant especially with its chemotherapic potential. Yassin and Mwafy [25] showed antilipidemic effect without toxicity at the dose Haeba et al.[26] determined with toxicological studies of N.O. leaflets. Similarly; Gayathri et al.[27] found that N.O. extract had an antilipidemic effect in high fat diet fed rats.

Hepatotoxicity of N.O. extract is tested by MTT, LDH leakage measurements in human hepatocytes and we did not find toxic effect or minimal toxicity is found with higher dosages. However, the action mechanism of N.O. is not known yet in the regulation of glucose metabolism. We found that N.O. increases glucose uptake in both cell type, especially in liver cells which is more important for glucose retrival from blood. Our data suggest that N.O. acts through increase in insulin binding and may effect glucose utilization in adipocytes, and hepatocytes.

In summary, we conclude that N.O. is a bioactive component in hepatocytes and adipocytes and able to regulate glucose metabolism and insulin sensitivity in cells. Further investigations are required to evaluate the underlying mechanisms of benefit of N.O. treatment.

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REFERENCES

1. WHO: Library Cataloguing-in-publication data, Diabetes Action Now. An initiative of the World Health Organization and the International Diabetes Federation 2004.

2. Alarcon-Aguilar FJ, Roman-Ramos R, Flores-Saenz JL, Aguirre-Garcia F: Investigation on the hypoglycaemic effects of extracts of four Mexican medicinal plants in normal and alloxan-diabetic mice. *Phytother Res*, 16 (4): 383-386, 2002.

3. Modak M, Dixit P, Londhe J, Ghaskadbi S, Paul A Devasagayam T: Indian herbs and herbal drugs used for the treatment of diabetes. *J Clin Biochem Nutr,* 40 (3): 163-173, 2007.

4. Dey L, Attele AS, Yuan CS: Alternative therapies for type 2 diabetes. *Altern Med Rev,* 7 (1): 45-58, 2002.

5. Dhanabal SP, Kokate CK, Ramanathan M, Kumar EP, Suresh B: Hypoglycaemic activity of *Pterocarpus marsupium* Roxb. *Phytother Res*,20 (1): 4-8, 2006.

6. Leung L, Birtwhistle R, Kotecha J, Hannah S, Cuthbertson S: Anti-diabetic and hypoglycaemic effects of *Momordica charantia* (bitter melon):

A mini review. *Br J Nutr,* 102 (12): 1703-1708, 2009.

7. Srivastava Y, Nigam SK, Bhatt HV, Verma Y, Prem AS: Hypoglycemic and life-prolonging properties of *Gymnema sylvestre* leaf extract in diabetic rats. *Isr J Med Sci*, 21 (6): 540-542, 1985.

8. Banz WJ, Iqbal MJ, Bollaert M, Chickris N, James B, Higginbotham DA, Peterson R, Murphy L: Ginseng modifies the diabetic phenotype and genes associated with diabetes in the male ZDF rat. *Phytomedicinei*, 14 (10): 681-689, 2007.

9. Kwon DY, Daily JW 3rd, Kim HJ, Park S: Antidiabetic effects of fermented soybean products on type 2 diabetes. *Nutr Res,* 30 (1): 1-13, 2010.

10. Jitomir J, Willoughby DS: Cassia cinnamon for the attenuation of glucose intolerance and insulin resistance resulting from sleep loss. *J Med Food*, 12 (3): 467-472, 2009.

11. Ishikawa A, Yamashita H, Hiemori M, Inagaki E, Kimoto M, Okamoto M, Tsuji H, Memon AN, Mohammadio A, Natori Y: Characterization of inhibitors of postprandial hyperglycemia from the leaves of Nerium indicum. *J Nutr Sci Vitaminol (Tokyo),* 53 (2): 166-173, 2007.

12. Bellakhdar J, Claisse R, Fleurentin J, Younos C: Repertory of standard herbal drugs in the Moroccan pharmacopoeia. *J Ethnopharmacol*, 35, 123-143, 1991.

13. Bnouham M, Mekhfi H, Legssyer A, Ziyyat A: Ethnopharmacology Forum Medicinal plants used in the treatment of diabetes in Morocco. *Int J Diabetes & Metabolism*, 10, 33-50, 2002.

14. Tahraoui A, El-Hilaly J, Israili ZH, Lyoussi B: Ethnopharmacological survey of plants used in the traditional treatment of hypertension and diabetes in south-eastern Morocco (Errachidia province). *J Ethnopharmacol*, 110 (1): 105-117, 2007.

15. Marshall S, Garvey WT, Traxinger RR: New insights into the metabolic regulation of insulin action and insulin resistance: Role of glucose and amino acids. *FASEB J,* 5 (15): 3031-3036, 1991.

16. Khan AH, Pessin JE: Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways. *Diabetologia*, 45 (11): 1475-1483, 2002.

17. Leira F, Louzao MC, Vieites JM, Botana LM, Vieytes MR: Fluorescent microplate cell assay to measure uptake and metabolism of glucose in normal human lung fibroblasts. *Toxicol In Vitro*, 16 (3): 267-273, 2002.

18. Gok E Olgaz S: Binding of Fluorescein Isothiocyanate to Insulin: A fluorimetric labeling study. *J Fluorescence*, 14 (2): 203-206, 2004.

19. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC Jr: International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; International Association for the Study of Obesity. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation*. 16, 1640-1645, 2010.

20. Thorens B: Glucose sensing and the pathogenesis of obesity and type 2 diabetes. *Int J Obes (Lond).* 6 (Suppl): S62-S71, 2008.

21. Gould GW, Holman GD: The glucose transporter family: Structure, function and tissue-specific expression. *Biochem J*, 95 (Pt 2): 329-341, 1993.

22. De Ferranti S, Mozaffarian D: The perfect storm: Obesity, adipocyte dysfunction, and metabolic consequences. *Clin Chem,*54 (6): 945-955, 2008.

23. Antuna-Puente B, Feve B, Fellahi S, Bastard JP: Adipokines: the missing link between insulin resistance and obesity. *Diabetes Metab*, 34 (1): 2-11, 2008.

24. Samuel VT, Petersen KF, Shulman GI: Lipid-induced insulin resistance: Unravelling the mechanism. *Lancet*, 375 (9733): 2267-2277, 2010.

25. Yassin MM, Mwafy SN: Protective potential of glimepiride and *Nerium oleander* extract on lipid profile, body growth rate, and renal function in streptozotocin-induced diabetic rats. *Turk J Biol*, 31, 95-102, 2007.

26. Haeba MH, Mohamed AI, Mehdi AW Nair GA: Toxicity of *Nerium oleander* leaf extract in mice. *J Environ Biol*, 23, 231-237, 2002.

27. Gayathri V, Ananthi S, Chandronitha C, Sangeetha MK, Vasanthi HR: Hypolipidemic potential of flowers of *Nerium oleander* in high fat diet-fed Sprague Dawley rats. *Nat Prod Res,* 25 (11): 1110-1114, 2011.

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RESEARCH ARTICLE

Age- and Sex-Related Changes in Mineral Density and Mineral Content of the Tibiotarsal Bone in Quails During Post-hatching Development

Anna CHARUTA [1] Małgorzata DZIERZĘCKA [2] Marcin KOMOSA [3]
Barbara BIESIADA- DRZAZGA [4] Ewa DZIAŁA-SZCZEPAŃCZYK [5] Ross G. COOPER [6]

1 Vertebrates Morphology Department, University of Natural Sciences and Humanities, 08-110 Siedlce, POLAND
2 Department of Morphological Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences - SGGW, 02 - 787 Warsaw, POLAND
3 Department of Animal Anatomy - Institute of Zoology, Poznań University of Life Sciences, POLAND
4 Department of Breeding Methods and Poultry and Small Ruminant Breeding, University of Natural Sciences and Humanities, 08-110 Siedlce, POLAND
5 Department of Zoology and Apiculture, Westpomeranian University of Technology, 71-466 Szczecin, 20 Judyma Street, POLAND
6 Physiologist, Eurohouse, Dog Kennel Lane, Walsall WS1 2BU, UK

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Summary

For the first time computed tomography has been used to analyse tibiotarsal bones volumetric mineral density (vBMD) and mineral content (BMC) in quails during their post-hatching development. The analysis was performed including eighty male and female quails aged 2, 4, 6 and 8 weeks, respectively. Statistical calculations were performed using two-way ANOVA. It was found that vBMD generally in the middle of the diaphyses in the tibiotarsal bones in quails was two-fold higher vs. the metaphyses. Decrease of vBMD and BMC in the metaphyses of the tibiotarsal bones in males occurred between the 4 wk and 6 wk of the post-hatching development; the lowest values of vBMD (217 mg/ccm) and BMC (3.68 mg/mm) were observed in 6 wk males. In turn, in females, the decrease of vBMD in the metaphyses occurred in the 4th and 8th wk. Pearson's correlation coefficient between the metaphyseal vBMD and body weight in males was negative and amounted to r = - 0.71 in the 4 wk and r = -0.44 in the 6 wk. In the middle of the diaphyses, decrease in vBMD was observed in both sexes in the 4 wk and 8 wk. Our research showed that changes in vBMD and BMC are disorders in the process of mineralisation. In both sexes, they caused reluctance to walk and increased the likelihood of deformities and fractures.

Keywords: *Bone mineral density, Bone mineral content, Tibiotarsal bone, Mineralisation, Quails*

Bıldırcınların Yumurtadan Çıkışını Takip Eden Dönemde Tibiorsal Kemikte Yaş ve Cinsiyet İle İlişkili Mineral Yoğunluk ve Mineral İçerik Değişiklikleri

Özet

Bıldırcınların yumurtadan çıkışını takip eden süreçte, ilk kez bilgisayarlı tomografi kullanılarak tibiotarsal kemik mineral yoğunluğu (vBMD) ve mineral içeriği (BMC) analizi gerçekleştirilmiştir. Analiz, yaşları 2, 4, 6 ve 8 haftalık seksen erkek ve dişi bıldırcın üzerinde gerçekleştirilmiştir. İstatistiksel hesaplamalar iki yönlü ANOVA kullanılarak yerine getirilmiştir. vBMD'nin genellikle, metafiz ile karşılaştırıldığında, bıldırcınların tibiotarsal kemik içi diyafizinde 2 kat daha fazla olduğu saptanmıştır. Erkek bıldırcınların tibiotarsal kemik içi metafizinde vBMD ve BMC azalması, yumurtan çıkışını takip eden gelişme döneminin 4. ve 6. haftalarında meydana gelmektedir; erkeklerde en düşük vBMD (217 mg/ccm) ve BMC (3.68 mg/mm) değerleri 6. haftada gözlemlenmiştir. Buna karşın, dişilerde metafiz içinde vBMD azalması 4. ve 8. haftalarda oluşmuştur. Metafiz vBMD ve erkek vücut ağırlığı arasındaki Pearson'ın korelasyon katsayısı negatif olup 4. haftada r = - 0.71 ve 6. haftada r = -0.44 değerine sahiptir. Diyafizde ise her iki cinsiyet için 4. ve 8. haftada vBMD'de azalma olduğu gözlemlenmiştir. Yaptığımız çalışma, vBMD ve BMC değişikliklerinin mineralizasyon sürecini bozduğunu göstermiştir. Her iki cinsiyete yürüme konusunda isteksizliğe neden olup şekil bozuklukları ve çatlama olasılıklarını yükseltmişlerdir.

Anahtar sözcükler: *Kemik mineral yoğunluğu, Kemik mineral içeriği, Tibiotarsal kemik, Mineralizasyon, Bıldırcın*

İletişim (Correspondence)
+49 256 431286
anna.charuta@neostrada.pl

INTRODUCTION

The Japanese quail *(Coturnix coturnix japonica)* is a subject of interest for poultry farmers for a very long time as it has been used for the production of both eggs and meat [1,2]. In both cases, the quail is very efficient by comparison with other species of poultry [3]. Due to its high yield efficiency, the quail, similarly to other poultry species in the research conducted by other authors [4-7], has problems with the skeleton. However, there are only few studies on skeleton formation, including the tibiotarsal bone during post-hatching development of quails [8]. In present literature, there is lack of data concerning mineral content BMC and bone density vBMD values in bone sections which are most exposed to deformities: proximal metaphysis and mid-diaphysis *in situ* of tibiotarsal bones in quails from the moment of hatching till slaughtering maturity in post hatching development. The bird is an experimental one used as an animal model in osteoporosis research and in mechanisms of bone turnover [9]. In some publications, post-hatching development of the skeleton of the pelvic limb of Japanese quails, including bone formation processes, are described in details, however, it is done till 16 day of living [10,11]. That is why it is important to learn about processes describing bone mineralisation in further stages of quail development of a tibiotarsal bone, particularly exposed to deformities. Thus, the aim of the study was to analyse the changes in mineral content and density of the tibiotarsal bones during post-hatching development in quails as influenced by sex and age.

MATERIAL and METHODS

The study was conducted on 80 quails. The birds were divided into the following age groups: 2, 4, 6, and 8 weeks (wk). The quails were kept in cages. 3-wk-old birds were moved to cages for adult birds. The height of the cage was 22 cm, the length - 90 cm, the width - 53 cm. The floor of the cages was honeycomb too with eyeholes not bigger than 1 cm. The birds had three birdbaths and the access to feed ad libitum. In one cage there were 15 females and 5 males. The quails were fed with full-portion feed prepared especially for quails. The feed has a consistence of crumble (crumbled granulate).

The feeding of nestlings (1-7 day), during this period 1 kg of the feed had 12.56MJ of the metabolic energy, 28% protein, 1.0% calcium. The second stage of feeding was 8-28 day (12.14 MJ metabolic energy, 24% protein, 0.80% calcium). The third stage was from 29 to 42 day and longer (11.72 MJ of metabolic energy, 20% of protein, 0.80% calcium.

Tibiotarsal bones to be analysed were obtained from 10 males and 10 females from each of the age groups. The bones were thoroughly cleaned from soft tissues (muscles were removed, the periosteum, epiphyseal and metaphyseal cartilages were left). Each bone was weighed. A laboratory weight was used to measure body weight and bone weight (AXIS, type AG500C, max loading 500 g with the read graduation of 0.001 g).

Then the bones were frozen in the temperatures from -25°C to -30°C. Next, XCT Research SA Plus Peripheral Quantitative Computed Tomography (pQCT) Scanner (Stratec Medizintechnik GmbH. Pforzheim Germany) was used to analyse the *in situ* structure of the proximal metaphyses and in the middle of the diaphyses of the tibiotarsal bones. Before the pQCT analysis, the bones were defrosted.

The following densitometry parameters were determined:

vBMD - Total volumetric bone mineral density (mg/ccm). The mean density of the total bone.

BMC - Total bone content per 1 mm slice (mg/mm). The mineral content of the total bone within a 1 mm slice.

The tomographic analysis of the proximal metaphysis was conducted at 18% of the bone length, whereas the analysis of the in the middle of the diaphyses of the tibiotarsal bone was performed at 50% of the bone length with the voxel size of 0.07 mm and scanning speed of 4 mm/min. The area of analysis was determined following preliminary scanning (20 mm/s) and the bone length was measured. Threshold coefficient, differentiating compact bone from trabecular bone, was determined at the level of 0.900 cm^{-1}.

The obtained results underwent statistical analysis. All calculations were performed using the Statistica 9.0 software (StatSoft, Inc. Tulsa, USA), at $P \leq 0.05$. The two-way ANOVA analysis was conducted in accordance with the model:

$$y_{ijl} = m + a_i + b_j + ab_{ij} + e_{ijl}$$

where: y_{ij}- value of the studied feature, m – population average, a_i- i- effect of the level of A factor, b_j- j- effect of the level of B factor, ab_{ij}-effect of interaction between i and j, e_{ijl}- random error.

T-Tukey's- Kramer test was applied to compare the averages at $P \leq 0.05$ and $P \leq 0.01$. The relations between the studied features, the body weight and the bone weight were also analysed. Furthermore, relationships between the examined features and body and bone weight were tested with the use of Pearson's correlation coefficient.

The analyses were conducted after the Local Ethics Committee had accepted and approved the planned procedures on the experimental birds (33/2009). There was no conflict of interest during the course of the study.

RESULTS

Mean values of BMC and vBMD for tibiotarsal sections and body weight depending on age and sex, are presented in the following tables and figures:

Differences in Bone Density vBMD and Mineral Content BMC in Males as Influenced by Age

Analysing mineral density vBMD in proximal metaphyses of tibiotarsal bones in post-hatching development of males, it was stated that the first decrease of vBMD occurred in proximal metaphyses in 4 wk, then between 4 and 6 wk vBMD in proximal metaphyses in males of tibiotarsal bones significantly attenuated by 102.68 mg/ccm, at P≤0.05 *(Table 1)*.

Another analysed parameter in the proximal metaphysis of males was mineral content BMC, which amounted to 4.18 mg/mm in 2 wk. During the whole post-hatching development, the lowest mineral content in the proximal metaphysis of males was observed in 6 wk, it was only 3.68 mg/mm. BMC attenuated by 1.52 mg/mm, at P≤0.01 in 6 wk (in comparison to 4 wk) *(Table 1)*. It is woth noting that BMC values attenuated in proximal metaphyses in 6 wk, whereas vBMD values in 4 and 6 wk. At that age, unwillingness to move and more bone fractures were observed, whereas at 6 wk – macroscopically deformed tibiotarsal bones.

Analysing densitometric parameters in the middle of the diaphyses of the tibiotarsal bone in males, it was stated that vBMD in the diaphyses was twice bigger than in the metaphyses *(Table 1)*. BMC in the diaphyses was lower than in the proximal metaphyses *(Table 1)*. The highest vBMD and BMC in the middle of the diaphyses was observed in 6 wk males and amounted to 669.83 mg/ccm and 3.95 mg/mm, respectively. The lowest vBMD in mid-diaphysis in males was observed in 4 wk, in relation to values achieved in 6 wk it was lower by 95 mg/ccm, at P≤ 0.05 *(Table 1)*.

Differences in Bone Density vBMD and Mineral Content BMC in Females as Influenced by Age

Bone density vBMD of the tibiotarsal bones in proximal metaphyses in 2 wk females amounted to 313.48 mg/cm^3. In 4 wk in proximal metaphyses, there was a slight decrease in vBMD values, whereas in 6 wk the highest values of vBMD in females in the whole post-hatching development in proximal metaphyses were observed in 6 wk (450 mg/ccm). Significant statistical differences were found between vBMD values between 4 and 6 wk, at P≤0.05.

Mineral content of BMC in proximal metaphyses in females was the lowest during the post-hatching development in 2 wk (3.65 mg/mm), and the highest in 6 wk (5.88 mg/mm). The values of densitometric parameters in the middle of the diaphyses were as follows: vBMD was twice bigger in relation to metaphyses. During the post-hatching development in females, the highest vBMD in the middle of the diaphyses was observed in 6 wk (748.00 mg/ccm, at P≤0.05 *(Table 1)*. The highest BMC values in mid-diaphysis were observed in 8 wk (5.04 mg/mm).

Differences in Bone Density vBMD and Mineral Content BMC between Males and Females as Influenced by Age

No significant differences in vBMD were observed between males and females in the proximal metaphyses in 2 wk vBMD increased both in males and females during the post-hatching development till 4 wk. There were statistically significant differences in vBMD in proximal metaphyses between 6 wk males and females at P≤0.01. vBMD amounted to 217.29 mg/mm for 6 wk males and 450 mg/mm for females at the same age.

During the analysis, it was stated that BMC was higher in males in the proximal metaphyses 2 and 4 wk *(Table 1)*. However, no statistically significant differences were observed. Statistically significant differences in BMC values were found between males and females in 6 wk, at P≤0.01.

Table 1. *Mean values (X) and standard deviation ± SD of the BMC and vBMD in the tibiotarsal bone sections, depending on age and sex*
Tablo 1. *Tibiotarsal kemik bölümlerinde, yaşa ve cinsiyete bağlı BMC ve vBMD'nin ortalama değerleri (X) ve standart sapması ± SD*

Item	**Section Within Bones**					
BMC mg/mm	**Proximal Metaphysis**			**Middle of the Shaft**		
Age	**Males ± SD**	**Females ± SD**	**Pooled Sexes ± SD**	**Males ± SD**	**Females ± SD**	**Pooled Sexes ± SD**
2wk	4.18±0.83 ^{aA}	3.65±0.66 ^{aA}	3.81±0.70 ^{aA*}	1.74±1.13 ^{aA}	1.93±0.31 ^{aA}	1.83±0.26 ^{aA*}
4wk	5.20±0.78 ^{aA}	4.23±0.85 ^{aA}	4.62±0.94 ^{aA*}	3.11±0.47 ^{bB}	3.16±0.51 ^{bB}	3.14±0.49 ^{bB*}
6wk	3.68±0.81 ^{bB**}	5.88±0.0 ^{bB**}	4.69±0.99 ^{aAbB*}	3.95±0.41 ^{bB}	4.15±0.00 ^{bB}	3.97±0.38 ^{cC*}
8wk	4.47±0.56 ^{aA}	4.48±0.49 ^{aA}	4.51±0.55 ^{bB*}	3.78±0.33 ^{bB**}	5.04±0.75 ^{cC**}	4.41±0.66 ^{cC}
BMD mg/ccm	**Proximal Metaphysis**			**Middle of the Shaft**		
Age	**Males ± SD**	**Females ± SD**	**Pooled Sexes ± SD**	**Males ± SD**	**Females ± SD**	**Pooled Sexes ± SD**
2wk	327.30±43.16 ^{a}	313.48±15.18 ^{a}	336.04±66.87 ^{c*}	628.34±39.23 ^{a}	650.61±37.31 ^{a}	638.82±38.85 ^{c*}
4wk	329.97 $^{a\pm}$ 33.09	285.15±36.57 ^{a}	346.04±58.90 ^{ab*}	574.60±55.13 ^{a}	609.78±100.93 ^{a}	597.47±87.69 ^{ab*}
6 wk	217.29 $^{b\pm}$ 60.36	450.10±0.00 ^{b}	371.10±64.49 ^{b*}	669.83±79.58 ^{a}	748.00±0.00 ^{b}	680.02±78.70 ^{b*}
8 wk	337.65 $^{a\pm}$ 62.36	340.92±30.90 ^{a}	338.31±57.13 ^{a*}	593.61±40.38 ^{a}	686.57±28.29 ^{a}	612.21±53.56 ^{a*}

^{a,b}Means within a column with different superscripts are significantly different (P≤0.05), ^{A,B} Means within a column with different superscripts are significantly different (P≤0.01), **Means within a row are significantly different (P≤0.01), *Means within a row are significantly different (P≤0.05)

***Table 2**. Pearson's correlation coefficient for vBMD and BMC of males and females, depending on body weight and bone weight*
***Tablo 2.** Dişi ve erkeklerde, vücut ağırlığı ve kemik yoğunluğuna bağlı olarak vBMD ve BMC için Pearson'ın korelasyonu*

Age	BMC							
	Proximal Metaphysis				Middle of the Shaft			
	Males		Females		Males		Females	
	BW	Bone Mass	BW	Bone Mass	BW	Bone Mass	BW	Bone Mass
2 wk	1.00*	1.00*	0.81*	0.77*	0.74*	0.74*	0.48	0.83*
4 wk	0.15	0.49	0.62*	0.80*	0.75*	0.40	0.20	0.63*
6 wk	-0.90*	-0.07	0.24	0.17	0.10	0.45*	0.44	0.21
8 wk	0.55*	0.24	-0.79*	-0.12	0.30	0.77*	0.78*	0.83*
Age	**BMD**							
2 wk	1.00*	1.00*	0.06	0.04	-0.06	-0.09	0.28	0.15
4 wk	-0.71*	-0.58*	0.45	0.46	0.31	-0.11	0.08	0.52
6 wk	-0.44*	-0.15	-0.95*	-0.34	-0.10	-0.02	0.23	0.38
8 wk	0.29	-0.12	-0.89*	-0.48	0.14	-0.21	0.66*	0.31

** significantly different P≤0.05, BW- Body Weight*

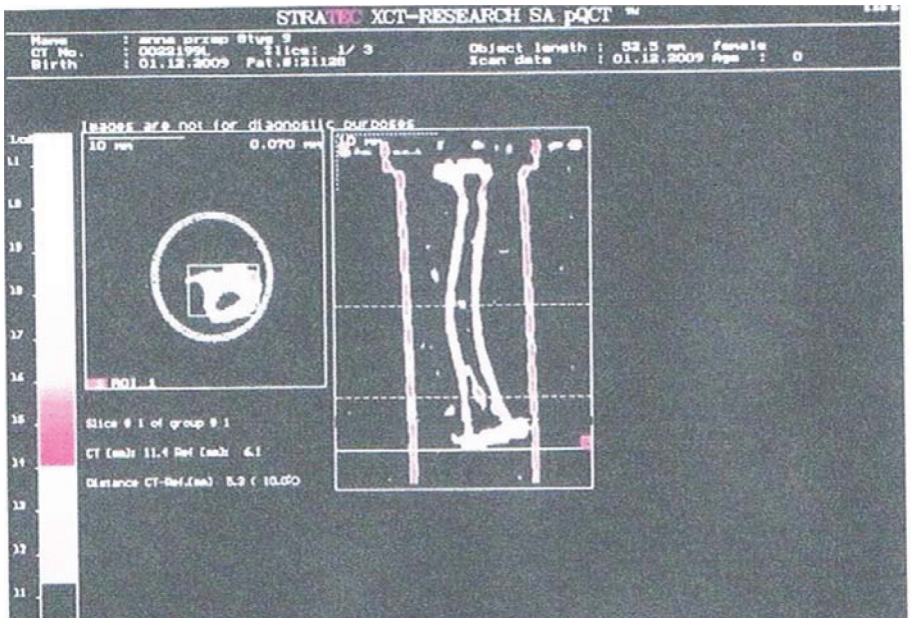

Fig 1. Tomographic analysis of a deformed tibiotarsal bone in quail male in 6 wk with marked analysis areas: proximal metaphysis and mid-diaphysis

Şekil 1. Bıldırcında işaretli analiz bölgesinde deforme tibiotarsal kemiğin tomografik analizi: proksimal metafiz ve orta diafiz

BMC in 6 wk was significantly higher in females (5.88 mg/mm) than in males (3.68 mg/mm). It is worth emphasising that statistically significant differences in BMC in the middle of the diaphyses between the two sexes were observed in 8 wk, at P≤0.01. BMC amounted to 3.78 mg/mm for males and 5.04 mg/mm for females *(Table 1)*. Statistically significant differences were also observed in vBMD between males and females in 6 and 8 wk (P≤0.05) *(Table 1)*.

Pearson's Correlation Coefficient

Pearson's correlation coefficient defined the relation between vBMD and BMC values and body and bone weight in particular bone sections. The BMC in the proximal metaphyses in 2 wk males depended on body weight r = 1.00 and bone weight r = 1.00. In 2 wk females, BMC was also related to body weight r = 0.81 and bone weight r = 0.77. Therefore, BMC increased in tibiotarsal bones when body weight and bone weight grew. A similar relation was observed in the middle of the diaphyses in 2 wk individuals of both sexes *(Table 2)*. The BMC in proximal metaphyses in 4 wk females depended on body weight (r = 0.62) and bone weight (r = 0.80). Thus, between 2 and 4 wk vBMD and vBMD values rose when body and bone weight increased.

In a group of males in proximal metaphyses, when BW increased in 6 wk, BMC decreased r = - 0.90.

While analysing Pearson's correlation coefficient in 2 wk males, a strong positive correlation was noticed between vBMD, and BW (r =1.0) and bone weight (r = 1.00) in proximal metaphyses. A strong negative s correlation between vBMD and body weight (r = -0.71) and bone weight (r = -0.58) was observed in 4 wk males. Thus when body weight and bone

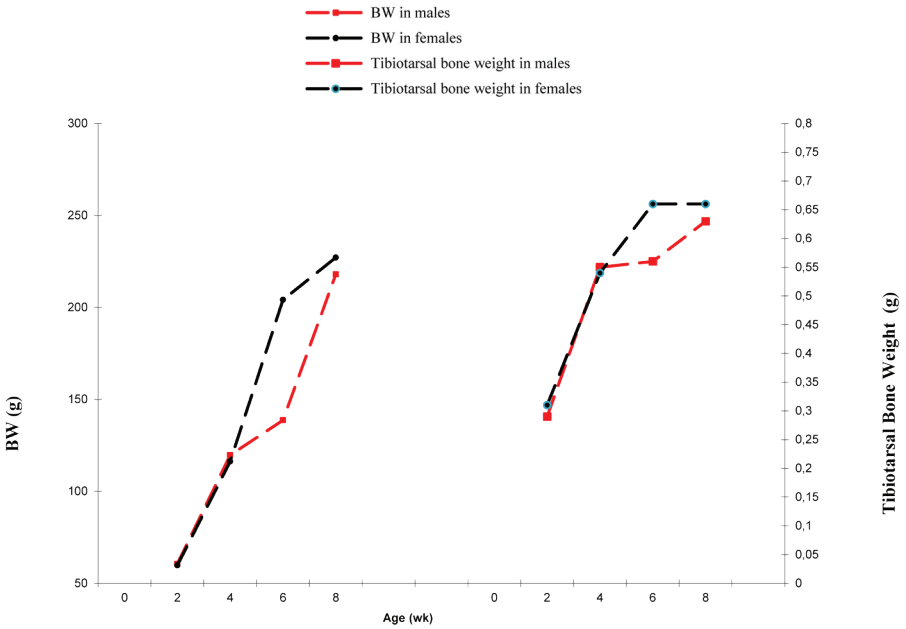

Fig 2. Mean values of BW (Body Weight g) and tibiotarsal bone weight in quails, depending on age and sex

Şekil 2. Yaş ve cinsiyete bağlı olarak bıldırcınlarda BW (vücut ağırlığı g) ve tibiotarsal kemik yoğunluğu ortalama değeri

weight increased in 4 wk males, the decrease of vBMD was observed. A negative correlation between vBMD and BW was also observed in a group of 6 wk males (r = - 0.44).

In females, it was observed that when BW rose, BMC (r = -0.79) decreased in 8 wk and vBMD attenuated in 8 wk (r = -0.89).Whereas, BMC and vBMD grew in the middle of diaphyses in 8 wk females when body weight increased, Pearson's correlation coefficient amounted to (r = 0.78) and (r = 0.66), respectively *(Table 2)*.

DISCUSSION

The tibiotarsal bone is the most frequently studied one in poultry, including quails and on influences of nutrition [12] and biology [13]. A lot of factors have an influence on shaping the structure of the skeleton and BMC and vBMD values in bones, such as food [14] or breeding method [15]. The authors suggest [16] that birds kept in aviary system had stronger bones than those kept in cages. According to Jedral [17], birds kept in cages had lower vBMD.

The conducted research analysed the influence of age and sex on vBMD and BMC in tibiotarsal bones in quails which were kept in cages.

The research confirmed that BMC was higher in proximal metaphyses and vBMD was higher in diaphyses (twice). It is a normal situation resulting from a bone structure. In proximal metaphyses, there is more mineral, as rebuilding processes take place faster there and metabolism is 8 times higher in the cancellous bone of proximal metaphyses.

As far as differences in densitometric parameters in tibiotarsal bone as influenced by sex were concerned, it is essential to emphasize that quails experience a significant (P≤0.01) sexual dimorphism, which is noticeable from 4 wk of age. The birds studied showed clear differences between sexes. It mainly concerned the body weight, which was higher in 6 wk females (200 g) than in 6 wk males (140 g).

The achieved results showed that from the 2 wk BMC increased in proximal metaphyses when body weight grew in both sexes, which was confirmed by a positive correlation r = 1.00 for males and r = 0.81 for females. In 2 wk, a positive correlation between BMC, BW and bone weight was observed in the middle of the diaphysis for both sexes. Also in males in 2 wk, a strong positive correlation was noticed between vBMD, and BW (r =1.00) and bone weight (r = 1.00). In the further post-hatching development vBMD values of proximal metaphyses of tibiotarsal bones in 4 and 6 wk males attenuated. It is worth noting that strong correlation between vBMD and body weight (r = - 0.71) and bone weight (r = -0.58) was observed in 4 wk males. Another analysed parameter, BMC, achieved the lowest values in proximal metaphyses in 6 wk (r = -90). Thus, decreasing BMC values in 6 wk males attenuated bone resistance to fractures. The values of both densitometric parameters increased in 8 wk males. Whereas in females, the decrease of vBMD values was observed in proximal metaphyses in 4 and 8 wk. The attenuation of BMC values in metaphyses occurred in 8 wk. It is worth adding that in females between 4 and 6 wk, body weight increased by approximately 88g and the values of densitometric parameters increased in 6 wk. In males, body weight increased by 21 g between 4 and 6 wk, whereas BMC and vBMD values attenuated. It was also observed that the decreasing values of both densitometric parameters along with the increase of body weight and bone weight in 6 wk in males were the cause of deformities of tibiotarsal bones. Three males out of 10 (30%) had deformed tibiotarsal bones in 6 wk. In a group of females,

limb problems were observed in 8 wk. Two out of 10 (20%) females had deformed tibiotarsal bones.

It is worth noting that a similar research was conducted in turkeys [18]. During the post-hatching development of turkeys, it was stated that vBMD attenuated in proximal metaphyses in 9 wk males (261.05 mg/ccm) and females (295.15 mg/ccm). In 9 wk turkeys, numerous fractures and bone deformities of tibiotarsal bones were observed, whereas in quails bone deformities appeared in 6 wk.

Using computed tomography, vBMD was also analysed in tibiotarsal bones of ducks [19]. It was stated that vBMD attenuated in proximal metaphyses between 4 and 6 wk in ducks of both sexes, which caused deformities in 6 wk, thus visible deformities of tibiotarsal bones of ducks and quails occurred at the same age. It should be emphasised that the decrease of vBMD in proximal metaphyses was observed earlier than in ducks, in 4 wk.

Trabecula® programme was used to analyse the structure of trabeculae in proximal metaphyses of tibiotarsal bones in ducks in post-hatching development. The number, volume and density of radiological trabeculae were determined. It was found that the density was the lowest in 6 wk ducks and amounted to 44.62%. Such a low density was the cause of numerous fractures of tibiotarsal bones [6]. Trabecula® programme was also used to study the structure of tibiotarsal bones in the development of geese as influenced by age and sex. It was found that the lowest number of trabeculae in proximal metaphyses was observed in 6 wk males (6.34 mm^2). Density (33.73%) and volume of trabeculae (1.50% mm) was also the lowest at that age group [7].

The achieved results concerning birds' development are a valuable source of information for poultry farmers because there is a critical moment in the development of the above mentioned species in which bone density BMD decreases causing bone deformities and making poultry breeding less profitable.

In conclusion, the presented reasearch determined the value of vBMD and BMC in quails during post-hatching development using a computed tomography. The gradual densitometric values in the proximal metaphyses were the cause of fractures and deformities bones in quails. Reduction of the densitometric values in the proximal metaphyses the ever increasing body weight caused deformation of the tibiotarsal bone. The results can provide valuable information for poultry farmers were the cause of fractures.

REFERENCES

1. Nowaczewski S, Kontecka H, Elminowska-Wenda G, Rosińska A, Bednarczyk M, Kucharska A: Egg quality traits of Japanese quail divergently selected for yolk cholesterol level. *Archiv fiùr Geflìùgelk*, 74, 141-144, 2010.

2. Vali N: The Japanese quail. *A Review Int J Poult Sci*, 7 (9): 925-931, 2008.

3. Nowaczewski S, Kontecka H, Rosinski A, Koberling S, Koronowski P: Egg quality of Japanese quail depends on layer age and storage time. *Folia Biol (Kraków)*, 58, 201-207, 2010.

4. Horbańczuk JO, Huchzermyer F, Parada R, Płaza K: Four-legged ostrich (*Struthio camelus*) chick. *Vet Rec*, 154 (23): 736, 2004.

5. Cooper RG, Naranowicz H, Maliszewska E, Tennett A, Horbańczuk JO: Sex- based comparison of limb segmentation in ostriches aged 14 months with and without tibiotarsal rotation. *J South African Vet Assoc*, 79 (3): 142-144, 2008.

6. Charuta A, Dzierzęcka M, Majchrzak T, Czerwiński E, Cooper RG: Computer- generated radiological imagery of the structure of the spongious substance in the postnatal development of the tibio-tarsal bones of the Peking domestic duck *(Anas platyrhynchos var. domestica)*. *Poult Sci*, 90 (4): 830-835. 2011.

7. Charuta A, Dzierzęcka M, Czerwiński E, Cooper RG, Horbańczuk JO: Sex-and age-related changes of trabecular bone of tibia in growing domestic geese *(Anser domesticus)*. *Folia Biol (Kraków)*, 60, 205-212, 2012

8. Starck JM, Chinsamy A: Bone microstructure and developmental plasticity in birds and other dinosaurs. *J Morphol*, 254, 232-246, 2002

9. Liu D, Veit HP, Wilson JH, Denbow DM: Long-term supplementation of varius dietary lipids alters bone mineral contetnt, mechanical properties and histological characteristics of Japanese quail. *Poult Sci*, 82, 831- 839, 2003.

10. Landsdown ABG: An investigation of the development of the wing skeleton in the quail *(Coturnix cotirnix japonica)*. *J Anat*, 104, 103-114, 1969.

11. Landsdown ABG: A study of the normal development of the leg skeleton of the quail *(Coturnix coturnix japonica)*. *J Anat*, 106, 147-160, 1970.

12. Liu D, Veit HP, Wilson JH, Denbow DM: Long-term supplementation of various dietary lipids alters bone mineral content, mechanical properties and histological characteristics of Japanese quail. *Poult Sci*, 82 (5): 831-839, 2003.

13. Cooper RG: Quail, biological importance and processing, *Poultry World* (in press) 2012.

14. Liu D, Veit HP, Wilson JH, Denbow DM: Maternal dietary lipids alter bone chemical composition, mechanical properties and histological characteristics of progeny of Japanese quail. *Poult Sci*, 82 (3): 463-473, 2003.

15. Knowles TG, Wilkins LJ: The problem of broken bones during the handling of laying hens -A review. *Poult Sci*, 77, 1798-1802, 1998.

16. Newman S, Leeson S: Effect of housing birds in cages or an aviary system on bone characteristics. *Poult Sci*, 77, 1492-1496, 1998.

17. Jendral MJ, Korver DR, Church JS, Feddes JJR: Bone mineral density and breaking strength of white leghorns housed in conventional, modified, and commercially available colony battery cages. *Poult Sci*, 87 (5): 828-837, 2008.

18. Charuta A, Cooper RG, Pierzchała M, Horbańczuk JO: Computed tomographic analysis of tibiotarsal bone mineral density and content in turkeys as influenced by age and sex. *Czech J Vet Sci*, (in press), 2012.

19. Charuta A, Cooper RG: Computed tomographic and densitometric analysis of tibiotarsal bone mineral density and content in postnatal Peking ducks *(Anas platyrhynchos var. domestica)* as influenced by age and sex. *Pol J Vet Sci*, 15 (3): 537-545, 2012.

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RESEARCH ARTICLE

Prevalence of Methicillin-Resistant Staphylococci in Dogs [1]

Özkan ASLANTAŞ * Süheyla TÜRKYILMAZ ** Mehmet Ali YILMAZ ***
Ebru Şebnem YILMAZ ****

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* Mustafa Kemal University, Faculty of Veterinary Medicine, Deparment of Microbiology, TR-31034 Hatay - TURKEY
** Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, TR-09010 Aydın - TURKEY
*** Susuz District Agricultural Directorate, TR-36760 Susuz, Kars - TURKEY
**** Mustafa Kemal University, Faculty of Art and Science, Department of Biology, TR-31034 Hatay - TURKEY

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Summary

The aim of the study was to investigate the occurence and species distribution of methicillin resistant staphylococci (MRS) in the nasal cavity of dogs. Nasal swabs were collected from 162 dogs entering private veterinary clinics in Hatay. Methicillin resistance was detected onto mannitol salt agar containing 2 µg/ml oxacillin and confirmed by *mec*A Polymerase Chain Reaction (PCR). Bacterial identification was done using 16S rRNA sequencing. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing of these isolates were determined by multiplex PCR. Antimicrobial susceptibility testing were performed disk diffusion method and antimicrobial resistance genes were determined by PCR. Methicillin-resistant coagulase negative staphylococci (MRCNS) harbouring *mec*A were isolated from 15.4% (25/162) of dogs. The species identified were *S. epidermidis* (n=12), *S. lentus* (n=6), *S. hominis* (n=4), *S. warneri* (n=1), *S. arlettae* (n=1) and *S. haemolyticus* (n=1). *mec*A-mediated methicillin resistance in *S. arlettae* was described for the first time. Methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant *Staphylococcus pseudintermedius* (MRSP) were not detected. SCC*mec* type I, II, III and IV were identified in 1, 10, 9 and 5 MRS isolates, respectively. The results indicate that continuous surveillance is necessary to determine the emergence of MRS including MRSA.

Keywords: *Dog, Methicillin resistance, Staphylococci*

Köpeklerde Metisilin Dirençli Stafilokokların Prevalansı

Özet

Bu çalışmanın amacı, köpeklerin nazal mukozalarında metisilin dirençli stafilokokların (MRS) varlığınının ve tür dağılımının belirlenmesidir. Bu amaçla, Hatay'da özel veteriner kliniklerine getirilen 162 köpekten nazal svablar alındı. Metisilin direncinin belirlenmesinde 2 µg/ml oksasillin içeren mannitollü tuzlu agar kullanıldı. Bakteriyel identifikasyon 16S rRNA dizi analizi ile gerçekleştirildi. Stafilokokal kromozomal kaset tiplendirmesi (SCC*mec*) için multipleks polimeraz zincir reaksiyonu (mPZR) yapıldı. Antimikrobiyal duyarlılıkları disk diffuzyon yöntemi ile ve antimikrobiyal direnç genleri PZR ile incelendi. Köpeklerin %15.42'ünden (25/162) *mec*A geni taşıyan MRS izole edildi. Yirmibeş MRS izolatı *S. epidermidis* (n=12), *S. lentus* (n=6), *S. hominis* (n=4), *S. warneri* (n=1), *S. arlettae* (n=1) ve *S. haemolyticus* (n=1) olarak identifiye edildi. *S. arlettae*'da *mec*A geni ilk kez belirlendi. Metisilin dirençli *S. aureus* (MRSA) ve *S. pseudintermedius* (MRSP) izole edilmedi. SCC*mec* tip I, II, III ve IV sırasıyla 1, 10, 9 and 5 MRS izolatında belirlendi. Sonuçlar, MRSA dahil MRS suşlarının ortaya çıkışını belirlemek için sürekli surveyansın gerekli olduğunu işaret etmektedir.

Anahtar sözcükler: *Köpek, Metisilin Direnci, Stafilokok*

INTRODUCTION

Emergence of methicillin resistant staphylococci (MRS), particularly methicillin resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus pseudointermedius* (MRSP) in pet animals, is public and animal health concern due to zoonotic transmission of these multidrug resistant bacteria. MRSA strains found in dogs in various countries have been shown to be same clones isolated from humans in the region [1]. However, methicillin resistance among coagulase

İletişim (Correspondence)
+90 326 2455845/1523
ozkanaslantas@yahoo.com, aslantas@mku.edu.tr

negative staphylococci (CNS) have been reported with increased frequency in human and veterinary medicine [2-6]. As there is limited data on the presence of MRSA, MRSP and other MRS in dog population in Turkey. This study investigated the prevalence of MRS carriage among dogs presenting private veterinary clinics in Hatay, Turkey.

MATERIAL and METHODS

Sample Collection

From December 2008 to June 2009, nasal swabs were obtained from 162 dog attending private veterinary clinics in Hatay, Turkey. This study was approved by the Animal Ethical Committee of Mustafa Kemal University (2008/78).

Sample Analysis

The swabs were placed in enrichment broth containing 10 g/l mannitol, 65 g/l sodium chloride, 2.5 g/l yeast extract and 10 g/l tryptone containing 2 µg/ml oxacillin and incubated at 35°C for 24 h. Subsequently, 10 µl inoculum was spread onto Mannitol Salt Agar containing 2 µg/ml oxacillin as above and incubated 35°C for 24-48 h. A single presumptive methicillin resistant staphylococcal colony was selected and identified phenotypically on the genus level by conventional biochemical tests.

DNA Extraction

Genomic DNA from individual pure cultures of MRCNS isolates were extracted with InstaGene matrix (Bio-Rad Laboratories, Canada) according to the manufacturer's instructions.

Identification and Characterisation of MRS Isolates

For the detection of *mec*A (methicillin resistance) gene, the oligonucleotide primers and PCR conditions used for this study were performed as reported previously by Oliveira and de Lencastre [7]. 16S rRNA gene amplification and sequence analysis were performed as described previously [8,9]. Thus, a large, 1371 bp fragment encoding 16S rRNA gene was amplified and subjected to sequence analysis for species discrimination. For the detection of 16S20 and 16S1390 universal rRNA, primers 5'- AGA GTT TGA TCC TGG CTC AG -3' and 5'- GAC GGG CGG TGT GTA CAA -3'were used as the forward and the reverse primer, respectively [10,11]. Nucleotide sequences were compared with the published sequences on National Center of Biotechnology Information (available online at http://www.ncbi.nlm.nih.gov), and sequences showing highest similarity score (>97%) to a type strain was considered as species identity.

SCCmec Typing

SCC*mec* types (I-IV) of the isolates were determined using methods and primers described by Oliveira and de Lencastre [7]. For the detection of *mec*A (methicillin resistance) gene and SCC*mec* typing, methicillin susceptible (*Staphylococcus aureus* ATCC 29213) and methicillin resistanat (*S. aureus* HPV107, *S. aureus* BK2464, *S. aureus* HUSA304, *S. aureus* GRE14) reference strains used as negative and positive control in PCR, respectively. Visualization of PCR products was performed on 1.5% agarose gel stained with ethidium bromide.

Animicrobial Susceptibility Testing

Antimicrobial susceptibility testing of MRS strains was performed according to the guideline of Clinical and Laboratory Standards Institute (CLSI) [12] using the following antimicrobial disks: erythromycin (15 µg), trimethoprim-sulfamethoxazole (1.25 µg/23.75 µg), vancomycin (30 µg), gentamicin (10 µg), quinopristin-dalfopristin (15 µg), ciprofloxacin (5 µg), mupirocin (5 µg), fusidic acid (10 µg), rifampicin (5 µg), amoxicillin-clavulanic acid (20 µg/10 µg), clindamycin (2 µg) and tetracycline (30 µg). Since standardized CLSI breakpoint for mupirocin and fusidic acid are not available, the disk diffusion testing of these antibiotics was performed as previously reported [13,14].

Determination of Antimicrobial Resistance Genes

PCR assays for the resistance genes *erm*A, *erm*B, *erm*C, *msr*A, *mph*C, *lun*A, *aac(6')/aph(2")*, *aph(3')-IIIa*, *ant(4')-Ia*, *tet*K, *tet*M, *ileS-2*, *fus*B, *fus*C was performed as previously reported [15-21].

RESULTS

Identification, Characterisation and SCCmec Types of MRS Isolates

MRS was isolated from 25 dogs (15.4%). Identification of isolates was done by sequencing a 1371 bp size PCR product by using universal 16S rRNA primers *(Fig. 1)*. 16S rRNA sequencing of isolates revealed the occurence of seven species: *S. epidermidis* (n=12), *S. lentus* (n=6), *S. hominis* (n=4), *S. warneri* (n=1), *S. arlettae* (n=1) and *S. haemolyticus* (n=1) *(Table 1)*. No dogs were colonized with MRSA and MRSP. The most prevalent SCC*mec* type were SCC*mec* II (40%), followed by SCC*mec* III (36%), SCC*mec* IV (20%), and SCC*mec* I (4%) *(Fig. 2, 3)*. While 20 isolates including type I, II and III were defined as hospital acquired methicillin resistant staphylococci (HA-MRS), 5 isolates including type IV were community acquired methicillin resistant staphylococci (CA-MRS).

Antimicrobial Susceptibility Testing

Ninety-two percent of isolates displayed resistance to at least one antimicrobial agent. Many MRCNS isolates were frequently resistant to erythromycin (14/25, 56%), tetracycline (13/25, 52%) and clindamycin (8/25, 32%). In addition, six (24%) of the isolates were resistant to ciprofloxacin and trimethoprim-sulfamethoxazole, five (20%) to gentamicin

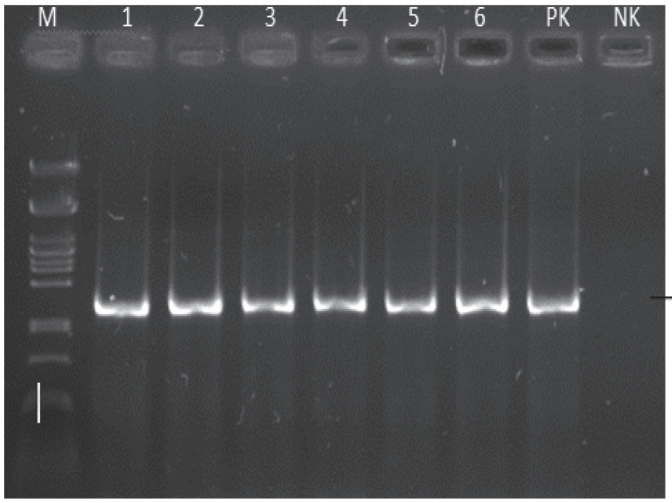

Fig 1. PCR performed by using 16S rRNA universal primers. M: Marker (Lambda phage DNA restricted with *Pst*I enzyme) 1-6: PCR performed by using isolated microorganism's DNA. PC: Positive control (*S. aureus* HPV107), NC: Negative control (master mix without DNA)

Şekil 1. 16S universal primerleri kullanılarak gerçekleştirilen PZR. M: Marker (*Pst*I enzimi ile kesilmiş lambda faj DNA'sı). PZR. 1-6: *Staphylococcus* izolatları, PK: Pozitif kontrol (*S. aureus* HPV107), NK: Negatif kontrol (DNA'sız master miks)

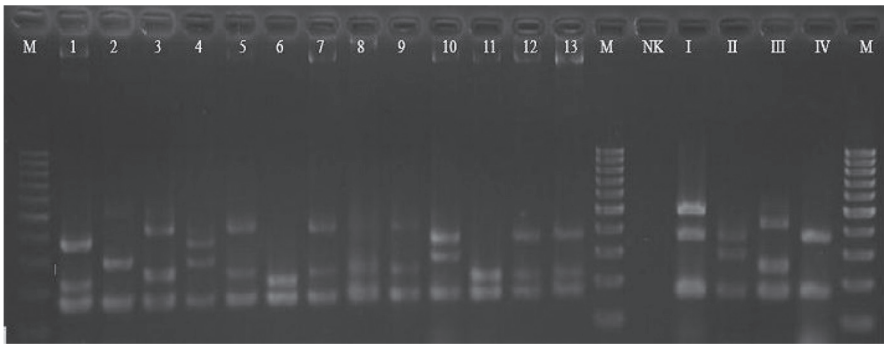

Fig 2. SCC*mec* types determined in MRS isolates. Lane M: 100 bp molecular marker. Lane 1-13: SCC*mec* types belong to different MRS isolates, Lane NC: Negative control (master mix without DNA), Lane I: *S. aureus* HPV107 (SCC*mec* type I), Lane II: *S. aureus* BK2464 (SCC*mec* type II), Lane III: *S.aureus* HUSA304 (SCC*mec* type III), Lane IV: *S. aureus* GRE14 (SCC*mec* type IV)

Şekil 2. MRS izolatlarında belirlenen SCC*mec* tipleri. M: 100 bp moleküler marker, 1-13: Farklı MRS izolatlarına ait SCC*mec* tipleri. NK: Negatif kontrol, I: *S. aureus* HPV107 (SCC*mec* tip I), II: *S. aureus* BK2464 (SCC*mec* tip II), III: *S. aureus* HUSA304 (SCC*mec* tip III), IV: *S. aureus* GRE14 (SCC*mec* tip IV)

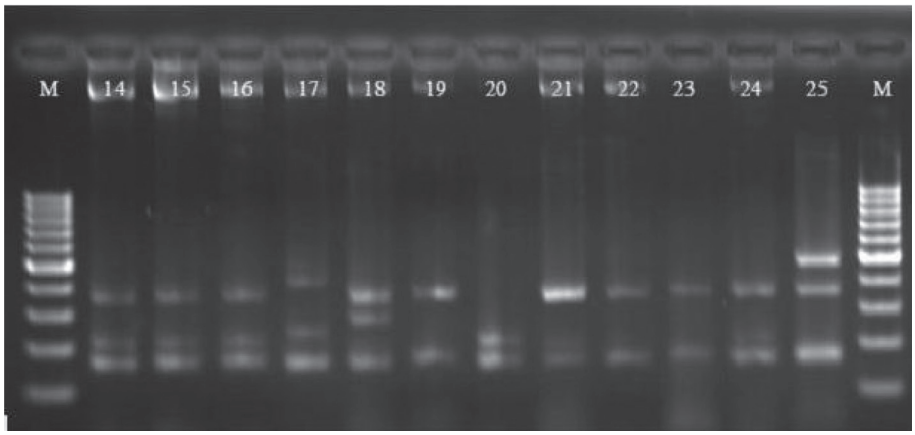

Fig 3. SCC*mec* types determined in MRS isolates. Lane M: 100 bp molecular marker. Lane 14-25: SCC*mec* types belong to different MRS isolates

Şekil 3. MRS izolatlarında belirlenen SCC*mec* tipleri. M: 100 bp moleküler marker. 14-15: Farklı MRS izolatlarına ait SCC*mec* tipleri

and mupirocin, three (12%) to rifampicin and one (4%) to quinopristin-dalfopristin, fusidic acid, and amoxicillin-clavulanic acid. But, all MRCNS isolates were found to be susceptible to vancomycin. All *S. hominis*, *S. warneri* and *S. haemolyticus* isolates displayed multiple antimicrobial resistance *(Table 1)*.

Prevalence of Resistance Genes

The *mec*A was detected in all strains. Of the 14 erythromycin-resistant (ER) isolates, 12 (85.7%) were positive for *erm*C, followed by *erm*B (9/14; 64.3%), *mph*C (9/14; 64.3%), *msr*A (7/14; 50.0%) and *erm*A (1/14, 7.1%). The *tet*K was the most prevalent gene among tetracycline resistant isolates, detected alone in 8 (61.5%) isolates, in combination with *tet*M in 3 (42.8%) isolates. The *tet*M was detected in two (15.4%) isolates. Among aminoglycoside-resistant isolates, *aac(6')/aph(2")* was detected in three (60%) strains, *aph(3')-IIIa* and *ant(4')-Ia* in one strain, and *ant(4')-Ia* in one isolate. Eight clindamycin resistant isolates were positive for *lnu*A gene. While only three isolates carried *ileS-2* gene among five mupirocin resistant isolates, *fus*B and *fus*C genes were not detected in one fusidic acid resistant isolate *(Table 1)*.

Table 1. *Antimicrobial resistance phenotypes, genotypes and SCCmec types of methicillin resistant coagulase negative staphylococci isolated from dogs*
Tablo 1. *Köpeklerden izole edilen metisilin dirençli koagülaz negatif stafilokokların antimikrobiyal direnç fenotipleri, genotipleri ve SCCmec tipleri*

MRCoNS Species	Phenotype*	Genotype	SCC*mec*Type
S. epidermidis	OXA, E	*mec*A, *erm*B, *erm*C, *mph*C	I
S. epidermidis	OXA, E, MUP, CIP	*mec*A, *erm*B	IV
S.epidermidis	OXA, TE, MUP	*mec*A, *tet*K, *ileS-2R*	IV
S. epidermidis	OXA	*mec*A	III
S.epidermidis	OXA, TE, E, DA, CIP	*mec*A, *tet*K, *erm*C, *msr*A, *mph*C, *lnu*A	II
S. epidermidis	OXA, SXT	*mec*A	IV
S. epidermidis	OXA, TE, E, SXT	*mec*A, *tet*K, *erm*C, *msr*A, *mph*C	IV
S. epidermidis	OXA, E, MUP	*mec*A, *erm*B, *erm*C, *msr*A, *ileS-2R*	III
S. epidermidis	OXA, TE, E, DA, FD	*mec*A, *tet*K, *erm*B, *erm*C, *mph*C	II
S. epidermidis	OXA, E, CN, DA, QD, MUP	*mec*A, *erm*B, *erm*C, *msr*A, *mph*C, *aac(6')/aph(2'')*, *lnu*A, *ileS-2R*	II
S. epidermidis	OXA	*mec*A	III
S. epidermidis	OXA, TE, E, DA, CIP	*mec*A, *tet*K, *erm*C, *msr*A, *mph*C, *lnu*A	II
S. lentus	OXA, TE	*mec*A, *tet*K, *tet*M	II
S. lentus	OXA, TE, CN, RD	*mec*A, *tet*M, *aac(6')/aph(2'')*	II
S. lentus	OXA, TE, DA	*mec*A, *tet*K, *tet*M, *lnu*A	II
S. lentus	OXA, TE, E, DA, SXT, CIP	*mec*A, *tet*K, *tet*M, *lnu*A, *erm*A, *erm*B, *erm*C, *mph*C	III
S. lentus	OXA, AMC, SXT, CIP	*mec*A	II
S. lentus	OXA, TE	*mec*A, *tet*K	IV
S. hominis	OXA, TE, CN, RD	*mec*A, *tet*K, *aac(6')/aph(2'')*	II
S. hominis	OXA, TE, E, CN, DA	*mec*A, *tet*M, *lnu*A, *erm*B, *erm*C, *msr*A, *mph*C, *aph(3')-IIIa*, *ant(4')-Ia*	III
S. hominis	OXA, E, MUP	*mec*A, *erm*C	III
S. hominis	OXA, TE, E, SXT	*mec*A, *tet*K, *erm*B, *mph*C	III
S. warneri	OXA, E, DA, CN	*mec*A, *lnu*A, *erm*B, *erm*C, *msr*A, *ant(4')-Ia*	III
S. arlettae	OXA, SXT	*mec*A	II
S. haemolyticus	OXA, E, RD, CIP	*mec*A, *erm*C	III

**** OXA:*** *oxacillin,* ***E:*** *erythromycin,* ***SXT:*** *trimethoprim-sulfamethoxazole,* ***QD:*** *quinopristin-dalfopristin,* ***CN:*** *gentamicin,* ***CIP:*** *ciprofloxacin;* ***MUP:*** *mupirocin,* ***FD:*** *fusidic acid,* ***RA:*** *rifampicin,* ***AMC:*** *amoxicillin-clavulanic acid,* ***DA:*** *clindamycin,* ***TE:*** *tetracycline*

The most prevalent SCC*mec* type were SCC*mec* II (40%), followed by SCC*mec* III (36%), SCC*mec* V (20%), and SCC*mec* I (4%) *(Fig. 2, 3)*.

DISCUSSION

Considering the high zoonotic potential of MRSA and MRSP, it is encouraging that MRSA and MRSP were not isolated from any dogs sampled in this study. This indicates that these agents have a very low in the total population of dogs admitted to clinics. Similar results have been reported in Turkey and Denmark [4,6].

CNS are recognised as a major cause of nosocomial infections, especially in immunocompromised patients [2]. An increase of MRCNS strains was reported from 38% in 1996 to 67.5% in 2007 in Turkey [23]. Although, importance of CNS in veterinary medicine or potential for zoonotic infection is not well known. In recent years, CNS has steadily gained importance as veterinary pathogens and implicated in mastitis, pyoderma, cystitis, arthritis and respiratory system infections in various animal species [8,9,24,25].

The most prevalent SCC*mec* types were II (40%, 10/25) and III (36%, 9/25), identified among all MRCNS. Type IV is predominant among *S. epidermidis* isolates. SCC*mec* type IV was more frequently acquired by *S. epidermidis*, which is in accordance with the enhanced mobility of this type of SCC*mec*. A majority of hospital-acquired MRSA (HA-MRSA) isolates harbor SCC*mec* type I-III [26], SCC*mec* type III were found to be more prevalent among human MRSA strains with a prevalence rate of 82.1% in Turkey [27]. Dominance of HA-MRSA SCC*mec* type II and III indicate that dogs are a large reservoir of SCC*mec* in MRCNS. It is reasonable to assume that CoNS of dog origin share a common pool of SCC*mec* with MRSA and thus pose a potential threat to public and animal health.

Among 25 MRCNS isolated, *S. epidermidis*, *S. lentus* and *S. hominis* were most prevalent species. To the best of our knowledge, this is the first report of *mec*A-mediated

methicillin resistance in *S. arlettae* in dogs. *S. arlettae* is one of the CoNS isolated from the skin of mammals and poultry [28]. Bagcigil et al.[4] reported *S. epidermidis* (n=7) and S. *haemolyticus* (n=3) as more prevalent species in dogs in Denmark. Another study carried out in Turkey, *S. hominis* was found to be the more prevalent among MRCNS in dogs [6]. Although no information is available on the frequency of nosocomial pathogens in veterinary hospitals, some species, mainly *S. epidermidis*, *S. haemolyticus*, *S. hominis* have been isolated from nosocomial infections in Turkey [23,29].

Methicillin resistant strains have high rates of resistance to other classes of antimicrobials than methicillin susceptible strains [2]. In this study, MRCNS strains were resistant to clinically relevant antimicrobial drugs such as mupirocin, fusidic acid, quinopristin-dalfopristin, rifampicin in various levels. These findings confirm that MRS may pose a major therapeutic challenge for veterinarians due to limited choise of antimicrobials. Taken into consideration of multiple resistance, antimicrobial selective pressure is likely to play a key role in the emergence and spread of MRCNS among dog population.

All except two MRCNS isolates carried more than one antimicrobial resistance gene. In particular, one *S. hominis* isolate carried nine resistance genes that confer resistance to five antimicrobials. Hanssen and Sollid [30] reported that resistant strains of CNS might serve as pool of antimicrobial resistance genes. Because majority of resistance determinants carried by mobile genetic elements, and this favors transfer of resistance genes within and across bacteriel species and even across genus borders

In conclusion, the results indicate that MRCNS are common in dogs in Turkey. Therefore, the resistance trends observed among staphylococci isolated from the nasal cavity of dogs seem to reflect the national and local patterns of antimicrobial usage in this animal species. However, further studies based on larger and more representative study populations are needed to determine the true prevalence of these agents.

REFERENCES

1. Weese JS, Van Duijkeren E: Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. *Vet Microbiol*, 140, 418-429, 2010.

2. Piette A, Verschraegen G: Role of coagulase-negative staphylococci in human disease. *Vet Microbiol*, 134, 45-54, 2009.

3. Pyörälä S, Taponen S: Coagulase-negative staphylococci-emerging mastitis pathogens. *Vet Microbiol*, 134, 3-8, 2009.

4. Bagcigil FA, Moodley A, Baptiste KE, Jensen VF, Guardabassi L: Occurrence, species distribution, antimicrobial resistance and clonality of methicillin- and erythromycin-resistant staphylococci in the nasal cavity of domestic animals. *Vet Microbiol*, 121, 307-315, 2007.

5. Vengust M, Anderson ME, Rousseau J, Weese JS: Methicillin-resistant staphylococcal colonization in clinically normal dogs and horses in the community. *Lett Appl Microbiol*, 43, 602-606, 2006.

6. Bağcıgil AF, İkiz S, Güzel Ö, Parkan Ç, Ilgaz A: Kliniğe getirilen köpeklerden ve klinik ortamından metisiline dirençli stafilokokların izolasyonu ve *mec*A geninin PCR ile araştırılması. *VII. Ulusal Veteriner Mikrobiyoloji Kongresi (Uluslararası Katılımlı), 26-28 Eylül 2006, Side-Antalya,* 2006.

7. Oliveira DC, de Lencastre H: Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Chemother*, 46, 2155-61, 2002.

8. Kaynarca S, Türkyılmaz S: Methicillin resistance and slime positivity of staphylococci isolated from bovine mastitis. *Kafkas Univ Vet Fak Derg,* 16 (4): 567-572, 2010.

9. İnegöl E, Türkyılmaz S: Determination of SCC*mec* types in methicilline resistant staphylococci isolated from cows and farm workers. *Ankara Üniv Vet Fak Derg,* 59, 89-93, 2012.

10. Suau A, Bonnet R, Sutren M, Godon JJ, Gibson G, Collins MD, Dore´ J: Direct rDNA community analysis reveals a myriad of novel bacterial lineages within the human gut. *Appl Environ Microbiol*, 65, 4799-4807, 1999.

11. Sghir A, Antonopoulos D, Mackie RI: Design and evaluation of a lactobacillus group-specific ribosomal RNA-targeted hybridization probe and its application to the study of intestinal microecology in pigs. *Syst Appl Microbiol*, 21, 291-296, 1998.

12. Clinical and Laboratory Standards Institute: Performance standards for Disc and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard-third Edition (M31-A3), CLSI, Wayne, PA, 2008

13. Toma E, Barriault D: Antimicrobial activity of fusidic acid and disk diffusion susceptibility testing criteria for Gram-positive cocci. *J Clin Microbiol*, 33, 1712-1715, 1995.

14. Fuchs PC, Jones RN, Barry AL: Interpretive criteria for disk diffusion susceptibility testing of mupirocin, a topical antibiotic. *J Clin Microbiol*, 28, 608-609, 1990.

15. Mclaws F, Chopra I, O'Neill AJ: High prevalence of resistance to fusidic acid in clinical isolates of *Staphylococcus epidermidis. J Antimicrob Chemother*, 61, 1040-1043, 2008.

16. Choi SM, Kim SH, Kim HJ, Lee DG, Choi JH, Yoo JH, Kang JH, Shin WS, Kang MW: Multiplex PCR for the detection of genes encoding aminoglycoside modifying enzymes and methicillin resistance among Staphylococcus species*,* 18, 631-636, 2003.

17. Strommenger B, Kettlitz C, Werner G, Witte W: Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. *J Clin Microbiol*, 41, 4089-4094, 2003.

18. Lüthje P, Schwarz S: Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. *J Antimicrob Chemother*, 57, 966-969, 2006.

19. Martineau F, Picard FJ, Lansac N, Ménard C, Roy PH, Ouellette M, Bergeron MG: Correlation between the resistance genotype determined by multiplex PCR assays and the antibiotic susceptibility patterns of *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrob Agents Chemother*, 44, 231-238, 2000.

20. Jensen LB, Frimodt-Møller N, Aarestrup FM: Presence of *erm* gene classes in gram-positive bacteria of animal and human origin in Denmark. *FEMS Microbiol Lett*, 170, 151-158, 1999.

21. Lina G, Quaglia A, Reverdy ME, Leclercq R, Vandenesch F, Etienne J: Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. *Antimicrob Agents Chemother*, 43, 1062-1066, 1999.

22. Nunes EL, dos Santos KR, Mondino PJ, Bastos Mdo C, Giambiagi-de Marval M: Detection of *ileS-2* gene encoding mupirocin resistance in methicillin-resistant *Staphylococcus aureus* by multiplex PCR. *Diagn Microbiol Infect Dis*. 34, 77-81, 1999.

23. Köksal F, Yasar H, Samasti M: Antibiotic resistance patterns of coagulase-negative staphylococcus strains isolated from blood cultures of septicemic patients in Turkey. *Microbiol Res*, 164, 404-410, 2009.

24. Taponen S, Pyörälä S: Coagulase-negative staphylococci as cause of bovinemastitis-Not so different from *Staphylococcus aureus*? *Vet Microbiol*, 134, 29-36, 2009.

25. van Duijkeren E, Box AT, Heck ME, Wannet WJ, Fluit AC: Methicillin-

resistant staphylococci isolated from animals. *Vet Microbiol*, 103, 91-97, 2004.

26. Martins A, Cunha de ML: Methicillin resistance in *Staphylococcus aureus* and coagulase negative staphylococci: epidemiological and molecular aspects. *Microbiol Immunol*, 51, 787-795, 2007.

27. Kılıç A, Güçlü AÜ, Şenses Z, Bedir B, Aydogan H, Başustaoğlu AC: Staphylococcal cassette chromosome *mec* (SCC*mec*) characterization and panton-valentine leukocidin gene occurrence for methicillin-resistant *Staphylococcus aureus* in Turkey, from 2003 to 2006. *Antonie van Leeuwenhoek*, 94, 607-614, 2008.

28. Schleifer KH, Kilpper-Balz R, Devriese LA: *Staphylococcus arlettae* sp. nov., S. *equorum* sp. nov. and *S. kloosii* sp. nov.: Three new coagulase-negative, novobiocin-resistant species from animals. *Syst Appl Microbiol*, 5, 501-509, 1984.

29. Biçer AT: Hastane izolatı Staphylococus aureus ve koagülaz negatif staphylococcus suşlarında metisilin direncinin farklı yöntemlerle araştırılması. *Uzmanlık Tezi.* Çukurova Üniversitesi Tıp Fakültesi Mikrobiyoloji Anabilim Dalı, Adana, 2009.

30. Hanssen AM, Ericson Sollid JU: SCC*mec* in staphylococci: genes on the move. *FEMS Immunol Med Microbiol*, 46, 8-20, 2006.

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RESEARCH ARTICLE

Productivity and Meat Nutrient in Fish: The Diet Effect

Dragana LJUBOJEVIĆ * Miroslav ĆIRKOVIĆ * Nikolina NOVAKOV *
Rade JOVANOVIĆ ** Snežana JANKOVIĆ ** Vesna ĐORĐEVIĆ ***
Zoran MAŠIĆ ****

* Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, 21000 Novi Sad, Republic of Serbia
** Institute for Science Application in Agriculture, 11000 Belgrade, Republic of Serbia
*** Institute of Hygiene and Meat Technology, 11000 Belgrade, Republic of Serbia
**** Scientific Veterinary Institute "Novi Sad", 21000 Novi Sad, Republic of Serbia

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Summary

Meat quality of carp varies by age and rearing system as well as feed consumed. The aim of the study was to determine the impact of the diet on the survival rate, yield per unit of area, chemical composition, the amount of total cholesterol and fatty acid profile of two-year old common carp (*Cyprinus carpio* L.) reared in the basic culture systems. Fish were grown in the similar ponds and subjected to 1 of 3 feeding systems: only natural food (extensive system), supplemental grain (semi-intensive system), and extruded formula consisting of soybean, sunflower kernel, wheat flour, corn and brewery yeast (intensive system). Feeding extruded formula doubled production per hectare of pond surface area, compared with feeding supplemental grain and almost thrice compared with feeding only natural food. The n-3/n-6 ratio varied widely by the diet. Carp fed extruded formula yielded the most preferable the unsaturated fatty acid:saturated fatty acids and polyunsaturated fatty acids:saturated fatty acids ratios. In conclusion, the provision of processed plant meals can be an important protein source for common carp to improve productivity and food quality.

Keywords: *Productivity, Common carp, Meat nutrients, Nutrition, Rearing system*

Balıklarda Verimlilik ve Et Besin Bileşimi: Yemin Etkisi

Özet

Sazan balığının et kalitesi yaş ve yetiştirme sisteminin yanı sıra tüketilen yeme göre de değişir. Çalışmanın amacı; temel kültür sistemlerinde yetiştirilen iki yaşlı sazanlarda (*Cyprinus carpio* L .) farklı diyetlerle sağkalım oranı, birim yetiştirme alanından elde edilen verimlilik ve et besin bileşimi, total kolesterol ile yağ asidi profiline etkisini araştırmaktı. Aynı gölette büyütülen ba balıklarda; 1-Sadece doğal gıda (ekstansif sistem), 2- Tahıl takviyesi (yarı-entansif sistem) veya 3-Soya, ayçiçeği, buğday unu, mısır, malt içeren ekstrude karışım (entansif sistem) ile beslendi. Ekstrude karışımla besleme verimliliği hektar olarak gölet yüzeyine göre; tahıl takviyesi yapılanlara kıyasla iki, sadece doğal gıda sağlananlara göre ise üç kat artırmış oldu. Etlerdeki n-3:n-6 oranı diyete bağlı olarak varyasyon gösterdi. İnsan tüketimi için tercih edilen doymamış: doymuş yağ asidi ve çoklu doymamış: doymuş yağ asidi oranı ekstrude karışımla beslenen balıkların etinden elde edildi. Sonuç olarak, yeterli miktarda işlenmiş bitkisel küspe ve ürünlerin kullanımı sazanlar için önemli protein kaynağı olabilir ve ürünün gıda kalitesini artırabilir.

Anahtar sözcükler: *Verimlilik, Sazan, Et besin bileşimi, Besleme, Yetiştirme sistemi*

INTRODUCTION

Common carp is the most widespread fish species in Serbia. Alike other fish species, common carp cannot synthesize the essential fatty acids of the n-6 and n-3 series. Hence, these fatty acids must be provided by the feed. Their original food sources are phytoplankton and zooplankton [1] that are rich in proteins, fats, free amino acids, fatty acids, oligopeptides and vitamins. Carp farms rely on natural food during the production cycle, which is known as extensive culture system and characterized by low yield. This production system is dependent on pond fertility and economically feasible [2]. The main type of fish production in Serbia is the semi-intensive system for cyprinid production, carp being as the major

İletişim (Correspondence)
+381 64 2745389
ljubojevic.ljubojevicd.dragana@gmail.com

species. In addition to natural food, cereals are supplemented to meet protein requirement. Some fish farms increase production by introducing extruded complete feed for carp [3]. The cost of inputs per unit of fish weight is higher than in extensive and semi-intensive farming, especially because of the high cost of fish feed that contains a high level of protein with a balanced amino acid composition. High cost can be overcome by replacing animal origin feedstuffs with local available vegetable-derived protein ingredients. Many cultured warm-water fish, including carp, require no meat or fish products in their diets [4-6].

The use of plant-derived materials such as legume seeds and different types of oilseed cake contain a wide variety of antinutritional substances, including phytates, glucosinolates, saponins, tannins, lectins, oligosaccharides and non-starch polysaccharides, phytoestrogens, alkaloids, antigenic compounds, gossypols, cyanogens, mimosine, cyclopropenoid fatty acids, canavanine, antivitamins, and phorbol esters [7] that limit feed utilization. Extrusion is used to make plant protein more available to animals through denaturing termolabile antinutritional factors, resulting in improved nutrient digestibility, palatability, pellet durability, water stability, and pellet storage life [8]. Common carp meat is rich in protein and n-3 polyunsaturated fatty acids (PUFA), including eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids [9]. The typical fatty acid composition of fish species is strongly affected by the diets, sex and environmental conditions [10]. Beside PUFA, fish fats contain cholesterol. Fish meat cholesterol content (490-920 mg kg^{-1}) is similar to pork or beef (450-840 mg kg^{-1}) [11]. The aim of the study was to determine the impact of the diet on the survival rate, yield per unit of area, chemical composition, amount of total cholesterol and fatty acids profile of two-year old common carp (*Cyprinus carpio* L.) reared in the extensive, semi- intensive and intensive systems.

MATERIAL and METHODS

Pond Management and Fish Samples

The growth trial was carried out at the experimental fish farm (Mošorin, Serbia) with common carp (*Cyprinus carpio* L.) obtained from a commercial fish farm. Fish were grown in three earthen ponds each of 1 ha where were left dry and untreated during winter. The initial density of carp per hectare was equal, 2500 individuals. The average initial live weight of all fish was 250 g *(Table 3)*. The production in first pond was based on natural food consisting of benthic and planktonic organisms (extensive system). In the second pond additional feeding was done with mixture of corn (80%) and wheat (20%) (semi-intensive system). In the last pond, the common carp was supplemented with extruded formulated feed mixture (intensive system). During the experiment, the water temperature, dissolved oxygen content and pH were measured biweekly in the morning hours (around 9.00 h). The water quality parameters did not differ significantly between the ponds. The content of dissolved oxygen was highly variable, ranging from 1.4 to 14.8 (mg $O_2 l^{-1}$). The pH varied from 7.04 to 8.62, while temperature of water ranged from 15 to 28.7°C.

Soybean meal, brewery yeast, wheat flour and corn were used as ingredients for extruded formula. Ingredients were mixed and extruded using a twin screw extruder in Animal Feed Manufactory (Komponenta, Ćuprija, Republic of Serbia). Composition of formulated feed is shown in *Table 1* and fatty acid compositions of supplemental grains and extruded formula are shown in *Table 2*.

The experimental carp were measured biweekly in order to adjust the daily feed rate that was 3 % of the total fish mass. Fish were hand-fed twice daily at 8:00 and 15:00 h.

Table 1. *Composition and proximate analysis of the extruded formula diet*
Tablo 1. *Ekstrude formulanın içerik ve besin madde kompozisyonu*

Ingredients	**g kg^{-1} dry diet**	**Chemical analysis**	**g kg^{-1} dry diet**
Soybean meal	450	Dry matter (DM)	897.1
Sunflower kernel	150	Crude protein (CP)	280.6
Brewery yeast	50	Crude fat (CF)	63.3
Wheat flour	146	Crude ash (CA)	41.6
Corn	180	NFE[3]	61.4
Methionin	1	Gross energy (MJ·kg^{-1} DM)[4]	102.5
Lysine L	3		
Vitamin mix[1]	10		
Mineral mix[2]	10		

[1] Vitamin mix (mg/kg^{-1} of diet): vitamin B_1, 15; vitamin B_2, 10; vitamin B_6, 20; vitamin B_{12}, 0,15; vitamin K_3, 15; inositol, 250; Ca-pantothenic acid, 80; nicotinic acid, 100; folic acid, 1; vitamin H (biotin), 1; vitamin E, 140; vitamin C, 500; vitamin A, 20.000 IU; vitamin D_3, 6.000 IU; choline chloride, 1.800, and cellulose was used as a carrier, [2] Mineral mix (mg kg^{-1} of diet): Cu 20, Fe 40, Mn 30, Se 0.4, Zn 125, and cellulose was used as a carrier , [3] NFE, nitrogen-free extract, g.kg^{-1} DM = 100 - (CP + CF + CA), [4] Calculated based on the following conversion factors: CP – 24 kJ g^{-1}, CL – 39 kJ g^{-1}, NFE - 17 kJ g^{-1} [13] (Jobling 1994)

Table 2. *Fatty acid composition of the experimental diets*
Tablo 2. *Deneme diyetlerinin yağ asidi bileşimi*

Fatty Acid (% of the Total Lipid Fatty Acids)	Grain Mixture (80% Corn + 20% Wheat)	Extruded Formula
Myristic acid, $C_{14:0}$	0.40	0.47
Palmitic acid, $C_{16:0}$	10.86	10.98
Palmitoleic acid, $C_{16:1}$	0.16	0.48
Stearic acid, $C_{18:0}$	2.02	2.74
Oleic acid, $C_{18:1cis-9}$	27.62	26.32
Vaccenic acid, $C_{18:1\ cis-11}$	0.16	0.82
Linoleic acid, $C_{18:2n-6}$	56.15	54.15
αLinolenic acid, $C_{18:3n-3}$	0.98	2.23
Arachidic acid, $C_{20:0}$	0.38	0.3
Eicosenoic acid, $C_{20:1}$	0.20	0.34
Behenic acid, $C_{20:2}$	0.26	0.22
Dihomogammalinolenic acid, $C_{20:3n-6}$	0.61	0.58
Eicosatrienoic acid, $C_{20:3n-3}$	-	0.32
Arachidonic acid, $C_{20:4}$	-	0.06
Lignoceric acid, $C_{24:0}$	0.17	0.19
SFA	13.83	14.55
MUFA	28.14	27.96
PUFA	58	57.56
n-6	57.02	55.01
n-3	0.98	2.55
n-3/n-6	0.02	0.05

Measurements

The fishponds were stocked in March and harvested in October. Growth-performance indicators [body weight gain (BWG), specific growth rate (SGR), feed conversion ratio (FCR), weight gain (WG) and survival rate (SR)] were measure using following formulas:

SGR = 100 (Ln (mean final body weight)-Ln (mean initial body weight))/time (days);

FCR = dry feed intake (g)/wet weight gain (g);

SR(%) = (Final fish number/initial fish number)*100;

WG = Final body weight (g)- initial body weight(g) (g $fish^{-1}$);

BWG(%) = 100*(mean final weight-mean initial weight)/ mean initial weight

All fish were reared under variable natural atmospheric conditions. Natural production in each pond was increased by application of agrotechnical measures such as drying of fish ponds during winter, soil treatment, fertilization and adding lime. Livestock manure (2.000 kg ha^{-1}) was applied to the bottom of each empty pond and later biweekly over the water surface (a total of 4.000 kg ha^{-1} during growing season). Agricultural limestone (250 kg ha^{-1}) was provided to increase total alkalinity and total hardness of pond water, to the bottom of each empty pond and over the water surface. The same methods of cultivation and fertilization were applied in all the ponds. The aeration of the fish ponds was continuously secured by using an aerator pre pond. The water flow was about 3.5 l s^{-1}, that provided that there were no adverse effects of carbon dioxide and ammonia on the carp.

Laboratory Analyses

Twelve samples of two years old carp were taken from each pond during the harvesting. Also, samples of supplemental grain and extruded formula were taken and stored at –18°C until analyses. The meat from dorsal muscles without skin was used for chemical analyses. Water content of fish fillets was determined after drying the samples at 105°C to constant weight for 24 h (SRPS ISO 1442:1997). Crude protein content was determined by Kjeldahl method (Manual book, Kjeltec Auto 1030 Analyzer, Tecator, Sweeden) and ash was determined after burning at 550±25°C (SRPS ISO 936:1998). Crude lipid in fish tissue was also analyzed using the Soxhlet System with ether as solvent (SRPS ISO 1443:1997). Fatty acids determination was performed according to Spirić et al.[12]. Cholesterol determination in carp fillets (from direct saponification) was performed by using HPLC/PDA system (Waters 2695 Separation module/Waters photodiode array detector, USA) on a Phenomenex Luna C18 reverse/phase column, according to Maraschiello et al.[13]. In quantification

of cholesterol, external standardization was used, along with Empower Pro software to control the HPLC system for data acquisition and data processing as described [14].

Statistics

The group effect was determined using one-way ANOVA (Statistica 10.0, StatSoft Inc.). Inter-group differences were attained by the Tukey HSD test at P≤0.01. The results were presented as means ± SE.

RESULTS

Performance

The harvesting weight, survival rate, harvesting density, and specific growth rate were the greatest in intensive system and lowest in extensive system. At the end of the rearing period in October the average final live weight of carp in the group that had been fed on natural food was 462.58 g. Final live weight of carp in the group that had been given grains supplementary feeding was 754.08 g and in the group that had been formula feed feeding was 1188.75 g. Total harvesting density was 994.5 kg/ha in extensive, 1696.68 kg/ha in semi-intensive and 2734.12 kg/ha in intensive system *(Table 3)*. Feeding extruded formula increased production per hectare of pond surface area almost doubled compared with feeding supplemental grains and almost thrice compared with feeding only natural food.

Nutrient Composition

Carp reared in intensive system had the greatest protein and moderate lipid, ash, and cholesterol contents *(Table 4)*. Expectedly, rearing in extensive system reduced total lipid and cholesterol contents.

Fatty Acid Profile

Rearing in extensive system resulted in the greatest total SFA level in meat of carp, particularly of palmitic and stearic acids *(Table 5)*. In the semi-intensive system, MUFA was the greatest, predominantly of oleic acid. Carp that ingested

Table 3. *Growth performance of common carp reared in three different culture systems*
Tablo 3. *Üç farklı kültür sisteminde yetiştirilen sazanların büyüme performansı*

Variable	Rearing system		
	Extensive (Only Natural Food)	Semi-intensive (Grain Mixture (80% Corn + 20% Wheat)	Intensive (Extruded Formula)
Initial number of fish (ind ha^{-1})	2500	2500	2500
IBW (g)	250±16.04	250±24.92	253±15.86
FBW (g)	462.58±31.32^{c}	754.08±28.12^{b}	1188.75±49.4^{a}
Final number of fish (ind ha^{-1})	2150	2250	2300
Survival rate (%) SR	86	90	92
Stocking density (kg ha^{-1})	625	625	632.5
Harvesting density (kgha^{-1})	994.5	1696.68	2734.12
WG (gfish^{-1})	212.58	504.08	935.75
BWG%	85.03	201.63	369.86
SGR (%day^{-1})	0.26	0.47	0.66
FCR (g·g^{-1})		2.7	1.86

*[1] Data are means ± SE (n = 12). Values within the same row with different letter supercripts differ at P<0.01, [2] **IBW,** initial body weight; **FBW,** final body weight; **SR,** survival rate; **SGR,** specific growth rate; **FCR,** feed conversion ratio; **WG,** weight gain; **BWG,** body weight gain*

Table 4. *Proximate analysis results of common carp reared in three different culture systems*
Tablo 4. *Üç farklı kültür sisteminde yetiştirilen sazanların besin madde kompozisyonu*

Variable	Rearing system		
	Extensive (Only Natural Food)	Semi-intensive (Grain Mixture (80% Corn + 20% Wheat)	Intensive (Extruded Formula)
Moisture (g kg^{-1})	814.9±3.2^{a}	764±1.8^{c}	783.5±0.4^{b}
Crude protein (g kg^{-1})	154.8±2.8^{b}	155.9±2.1^{b}	171.7±0.5^{a}
Crude lipid (g kg^{-1})	20.7±1.1^{c}	68.5±1.4^{a}	31.9±0.5^{b}
Crude ash (g kg^{-1})	09.6±0.9^{c}	11.6±0.7^{a}	10.3±0.1^{b}
Total cholesterol (mg kg^{-1})	379.4±0.2^{c}	578±1.1^{a}	513.1±1.2^{b}

[1] Data are means ± SE (n = 12). Values within the same row with different letter supercripts differ at P<0.01

only natural food had higher n-3 fatty acids in the muscle than carp that received supplemental wheat or extruded formula *(Table 5)*. However, common carp reared in intensive culture system had higher n-6 fatty acids than carp reared other two culture systems. Thus, the total amount of PUFA was higher in muscle triacylglycerol of carp fed with extruded formulated feed compared to carp fed only on natural food. This is reflection of dietary fat being transferred to body tissues.

The n-3/n-6 ratio of the fish muscle was the highest in carp fed only on natural food, followed by carp fed extruded formula and the lowest value was observed in carp that received supplemental wheat. The highest level of n-3 fatty acids was found in the muscle of carp that received only natural food and the lowest in carp fed supplemental grains.

In two year-old carp fed extruded formula was observed the best ratio UFA/SFA, PUFA/SFA, the highest content of PUFA, the lowest content of SFA compared with other two groups. Lipids of carp in intensive production contained less MUFA (45%) than carp from the semi-intensive production (64%).

DISCUSSION

Performance

Supplementary feeding with grains leads to improved growth performance in common carp, and especially feeding with extruded formula. Survival rate in carp that ingested only natural food was lower than in carp fed grains, as well as than in carp fed formulated feed. In general, in all groups the survival rate was satisfactory and it was within the range considered normal for carp pond production in Republic of Serbia. Additional feeding with grains almost doubled average final body weight, while carp that received extruded formula showed three fold higher final body weight than carp fed only natural food and that lead to doubled harvesting density in semi-intensive system and threefold higher harvesting density in intensive system compared with extensive. Consequently, all growth parameters were the highest in intensive system and lowest in extensive system. That fact justified the use of supplemental feed in the rearing of carp and it represents a major opportunity to increase production in carp ponds. The growth parameters and total production of the carp were quite affected by that of the diets. Using adequately prepared extruded formula can further improve growth performance and yield of fish per unit of area. Administered extruded feed results in good growth and feed conversion. Higher temperature influenced the significantly higher growth rate of common carp in region of Republic of Serbia compared with Central Europe [9]. Besides the direct effect of using feed containing high protein indirect effect was achieved through nitrogen and phosphorus, which are released during digestion of formulated feed and increased development of natural food in the pond [3]. The positive effect is certainly significant in maintaining a better quality of water environment.

Nutrient Composition

In the literature, depending on age, rearing system, and food, fat content varies from 23 to 168 g kg^{-1}DM and protein content varies from 140 to 180 g kg^{-1} in carp [15-17]. In the present trial nutrient composition was highly depended on the diet. Supplementary feeding with grains leads to enlarged amounts of crude lipid in fish meat and it was doubled higher compared to supplementary feeding with extruded formula and three-fold higher compared to carp which ingested only natural food. The fillets obtained from the experimental fish were characterized by a varied content of fat and water. The same regularity was observed by Ćirković et al.[18]. The varied content of fat was compensated by the content of water, which is in agreement with the results obtained by Żmijewski et al.[19] who found a reverse correlation between the fat and water contents, which is common for many fish species. Crude protein level was the highest in the fillets of carp from intensive system, while there were no significant difference in the amount of protein in fillets of carp from extensive and semi-intensive systems. Cholesterol content in fish meat is not correlated with fat content [11]. Trbović et al.[16] reported that the amounts of total cholesterol in lipids were 490 mg kg^{-1} in one-year old carp harvested in April and 540 mg kg^{-1} in one-year old carp harvested in June. Data about influence of diet and rearing systems on cholesterol content in carp are limited. However, it is known that cholesterol content in lipids of carp varies considerably, within the range of 470-1.200 mgkg^{-1} [20,21]. Total cholesterol content in the present research was the highest in semi-intensive system and the lowest in extensive system, but in all groups was favourable and within the previously mentioned [20,21]. This great variability could be related to harvest season and age as well as rearing system.

The present results confirms that proximate composition of common carp highly depends of diet [22]. The fat content in fish meat contributes to its juiciness, tastefulness and texture, as well as organoleptic properties. Lipid content in fillets from extensive system was very low and such lean tissue is dry and perceived as thickly fibrous. On the other hand, there are certain groups of people who require meat with minimal fat and cholesterol content.

Fatty Acid Profile

The preference for a feed rich in saccharides leads to an increase in the percentage of the oleic acid in body lipids of the fish, which is produced in the organism by desaturation and elongation of SFA [23]. At the same time, proportion of PUFA n-3 decreases [23,24]. Supplementary feeding with grains leads to reduced amounts of essential fatty acids in fish meat and this is due to the lower proportion of natural food in the diet of the carp which received additional grains.

The two fatty acids 18:2n-6 and 18:3n-3 are precusors for synthesis of n-6 and n-3 PUFAs, recpectively [25]. Carp that received extruded formula showed high values of n-6 fatty

Table 5. *Fatty acid composition of common carp reared in three different culture systems*
Tablo 5. *Üç farklı kültür sisteminde yetiştirilen sazanların yağ asidi profili*

Fatty Acids (% of the Total Lipid Fatty Acids)²	**Rearing system**		
	Extensive (Only Natural Food)	**Semi-intensive (Grain Mixture (80% Corn + 20% Wheat)**	**Intensive (Extruded Formula)**
Lauric acid, $C_{12:0}$	0.05±0.02^{c}	0.14±0.01^{a}	0.10±0.01^{b}
Myristic acid, $C_{14:0}$	1.14±0.07^{a}	0.71±0.03^{b}	0.73±0.01^{b}
Pentadecylic acid, $C_{15:0}$	0.49±0.12^{a}	0.01±0.01^{b}	0.23±0.01^{b}
Palmitic acid, $C_{16:0}$	20.99±0.28^{a}	17.31±0.09^{b}	16.89±0.03^{b}
Margaric acid, $C_{17:0}$	0.69±0.09^{a}	0.12±0.01^{b}	0.18±0.005^{b}
Stearic acid, $C_{18:0}$	5.26±0.2^{b}	5.79±0.02^{a}	4.16±0.01^{c}
Arachidic acid, $C_{20:0}$	0.19±0.05^{a}	0.11±0.02^{b}	0.10±0.005^{b}
SFA	28.82±0.36^{a}	24.19±0.11^{b}	22.4±0.03^{c}
Palmitoleic acid, $C_{16:1}$	5.15±0.07^{b}	6.23±0.01^{a}	5.2±0.04^{b}
Oleic acid, $C_{18:1cis-9}$	32.58±0.42^{c}	51.35±0.04^{a}	34.45±0.01^{b}
Vaccenic acid,$C_{18:1cis-11}$	4.26±0.13^{b}	4.54±0.04^{a}	2.93±0.01^{c}
Eicosenoic acid, $C_{20:1}$	1.51±0.18^{c}	2.19±0.05^{b}	2.54±0.01^{a}
MUFA	43.49±0.42^{c}	64.31±0.09^{a}	45.12±0.03^{b}
Linoleic acid, $C_{18:2, n-6}$	13.49±0.25^{b}	8.7±0.13^{c}	22.57±0.01^{a}
Linolenic(GLA)$C_{18:3, n-6}$	0.19±0.07^{b}	0.11±0.02^{c}	0.25±0.01^{a}
α-Linolenic, $C_{18:3, n-3}$	4.59±0.19^{a}	0.61±0.06^{c}	2.12±0.01^{b}
Behenic acid, $C_{20:2}$	0.7±0.08^{a}	0.27±0.05^{b}	0.73±0.01^{a}
Dihomo-gamma-linolenic acid, $C_{20:3, n-6}$	0.77±0.12^{b}	0.43±0.04^{c}	1.02±0.01^{a}
Eicosatrienoic acid, $C_{20:3, n-3}$	0.86±0.1^{a}	0.06±0.02^{c}	0.71±0.01^{b}
Arachidonic acid, $C_{20:4}$	2.79±0.21^{a}	0.73±0.04^{c}	1.44±0.01^{b}
Eicosapentaenoic acid, $C_{20:5, n-3}$	1.17±0.1^{a}	0.2±0.02^{c}	0.93±0.01^{b}
Docosapentaenoic acid, $C_{22:5, n-3}$	0.91±0.11^{a}	0.18±0.03^{b}	0.85±0.02^{a}
Docosahexaenoic acid, $C_{22:6, n-3}$	2.22±0.3a	0.25±0.04^{c}	1.86±0.04^{b}
PUFA	27.69±0.39^{b}	11.53±0.15^{c}	32.48±0.03^{a}
n-6	17.93±0.36^{b}	10.24±0.13^{c}	26.01±0.04^{a}
n-3	9.75±0.35^{a}	1.29±0.1^{c}	6.48±0.04^{b}
n-3/n-6	0.54±0.03^{a}	0.13±0.01^{c}	0.25±0.001^{b}
n-6/n-3	1.84±0.09^{c}	7.99±0.66^{a}	4.02±0.03^{b}
PUFA/SFA	0.64±0.01^{b}	0.18±0.002^{c}	0.72±0.001^{a}
UFA/SFA	0.96±0.02^{b}	0.48±0.008^{c}	1.45±0.003^{a}
PUFA/MUFA	2.47±0.04^{c}	3.13±0.02^{b}	3.46±0.01^{a}

*¹ Data are means ± SE (n = 12). Values within the same row with different letter supercripts differ at P<0.01, ² **SFA** = saturated fatty acids; **MUFA** = monounsaturated fatty acids; **USFA** = unsaturated fatty acids, **PUFA** = polyunsaturated fatty acids from the n3 (n3 PUFA) and n6 (n6 PUFA) families*

acids in their muscle, based on the high content of linoleic acid in the diets. However, although grain mixture contained slightly higher amount of linoleic acid than formulated feed, percentage of this fatty acid was lower than in carp fed formulated feed, as well as than in carp which ingested only natural food, but the absolute content of linoleic acid was 2-3 folds greater in muscle of carp fed grains compared with muscle of carp fed only natural food. In general, in all groups the content of the n-6 fatty acids was higher than the content of n-3 fatty acids. The fatty acid composition of the carp muscle triacylglycerols was quite affected by that of the diets. Diets containing soybean or corn were characterized by high linoleic acid content. High contents of n-6 fatty acids in the grain based and extruded formulated (soybean meal, sunflower cernel, wheat flower and corn) diet resulted in high levels of these fatty acids in the carp meat.

The fatty acid composition of common carp reflects, to a large extent, that of the diet. The n-3/n-6 ratio varies between 0.8 and 2.4 [9]. There are reports indicating this ratio is about 0.5 [17,21], even less, about 0.2 [16,26]. Ackman [27] reported EPA and DHA acid concentrations in farmed carp as low as 0.35. In the

present study, feeds did not contain highly USFAs. Freshwater fish possess the bioconversion capacity to elongate and desaturate C_{18} PUFA to n-3 and n-6 fatty acids such as arachidonic acid, EPA and DHA [28].

Fish meal is expensive and mostly imported feedstuff. The use of plant-derived materials as fish feed ingredients are limited by the presence of a wide variety of antinutritional substances, so appropriate heat treatment is necessary.

In conclusion, the ratio of n-3/n-6 in common carps varied by feed and or rearing system. Using adequately processed plant meals as replacement protein sources can further improve productivity and nutritive value of carp, as reflected by n-6 polyunsaturated fatty acids, especially linoleic and arachidonic acids and favorable content of total cholesterol.

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REFERENCES

1. Adámek Z, Sukop I, Rendón PM, Kokil J: Food competition between 2+ tench: (*Tinca tinca* L.), common carp (*Cyprinus carpio* L.) and bigmouth buffalo (*Ictiobus cyprinellus* Val.) in pondpolyculture. *J Appl Ichthyol*, 19, 165-169, 2003. DOI: 10.1046/j.1439-0426.2003.00467.x

2. Milstein A: Ecological aspects of fish species interactions in polyculture ponds *Hydrobiologia*, 231 (3): 177-186, 1992. DOI: 10.1007/BF00018201

3. Marković G, Ćirković M: The possibilities for the intensification of commercial fish production in Central Serbia. *50th Jubilee Georgikon Scientific Conference, Proceedings,* p. 71, 2008.

4. Viola S, Mokady S, Rappaport U, Arieli Y: Partial and complete replacement of fishmeal by soybean meal in feeds for intensive culture of carp. *Aquaculture*, 26 (3-4): 223-236, 1982. DOI:10.1016/0044-8486(82)90158-2

5. Garg SK, Kalla A, Bhatnagar A: Evaluation of raw and hydrothermically processed leguminous seed as supplementary feed for the growth of two Indian major carp species. *Aquac Res*, 33, 151-163, 2002.

6. Khan MA, Jafri AK, Chadha NK, Usmani N: Growth and body composition of rohu *(Labeo rohita)* fed diets containing oilseed meals: partial or total replacement of fish meal with soybean meal. *Aquac Nutr*, 9, 391-396, 2003.

7. Francis G, Harinder-Makkar PS, Becker K: Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, 199 (3-4): 197-227, 2003. DOI:10.1016/S0044-8486(01)00526-9

8. Barrows FT, Hardy RW: Feed manufacturing technology. **In,** Stickney RR (Ed): Encyclopedia of Aquaculture. pp. 354-359, John Wiley & Sons Inc, New York, 2000.

9. Steffens W, Wirth M, Füllner G: Freshwater fishan important source of n3 polyunsaturated fatty acids: A review. *Arch Pol Fish*, 13, 15-16, 2005. http://www.infish.com.pl

10. Alasalvar C, Taylor KDA, Zubcov E, Shahidi F, Alexis M: Differentiation of cultured and wild sea bass *(Dicentrarchus labrax)*: Total lipid content, fatty acid and trace mineral composition. *Food Chem*, 79, 145-150, 2002.

11. Piironen V, Toivo J, Lampi AM: New data for cholesterol contents in meat, fish, milk, eggs and their products consumed in Finland. *J Food Compos Anal*, 15, 705-713, 2002. http://dx.doi.org/10.1006/jfca.2002.1095

12. Spirić A, Trbović D, Vranić D, Đinović J, Petronijević R, Matekalo-Sverak V: Statistical evaluation of fatty acid profile and cholesterol content in fish (common carp) lipids obtained by different sample preparation procedures. *Analytica Chimica Acta*. 672 (1-2): 66-71, 2010.

13. Maraschiello C, Diaz I, Regueiro JAG: Determination of cholesterol in fat and muscle of pig by HPLC and capillary gas chomatography with solvent venting injection. *J High Resol Chromatogr*, 19, 165-168, 1996. DOI: 10.1002/jhrc.1240190309

14. Spirić A, Trbović D, Vranić D, Đinović J, Petronijević R, Milijašević M, Janković S, Radičević T: Fatty acid composition, cholesterol and total fat content in rainbow trout *(Oncorhynchus mykiss)* as influenced by fatty acids in diet. *Tehnologija mesa*, 50 (3-4): 179-188, 2009. http://www.inmesbgd.com

15. Vladau VV, Bud I, Stefan R: Nutritive value of fish meat comparative to some animals meat. *Bull UASVM Anim Sci Biotech*, 65 (1-2): 301-305, 2008.

16. Trbović D, Vranić D, Đinović J, Borović B, Spirić D, Babić J, Spirić A: Fatty acid profile and cholesterol content in muscle tissue of one year old common carp *(Cyprinus carpio)* during growth. *Tehnologija Mesa*, 50 (5-6): 276-286, 2009. http://www.inmesbgd.com/

17. Ćirković M, Trbović D, Milošević N, Đorđević V, Janković S, Ljubojević D: Meat quality of two yearsold tench and carp grown in extensive conditions. *XIV International Symposium Feed Technology, Proceedings,* pp. 400-404, 2010.

18. Ćirković M, Ljubojević D, Đorđević V, Novakov N, Petronijević R, Matekalo-Sverak V, Trbović D: The breed effect on productivity and meat nutrient compsition of fish. *Kafkas Univ Vet Fak Derg*, 18 (5): 775-780, 2012.

19. Żmijewski T, Kujawa R, Jankowska B, Kwiatkowska A, Mamcarz A: Slaughter yield, proximate and fatty acid composition and sensory properties of rapfen (*Aspius aspius* L) with tissue of bream (*Abramis brama* L) and pike (*Esox lucius* L). *J Food Compos Anal*, 19, 176-181, 2006.

20. Bieniarz K, Koldras M, Kaminski J, Mejza T: Fatty acids. fat and cholesterol in some lines of carp *(Cyprinus carpio)* in Poland. *Arch Pol Fish*, 9, 5-24, 2001. http://www.infish.com.pl

21. Kopicova Z, Vavreinova S: Occurrence of squalene and cholesterol in various species of Czech freshwater fish. *Czech J Food Sci*, 25, 195-201, 2007. http://agriculturejournals.cz/publicFiles/00312.pdf

22. Steffens W, Wirth M: Influence of nutrition on the lipid quality of pond fish: Common carp *(Cyprinus carpio)* and tench *(Tinca tinca)*. *Aquac Int*, 15, 313-319, 2007.

23. Buchtova H, Svobodova Z, Križek M, Vacha F, Kocour M, Velišek J: Fatty acid composition in intramuscular lipids of experimental scaly crossbreds in 3yearold common carp (*Cyprinus carpio* L.). *Acta Veterinaria Brno*, 76, S73-S81, 2007. doi:10.2754/avb200776S8S073

24. Fajmonova E, Zelenka J, Komprda T, Kladroba D, Sarmanova I: Effect of sex. growth intensity and heat treatment on fatty acid composition of common carp *(Cyprinus carpio)* fillets. *Czech J Anim Sci*, 48, 85-92, 2003. http://www.cazv.cz

25. Sargent JR, Tocher DR, Bell JG: The lipids. **In,** Halver J, Hardy E. (Eds): Fish Nutrition. 3rd ed., pp. 181-257, Academic, San Diego, 2002.

26. Ljubojević D, Ćirković M, Đorđević V, Trbović D, Novakov N, Vranić D, Babić J: Lipids and total cholesterol content in meat of common carp of various ages. *22nd International Symposium Food Safety Production, Proceedings,* pp. 46-49, 2011.

27. Ackman RG: Nutritional composition of fats in seafoods. *Progr Food Nutr Sci*, 13, 161-241, 2000.

28. Bell JG, Tocher DR, Farndale BM, Cox DI, McKinney RW, Sargent JR: The effect of dietary lipid on polyunsaturated fatty acid metabolism in Atlantic salmon *Salmo salar* undergoing parr-smolt transformation. *Lipids*, 32, 515-525, 1997.

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RESEARCH ARTICLE

Comparison of Fixation Methods for Peripheral Nerve Fiber [1] [2]

M. Başak ULKAY * Abit AKTAŞ * Özgür AKSOY **
Damla HAKTANIR *** H. Hakan BOZKURT *

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[2] *This study was presented as a poster at Second International Neural Regeneration Symposium (INRS2012), 21-25 September 2012, Shenyang - China*

* İstanbul Üniversitesi, Veteriner Fakültesi, Histoloji ve Embriyoloji Anabilim Dalı, TR-34310 İstanbul - TÜRKİYE
** Kafkas Üniversitesi, Veteriner Fakültesi, Cerrahi Anabilim Dalı, TR-36000 Kars - TURKEY
*** İstanbul Üniversitesi, Veteriner Fakültesi, Patoloji Anabilim Dalı, TR-34310 İstanbul - TÜRKİYE

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Summary

Accurate fixation is a must for the assessment of myelinated nerve fiber morphology and transcardial perfusion and immersion methods are the most commonly used fixation techniques. In the present study we designed a new fixation technique for the histomorphometric and stereological evaluation of sciatic nerve fiber and referred it as instillation fixation. The method involved preliminary in situ fixation of the nerve sample without dissecting it from the animal body followed by a complete conventional fixation protocol. The objective of this study was to compare the three fixation techniques with each other for fixation artifacts. Eighteen female Wistar albino rats constituted the study material. The animals were allocated into three experimental groups corresponding to different fixation methods (immersion, instillation and transcardial perfusion, respectively). Quantitative assessments of nerve samples harvested from the animals of each group included the number of total myelinated axons, normal myelinated axons, alterations in myelin compaction, myelinated axons with irregular fiber shape, and with myelin loops and g ratio. Results revealed that normal myelinated axons were markedly lower in the immersion fixation group compared to those of instillation and transcardial perfusion. Moreover a significant decrease was noted with respect to alterations in myelin compaction in the instillation fixation group. In contrast, no significant difference was observed in myelin thickness and axon cross sectional area. In conclusion instillation fixation technique proved to be a valid and simple method for the assessment of peripheral nerve morphology for further analyses in a rat model.

Keywords: *Rat, Sciatic nerve, Fixation method*

Periferal Sinir Tespit Yöntemlerinin Karşılaştırılması

Özet

Doğru bir tespit işlemi miyelinli periferik sinirlerin morfolojik değerlendirmesi için ön koşuldur. Kalp perfüzyonu ve daldırma en yaygın kullanılan tespit yöntemlerindendir. Çalışmamızda bu metotlara ek olarak damlatma tekniği olarak adlandırılan yeni bir tespit metodu uygulandı. Sinir doku örneği canlı hayvan üzerinden çıkarılmadan ön tespit işlemine tabi tutulduktan sonra rutin tespit işlemleri tamamlandı ve üç tespit yönteminin tespit artefaktları açısından birbirleriyle histomorfometrik ve stereolojik olarak karşılaştırılması gerçekleştirildi. On sekiz adet dişi Wistar albino sıçan kullanıldı. Hayvanlar sırasıyla daldırma, damlatma ve kalp perfüzyonu olmak üzere üç eşit gruba ayrıldı. Her bir gruptaki hayvanlara ait siyatik sinir örneklerinin kantitatif değerlendirilmesi toplam miyelinli akson sayısı, normal miyelinli akson sayısı, miyelin kompaksiyonundaki değişimler içeren aksonların sayısı, düzensiz demet yapısı ile kıvrımlı miyelin halkaları içeren miyelinli aksonların sayısı ve g oranları hesaplanarak gerçekleştirildi. Elde edilen bulgulara göre daldırma tespit grubundaki normal miyelinli akson sayısında, damlatma ve kalp perfüzyonu grupları ile karşılaştırıldığında belirgin bir düşüş belirlendi. Bununla beraber damlatma tespit grubunda miyelin kompaksiyonunda değişimler içeren akson sayısında istatistiksel olarak anlamlı bir düşüş kaydedildi. Buna karşın miyelin kalınlığı ve ortalama akson alanında farklılık gözlenmedi. Sonuç olarak sıçan modelinde damlatma tespit tekniğinin periferik sinir morfolojisinin değerlendirilmesinde geçerli ve pratik bir metot olduğu gösterildi.

Anahtar sözcükler: *Sıçan, Siyatik sinir, Tespit metotları*

İletişim (Correspondence)
+90 212 4737070/17202
bulkay@istanbul.edu.tr

INTRODUCTION

Studies concerning peripheral nerve injuries and diseases have been drawing growing attention among researchers with the goal of improving diagnosis and treatment. Assessment of peripheral nerve morphology or in other words the morphology of myelinated axons is the cardinal of the investigation of nerve damage and regeneration [1-3].

Histomorphometric analysis of peripheral nerves is an intriguing topic for researches. Such methods involving computer-based nerve histomorphometry provide relatively unbiased and accurate assessment of quantitative features of nerve fibers, such as myelinated axon number, axonal cross-sectional area and myelin thickness and abnormalities in myelin structure when compared with conventional qualitative/visual examination methods and have been widely utilized both to determine the morphological characterizations of uninjured nerve samples and to investigate the response of the nerve tissue following several types of pathological conditions including injuries or any kind of therapeutic and surgical approaches [2,4]. Accurate analysis, however, depends on high-quality fixation of peripheral nerve tissue. The objective of fixation is to preserve the tissue sample as close to its natural form as possible, though even subtle changes in the intrinsic structure of the tissue sample, inevitably give rise to artifacts [5].

Transcardial perfusion and immersion are the two most common methods used for fixation, the former being accepted as the gold standard for the majority of the studies of neural tissue although some researchers pointed out the non-essentiality of transcardial perfusion, which was considered to be replaced by immersion of nerve specimens in the fixative agent after being dissected [5].

In this study we designed a novel fixation technique, which was termed as instillation fixation in a rat model and investigated the accuracy of this method in terms of several quantitative features of nerve fibers such as total axon number, the number of axons with no morphological alterations (number of normal axons), mean cross sectional area of axon, myelin thickness, alterations of myelin compaction, number of axons with irregular shape and myelin loops in the axoplasm (infoldings) as well as g ratio.

MATERIAL and METHODS

Experimental Design

Eighteen female Wistar albino rats, weighing between 200-250 g, obtained from the Experimental Medical Research Institute of Istanbul University, Istanbul, Turkey, were used in the study. The experimental animal protocol was carried out at Kafkas University, Faculty of Veterinary Medicine, Kars, Turkey and the tissue specimens were submitted to our laboratory for the whole tissue processing and assessment methods. The experiment was approved by The Animal Experiments and Ethics Committee of Kafkas University (No: 2011-46).

Animals were randomized into three groups (n=6 per group) and subjected to different fixation techniques: group I. Immersion fixation, group II. Instillation fixation and group III. Transcardial perfusion.

Before any manipulation, animals were anesthetized by single intramuscular injection of ketamine HCl (Ketalar, 50 mg/ml, Pfizer-Istanbul).

An oblique gluteal skin incision and a muscle-splitting incision were used to expose the sciatic nerve. The procedures in all groups were performed on the right sciatic nerve and the nerve was dissected after the whole experiment was completed and then the rats were sacrificed.

For all fixation procedures 2.5% glutaraldehyde in 0.1M sodium cocadylate phosphate buffer (pH 7.4) was used.

Immersion group: After being dissected the nerve specimen was immersed in the fixative solution.

Instillation group: Prior to the dissection, sciatic nerve was prefixed in situ through being embedded in a pool of the fixation solution formed by slightly lifted surrounding muscle tissues for 5 min. Then the nerve specimen was dissected and immersed in the fixative solution to complete fixation.

Perfusion group: Unlike the above mentioned procedures the animals in this group were heparinized before being anesthetized. The chest wall was opened to expose the beating heart, and a needle is then inserted into the left ventricle with an incision concurrently made in the right atrium. Perfusion was initiated with 400 ml of 0.1M sodium cocadylate phosphate buffer until the liver has become pale, assuring that the blood was rinsed off and the procedure was sustained with 400ml of 2.5% gluteraldehyde as the fixative. Finally the sciatic nerve was dissected and immersed in the fixation solution.

Tissue Processing

Nerve specimens belonging to the animals in all three groups were kept in the fixation solution for 24 h in 4ºC. Following fixation, the tissues were rinsed three times in sodium cocadylate phosphate buffer (pH 7.4) for 10 min. Then the tissues were postfixed in 1% osmium tetraoxide for 2 h. The tissues were rinsed once more in sodium cocadylate phosphate buffer (pH 7.4) for 10 min. The specimens were then dehydrated for 10 min in each of the following solutions using the following concentrations of ethanol; 3x70%, 3x 80%, 3x 90% and 3x 100%, respectively. Then the specimens were treated three times for 10 min each with pure propylene oxide (Sigma) and placed overnight in a sealed bottle including 1:1 mixture of propylene oxide and resin (Epon embedding kit/ Fluka Chemie Gmbt, Switzerland). The following day, the bottles were unsealed and remained intact for 3-4 h allowing the solution to evaporate. Then the specimens were

treated with epoxy resin for 24 h at room temperature. The whole procedure was completed by embedding the tissues in Epon Embedding Kit for 48 h at 60°C. For embedding, we used a silicon embedding mold that has 21 consecutively numbered, bullet-shaped cavities with a depth of 5 mm each. Semi-thin and ultra-thin sections (of 1 mm and 90 nm thickness, respectively) were cut by an ultramicrotome (Super Nova Reichert-Yung,Austria) and stained with 1% toluidine blue (semi-thin sections) and uranyl acetate-lead citrate (for ultra-thin sections), respectively. Ultra-thin sections were analyzed using a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a Mega-View III digital camera and Soft-Imaging System (SIS, Munster, Germany) [6]. Semi-thin sections were evaluated by light micro-scopy.

Stereological Analysis

Stereological analyses of sciatic nerves were conducted according to principles described previously [3,7,8]. A stereological workstation composed of a digital camera (mbf/Bioscience, Qimaging), otomatic controlled specimen stage, a light microscope (Leica, DM400B) and a software program (mbf Bioscience, Stereo investigator, version 9) was used to count axons. To obtain an estimation of total myelinated axon number in an unbiased manner, the axon profiles in the nerve cross-section were sampled with equal probability regardless of shape, size, orientation and location, which meant that each sampled item was selected with a systematic random manner [6]. For this aim, we chose an area fraction approach with an area of unbiased counting frame of 900 mm^2. Meander sampling of each sectioned nerve profiles was done in 70 µm x70 µm step size in a systematic-random manner, as well, ensuring that all locations within a nerve cross-section were equally represented and that all axon profiles were sampled with an equal probability regardless of shape, size, orientation and location [6,9].

The same stereological workstation was also used for stereological analyses of myelin thickness and axon cross-sectional area. A two-dimensional isotropic uniform random nucleator [10,11] was used for estimation of cross-sectional axon area and the thickness of myelin sheet using an oil objective (100x, NA 1.40). Meander sampling of each sectioned nerve profiles for axon cross-section area and myelin sheet thickness was done over successive, systemic-random steps of 70 µm-70 µm. Two dimensional nucleator at isotropic uniform random positions was used for estimation of axonal areas and the thickness of myelin sheet using an oil objective (100x, NA 1.40) [12].

After the tissue processing methods, all the nerve samples belonging to the animals in each group were histomorphometrically evaluated with the aid of a video monitor connected to the microscope at a final magnification of 100× NA 1.40 [13] according to the following parameters, which were previously obtained on the basis of stereological analysis: total axon number, the number of axons with no morphological alterations (number of normal axon/undamaged axon), mean cross sectional area of axon, myelin thickness, alterations of myelin compaction, number of axons with irregular shape and myelin loops in the axoplasm (infoldings) as well as g-ratio. G-ratio was calculated as the quotient between the axon perimeter and the myelin perimeter.

Statistical Analysis

At least six rats were studied for each experimental group. The "n" used for statistical analysis was the number of animals. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by a Duncan test using the SPSS 13.0 programs. Statistical significance was established as $P<0.05$.

RESULTS

Histomorphometric Evaluation

Several parameters were histomorphologically identified for the assessment of alterations in the size and shape of myelinated fibers corresponding to different fixation methods. Myelin invaginations in the axoplasm (infoldings), axons with irregular shapes, alterations in myelin compaction, as well as normal (undamaged axonal structures were recognized and evaluated on semi-thin sections by light microscopy. Electron micrographs of these abnormalities in myelin compaction revealed incisures and general lamellar separation of myelin sheath *(Fig. 1)*.

Stereological Analysis

Total and normal myelinated axon numbers: No statistically significant difference ($P>0.05$) was noted between the groups with respect to total myelinated axon number although a statistically significant difference was found in the immersion group in terms of normal myelinated axon number ($P<0.01$) *(Fig. 2)*.

Estimation of Myelin thickness and axonal cross section area: The groups were found to be identical in terms of mean cross sectional area of axon and thickness of myelin sheath, thus no significant difference was statistically detected in parallel with the histomorphometric evaluation ($P>0.05$) *(Fig. 3)*.

Alterations in myelin structure: The most frequent of these abnormalities was the presence of alterations in myelin compaction. A prominent decrease was detected in the instillation group with respect to the number of the axons with alterations in myelin compaction, which was statistically significant ($P<0.05$). However, no statistically significant difference was detected between the groups in terms of the number of axons with irregular shapes and myelin loops ($P>0.05$) *(Fig. 4)*. Percent values of abnormalities of myelin structure remained unchanged within the total myelinated axon number and these values were summarized in *Fig. 5*.

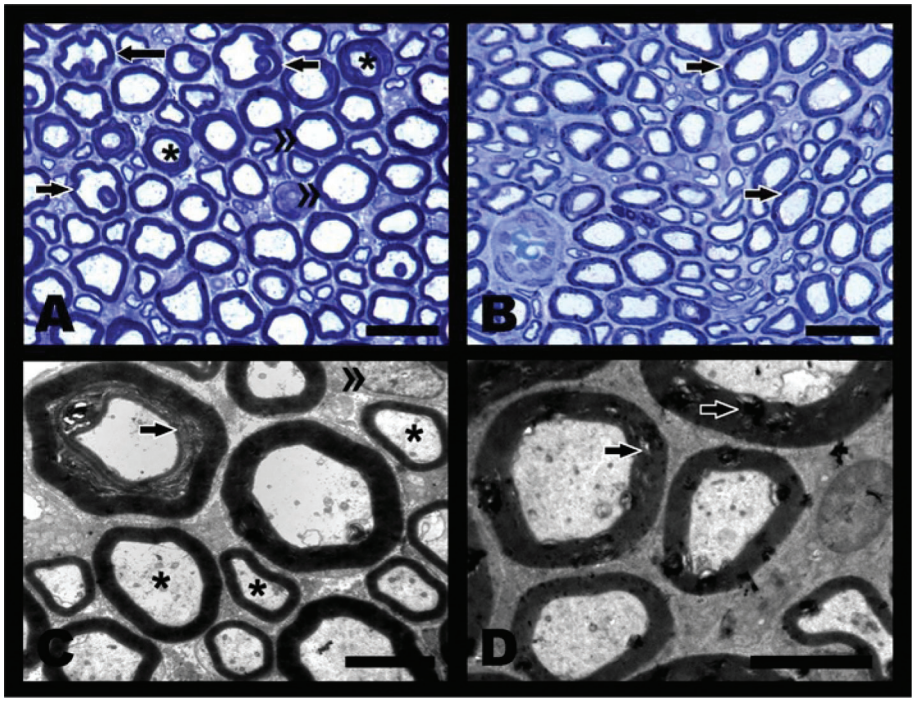

Fig 1. Light and electron microscopic views of sciatic nerve fibers. **A-** Recognition of myelin fiber abnormalities in the sciatic nerve. *Asterisks:* Alterations in myelin compaction (General lamellar separation). *Long arrow:* Irregular fiber shape. *Short arrows:* Myelinated fiber with a myelin loop (infoldings). *Arrowheads:* Normal myelinated axon **B-** Section from Immersion Group. *Arrows:* Alterations in myelin compaction. Semi-thin section. Toluidine blue Stain. Scale bar, 20 µm. **C-** Section from Instillation Group. *Asterisks:* Normal myelinated axons. *Arrows:* General lamellar separation. *Arrowhead:* Schwann cell nuclei **D-** Section from Immersion Group. *Arrows:* Incisures in myelin sheath. Ultra thin sections. Uranyl acetate-lead citrate stain. Scale bar, 5 µm

Şekil 1. Siyatik sinir demetlerinin ışık ve elektron mikroskobik görüntüleri

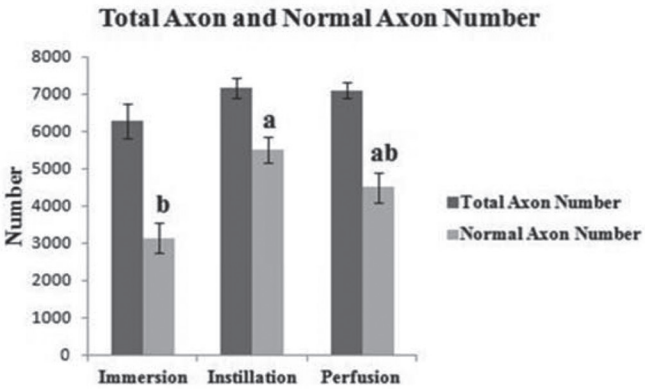

Fig 2. A comparison of the numbers of total myelinated axons and normal myelinated axons in the sciatic nerve. The graphic reveals a homogenous distribution of total axon number in all experimental groups (P>0.05). a, b, ab indicates the differences between the groups. ** P<0.01

Şekil 2. Siyatik sinirdeki toplam miyelinli akson ve normal miyelinli akson sayılarının karşılaştırılması

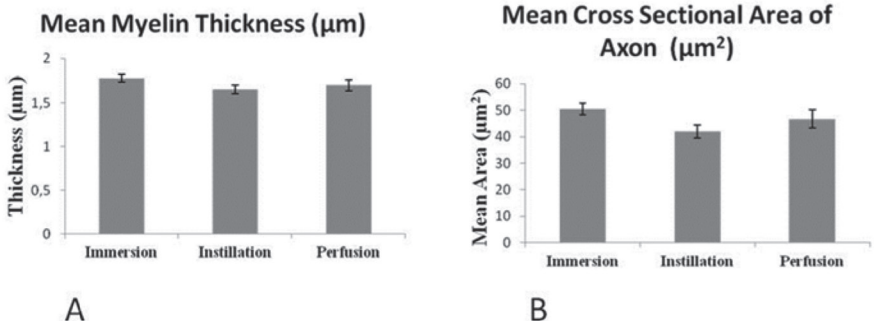

Fig 3. A comparison of the mean myelin thickness **(A)** and cross sectional area of axon in the sciatic nerve **(B)**. No statistically significant difference was noted between the groups (P>0.05)

Şekil 3. Siyatik sinirdeki miyelin kalınlığının **(A)** ve ortalama akson alanlarının **(B)** karşılaştırılması

Quantitative assessments of shrinkage for the three experimental groups were performed by Axon to myelin ratio (g-ratios). This ratio did not differ between groups, indicating no differential shrinkage was present *(Fig. 6)*.

DISCUSSION

Tissue fixation is the most essential step of all tissue processing protocols allowing further analyses. The goal of fixation is to preserve cells and tissue constituents in as close a life-like state as possible by preventing postmortem decay. However, the dilemma of fixation has always been that it introduces some artifact, which is observed as alterations of the original chemical and physical compositions of tissues [14,15]. A proper fixation protocol is crucial, as well for the studies on histomorphometric evaluation of peripheral nerve fibers particularly concerning the quantitative features such as axon number, axonal cross-sectional area and

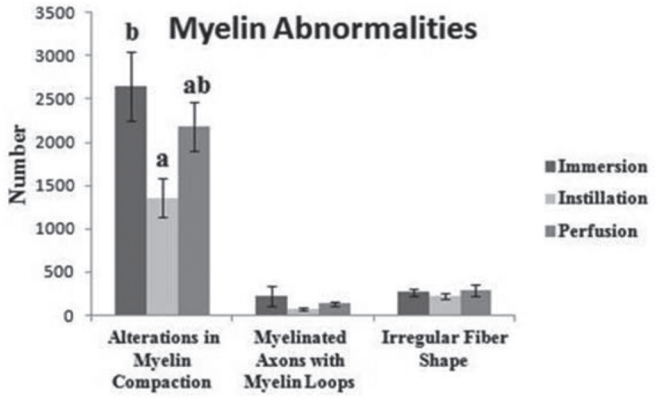

Fig 4. A comparison of myelin abnormalities corresponding to all three groups. a, b, ab indicates the differences between the groups in terms of alterations in myelin compaction * P<0.05 reveals immersion group. The number of myelinated axons with myelin loops and irregular fiber shape are non-significant (P>0.05)

Şekil 4. Tüm gruplar arasındaki miyelin anomalilerinin karşılaştırılması

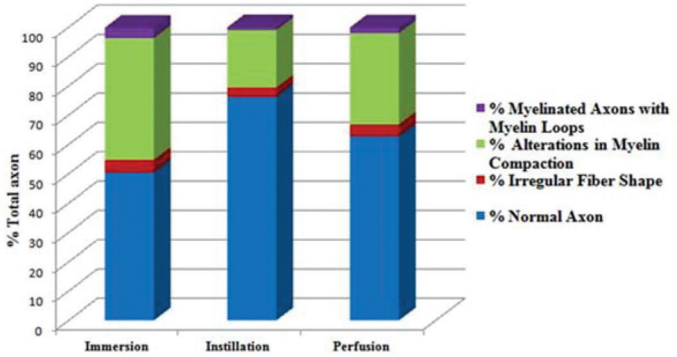

Fig 5. Percent values of myelin abnormalities within the total myelinated axon number

Şekil 5. Toplam miyelinli akson sayısı içindeki miyelin anomalilerinin yüzde değerleri

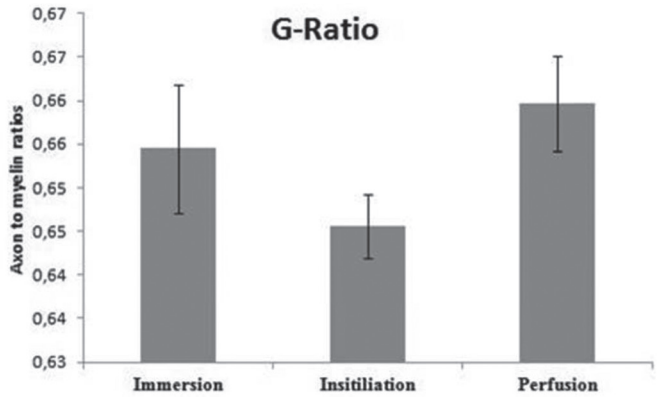

Fig 6. G-ratio (axon to myelin ratios)

Findings were similar across all groups, indicating that no preferential shrinkage artifact occurred with any of the fixation regimens (P>0.05)

Şekil 6. G-oranı

myelin thickness [2,4]. Most commonly used fixation protocols in these studies are immersion and perfusion fixation techniques [5,14,16]. Although transcardial perfusion has been approved as the most effective method it indeed has several disadvantages. It occupies plenty of time, labor and definitely should be performed by expertised staff. Biosafety of the procedure is subject to interrogation, as well, since large amount of fixatives including suspected carcinogens are applied [5,17]. A shortfall of transcardial perfusion is that the peripheral nerve can be curved by muscular contractions during the procedure and may thus be fixed in a curved shape, which makes sectioning tricky [1]. Immersion has lately been proved to be superior to conventional perfusion method due to its less time and labor intensiveness, practicality and cheapness. The staff is exposed to less volume of fixative with lesser time, as well [14]. It was considered to be more accurate in an aspect that structural distortions of the nerve samples due to muscular contractions during the transcardial perfusion method could be avoided in the immersion protocol since the nerve samples, immediately after having been dissected, were immersed in a small drop of fixative solution, kept in a straight position for a few minutes followed by immersion in fixative solution for complete fixation [15,18].

In this study we developed a novel technique, which was considered to be an alternative also to immersion and referred it as instillation fixation. Our experience of the studies we carried out with respect to peripheral nerve tissues revealed that metachromasie, observed as the pale staining of the nerve tissue samples, particularly in the middle sections was the handicap of the immersion method due to the insufficient diffusion of the fixative agent throughout the sample. In the immersion method, though superior to perfusion in above mentioned aspects, postmortem decay process was already initiated during the period the nerve was dissected

until immersed in the fixation solution, which took approximately 1.5-2 min and this was an *in vitro* process [5]. In the instillation fixation since the fixation process has already started while the animal was still alive, an adequate diffusion of the fixative through the entire sampled nerve was achieved, which eliminated the artifacts such as pale staining and therefore the quality of fixation was improved for further protocols. Another striking point was that no structural changes such as curves occurred in the sciatic nerve since the natural position of the nerve was preserved while the fixative solution properly penetrated into the tissue concurrently.

Consistent g-scores proved that no quantitative difference was found between the groups with respect to shrinkage and thus deceptive results were eliminated in terms of myelin thickness and axonal cross-sectional area.

No statistically significant difference was detected between the groups with respect to total axon number. However, normal axon number was found to be reduced in the immersion group. On the other hand, number of axons with alterations in myelin compaction significantly increased in the same group. This might be associated with the delayed and insufficient penetration of the fixative solution during the process, which resulted in artifacts as separation of lamellae of myelin sheaths. The mechanic pressure upon the inadequately fixed tissue samples during the sectioning might have caused separation of myelin sheaths. The electron microscopy data served to further demonstrate the flaws of the immersion technique. This difference might be associated with the individual diversity of each animal with respect to total myelinated axon number in the sciatic nerve. However the statistical insignificance of this parameter between the groups and identical percent values for alterations in myelin structure in all groups ruled out this conception. On the contrary, perfusion and instillation groups were equivalent in terms of all parameters investigated due to the constancy of the percent values of the parameters concerning the alterations in myelin structure within the number of total axons. In the previous studies [1,5] immersion technique was introduced as an alternative and in some aspects superior to perfusion method. However, our results pointed out the inadequacy of immersion technique parti-cularly demonstrated as a prominent increase of alterations in myelin compaction and a decrease of number of normal axons. Other myelin abnormalities such as irregular arrangement of myelin sheaths, formation of loops between layers and infoldings into the axoplasm reflected no significant differences among the groups since these parameters were known to be observed also with aging without any pathological condition [19,20] regardless of the method of fixation.

In conclusion, our data indicated that instillation fixation technique is capable of reducing morphological abnormalities of myelin in the sciatic nerve. Therefore, this technique was considered to be a practical method allowing accurate assessment of peripheral nerve morphology in a rat model.

REFERENCES

1. Di Scipio F, Raimondo S, Tos P, Geuna S: A simple protocol for paraffin-embedded myelin sheath staining with osmium tetroxide for light microscope observation. *Microsc Res Tech,* 71, 497-502, 2008.

2. Geuna S, Raimondo S, Fornaro M, Robecchi MG: Morpho-quantitative stereological analysis of peripheral and optic nerve fibers. *Neuro Quantology,* 10, 76-86, 2012.

3. Kaplan S, Geuna S, Ronchi G, Ulkay MB, von Bartheld CS: Calibration of the stereological estimation of the number of myelinated axons in the rat sciatic nerve: A multicenter study. *J Neurosci Methods,* 187, 90-99, 2010.

4. Canan S, Bozkurt HH, Acar M, Vlamings R, Aktas A, Sahin B, Temel Y, Kaplan S: An efficient stereological sampling approach for quantitative assessment of nerve regeneration. *Neuropathol Appl Neurobiol,* 34, 638-649, 2008.

5. Kasukurthi R, Brenner MJ, Moore AM, Moradzadeh A, Ray WZ, Santosa KB, Mackinnon SE, Hunter DA: Transcardial perfusion versus immersion fixation for assessment of peripheral nerve regeneration. *J Neurosci Methods,* 184, 303-309, 2009.

6. Canan S, Aktas A, Ulkay MB, Colakoglu S, Ragbetli MC, Ayyildiz M, Geuna S, Kaplan S: Prenatal exposure to a non-steroidal anti-inflammatory drug or saline solution impairs sciatic nerve morphology: A stereological and histological study. *Int J Dev Neurosci,* 26, 733-738, 2008.

7. Geuna S, Tos P, Guglielmone R, Battiston B, Giacobini-Robecchi MG: Methodological issues in size estimation of myelinated nerve fibers in peripheral nerves. *Anat Embryol (Berl),* 204, 1-10, 2001.

8. Larsen JO: Stereology of nerve cross sections. *J Neurosci Methods,* 85, 107-118, 1998.

9. Piskin A, Kaplan S, Aktas A, Ayyildiz M, Raimondo S, Alic T, Bozkurt HH, Geuna S: Platelet gel does not improve peripheral nerve regeneration: An electrophysiological, stereological, and electron microscopic study. *Microsurgery,* 29, 144-153, 2009.

10. Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A: Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS,* 96, 379-394, 1988.

11. Gundersen H J, Jensen EB, Kieu K, Nielsen J: The efficiency of systematic sampling in stereology-reconsidered. *J Microsc,* 193, 199-211, 1999.

12. Kaplan S, Piskin A, Ayyildiz M, Aktas A, Koksal B, Ulkay MB, Turkmen AP, Bakan F, Geuna S: The effect of melatonin and platelet gel on sciatic nerve repair: An electrophysiological and stereological study. *Microsurgery,* 31, 306-313, 2011.

13. Azcoitia I, Leonelli E, Magnaghi V, Veiga S, Garcia-Segura LM, Melcangi RC: Progesterone and its derivatives dihydroprogesterone and tetrahydro-progesterone reduce myelin fiber morphological abnormalities and myelin fiber loss in the sciatic nerve of aged rats. *Neurobiol Aging*, 24, 853-860, 2003.

14. Palay SL, Mc Gee RS, Gordon S, Jr. Grillo MA: Fixation of neural tissues for electron microscopy by perfusion with solutions of osmium tetroxide. *J Cell Biol,* 12, 385-410, 1962.

15. Raimondo S, Fornaro M, Di Scipio F, Ronchi G, Giacobini-Robecchi MG, Geuna S: Essays on peripheral nerve repair and regeneration. Chapter 5: Methods and protocols in peripheral nerve regeneration experimental research: Part II-morphological techniques. **In,** Geuna S, Tos P, Battiston B (Eds): Internatinal Review of Neurobiology. Vol. 87, pp. 81-103, Academic Press, New York, USA, 2009.

16. Williams TH, Jew JY: An improved method for perfusion fixation of neural tissues for electron microscopy. *Tissue Cell,* 7, 407-418, 1975.

17. Eichenbaum KD, Eichenbaum JW, Fadiel A, Miller DC, Demir N, Naftolin F, Stern A, Pevsner PH: Minimally invasive method for murine brain fixation. *Biotechniques,* 39, 487-488, 490, 2005.

18. Geuna S, Tos P, Battiston B, Guglielmone R, Giacobini-Robecchi MG: Morphological analysis of peripheral nerve regenerated by means of vein grafts filled with fresh skeletal muscle. *Anat Embryol (Berl),* 201 (6): 475-482, 2000.

19. Ceballos D, Cuadras J, Verdu E, Navarro X: Morphometric and ultra-structural changes with ageing in mouse peripheral nerve. *J Anat,* 195 (4): 563-576, 1999.

20. Shen D, Zhang Q, Gao X, Gu X, Ding F: Age-related changes in myelin morphology, electrophysiological property and myelin-associated protein expression of mouse sciatic nerves. *Neurosci Lett,* 502, 162-167, 2011.

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RESEARCH ARTICLE

Effects of Live Yeast Supplementation on Ruminal Parameters and Lactation Performance of Dairy Cows Fed Medium or High Levels of Dietary Concentrate [1]

Mehmet Ali BAL * Şerife GÖKSU *

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* Department of Animal Science, Faculty of Agriculture, Kahramanmaras Sutcu Imam University, TR-46100 Kahramanmaraş - TURKEY

Makale Kodu (Article Code): KVFD-2012-7124

Summary

The objectives of this study were to determine the effect of live yeast (LY) supplementation and dietary concentrate level interaction on ruminal parameters, dry matter intake (DMI), milk yield, and milk composition of lactating dairy cows. Four multiparous Holstein cows were assigned to one of four dietary treatments in a 4×4 Latin Square design in a 2×2 factorial arrangement with 21-d periods. The dietary treatments were: 1) 50% concentrate + live yeast (10 g/cow/d; 50LY), 2) 50% concentrate + no live yeast (50NLY), 3) 70% concentrate + live yeast (10 g/cow/d; 70LY), and 4) 70% concentrate + no live yeast (70NLY). A more distinct effect of the LY supplementation on ruminal pH was observed at 9 h of post-feeding, where cows that received 70NLY had the lowest ruminal pH (5.81) compared to cows that received 70LY (6.40; P< 0.05). The LY supplementation decreased the sum of ruminal isobutyrate, isovalerate, and valerate concentrations (4.3 vs. 4.6 mol/100 mol and 4.7 vs. 4.8 mol/100 mol) in both 50 and 70% concentrate diets compared to NLY (P= 0.02). Overall, the LY supplementation had only numerically higher on DMI (18.0 vs. 17.5 kg/d), milk yield (20.2 vs. 19.1 kg/d), and 3.5% fat corrected milk (19.4 vs. 18.8 kg/d) compared to NLY supplementation, respectively. The LY supplementation tended to increase (P= 0.06) milk fat yield in 50LY (0.66 kg/d) compared to 50NLY (0.62 kg/d). Similarly, the LY supplementation tended to increase (P= 0.08) solid non-fat (SNF) percentage in 50LY (9.83%) compared to 50NLY (9.63%). Although there were only numerical increases in DMI, milk yield, and 3.5% fat corrected milk with the supplementation of the LY, results indicated that the LY supplementation in the 50% concentrate diet would increase milk protein, SNF, and lactose percentages. In conclusion, ruminal pH reductions associated with feeding high dietary concentrate (70%) diets in dairy cows can be prevented with the LY supplementation.

Keywords: *Live yeast, Dairy cow, Lactation performance, Ruminal pH*

Canlı Maya İlavesinin Orta veya Yüksek Düzeyde Konsantre Yemle Beslenen Süt İneklerinde Ruminal Parametreler ve Laktasyon Performansı Üzerine Etkisi

Özet

Bu çalışmanın amacı, laktasyondaki süt ineklerinde canlı maya (CM) ilavesi ve rasyon konsantre yem düzeyi etkileşimlerinin ruminal parametreler, kuru madde tüketimi (KMT), süt verimi, ve süt komposizyonu üzerine olan etkilerinin belirlenmesi olmuştur. Dört adet 2 ve üstü laktasyondaki Holstein süt ineği 4x4 Latin kare deneme deseni içinde 2x2 faktöriyel düzenlemede 21-günlük periyotta 4 farklı muameleye tayin edilmiştir. Muamele grupları: 1) %50 konsantre yem + canlı maya ilavesi (10 gr/inek/gün; 50CM), 2) %50 konsantre yem + canlı maya ilavesi yok (50CMY), 3) %70 konsantre yem + canlı maya ilavesi (10 gr/inek/gün; 70CM), ve 4) %70 konsantre yem + canlı maya ilavesi yok (70CMY) olarak düzenlenmiştir. Canlı maya ilavesinin belirgin etkisi ruminal pH'da gözlenmiş olup, yemlemeden sonraki 9. saatte rumen pH'sı 70CMY grubundaki (5.81) ineklerde 70CM grubundakilere (6.40) göre en düşük düzeyde olmuştur (P<0.05). Ayrıca, CM ilavesi CMY'ye göre toplam ruminal izobütirat, izovalerat ve valerat (4.3 vs. 4.6 mol/100 mol ve 4.7 vs. 4.8 mol/100 mol) konsantrasyonlarını hem %50 hem de %70 konsantre yem rasyonlarında azaltmıştır (P= 0.02). Genel olarak, CM ilavesi CMY ile karşılaştırıldığında sırasıyla KMT (18.0 vs. 17.5 kg/gün), süt verimi (20.2 vs. 19.1 kg/gün), ve %3.5 yağı düzeltilmiş süt verimi (19.4 vs. 18.8 kg/gün) yönünden sadece rakamsal bir artışa sahip olmuştur. Canlı maya ilavesi, 50CM grubunda (0.66 kg/gün) 50CMY grubuna (0.62 kg/gün) göre süt yağı verimini artırma eğiliminde olmuştur (P= 0.06). Benzer şekilde, CM ilavesi süt yağsız kuru madde (YKM) yüzdesini 50CM grubunda (%9.83) 50CMY grubuna (%9.63) göre artırma eğiliminde olmuştur (P= 0.08). Her ne kadar CM ilavesiyle KMT, süt verimi, ve %3.5 yağı düzeltilmiş süt veriminde rakamsal artışlar gözlenmiş olsa da, sonuçlar CM ilavesinin %50 konsantre yem rasyonunda süt proteini, YKM ve laktoz yüzdelerini artırabildiğini göstermiştir. Sonuç olarak, süt ineklerinde yüksek konsantre yem düzeyiyle (%70) beslenmenin ilişkili olduğu ruminal pH düşüklükleri CM ilavesiyle önlenebilecektir.

Anahtar sözcükler: *Canlı maya, Süt ineği, Laktasyon performansı, Ruminal pH*

İletişim (Correspondence)
+90 344 2802107
malibal40@gmail.com

INTRODUCTION

Yeast (*Saccharomyces cerevisiae*) and yeast by-products are used in livestock nutrition as feed additives because of their beneficial effects on animal performance. They are used mainly in high producing dairy and beef cattle rations to compensate for the ruminal fermentation disturbances associated with the feeding of high dietary concentrate diets. *Saccharomyces cerevisiae (S. cerevisiae)* are able to grow rapidly in the rumen and facilitate fiber digestion [1]. Micro-nutrients found in *S. cerevisiae* also stimulate cellulolytic bacteria growth [2]. In addition, *S. cerevisiae* also protect ruminal fermentation from lactic acid accumulation [3]. Based on the theory proposed by Newbold et al.[4], *S. cerevisiae* in the rumen environment can utilize the remaining dissolved oxygen and save anaerobic microorganisms from the toxic effect of oxygen.

Previous researches focusing on feeding dairy cows with yeast and yeast by-products have had variable results. Several reasons may account for these variations including composition of the ration used (forage to concentrate ratio, quality of the forage, nutrient composition of the diet etc.), amount of yeast supplemented, type and number of viable yeast, number of cows used, and lactation stage of the cows. Robinson and Erasmus [5] summarized how nutrient composition of the diet would affect cow production variables in yeast supplemented rations. They performed a correlation analysis from the data of 22 different yeast supplemented lactation studies and found that an increase in the crude protein (CP) content of the diets supplemented with yeast was positively correlated with the yields of milk (r= 0.24), milk protein (r = 0.35), and DMI (r = 0.14). However, an increase in the acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents of diets supplemented with yeast were negatively correlated with milk yields (r= -0.54 and -0.55), milk fat (r = -0.23 and -0.19) and milk protein (r = -0.53 and -0.37), as well as DMI (r = -0.45 and -0.40). Similarly, Desnoyers et al.[6] summarized the meta-analysis of yeast and yeast by-product feeding studies (n = 110) from the literature and found that yeast supplementation increased the DMI by 0.44 g/d of kg BW in dairy cows. This positive response was related to an increase of the concentrate portion in the diet not affected by the NDF and CP contents of the diet. Results from this meta-analysis also found that milk production was 1.2 g/kg of BW higher in cows supplemented with yeast compared to cows not supplemented with yeast. It was concluded that yeast supplementation in the diets of dairy cows increased the ruminal pH and VFA concentrations by 0.03 point and 2.17 m*M*, respectively, while reducing the lactate concentration by 0.9 m*M*.

The objectives of this experiment were to test the interactions between the LY (*S. cerevisiae* NCYC R618) supplementation and dietary concentrate level on ruminal fermentation characteristics and lactation performance of lactating dairy cows.

MATERIALS and METHODS

Animals and Diets

This experiment was conducted at the Kahramanmaras Sutcu Imam University, Livestock Research Farm in 2008. Four multiparous lactating Holstein cows averaging 83 days in milk at trial initiation were assigned to each experimental diet in a 4×4 Latin Square design with 2×2 factorial arrangements of treatments for 21-d periods. Each period lasted 21 d with 14 d of dietary adaptation and 7 d of data collection. The treatments contained either 50 or 70% dietary concentrate with or without the LY supplementation. The respective dietary treatments were top-dressed with 10 g/cow/d LY supplementation (*S. cerevisiae* NCYC R618; BeneSacc™; Global Nutritech Ltd., Kocaeli, Turkey). Chemical composition of the LY was as follows; $4x10^9$ cfu/g of viability, 26.8% CP, 2.4% EE, 13.0% ADF, 25.1% NDF, 1.66 Mcal/kg NE_m, 1.47 Mcal/kg NE_l, 1.10 Mcal/kg NE_g, 0.09% Ca, 0.27% Mg, 0.78% P, and 0.33% S. The ingredient and nutrient compositions of the TMR's are presented in *Table 1*. The amounts of TMR offered and

Table 1. *Ingredient and nutrient compositions of total mixed rations (TMR)*
Tablo 1. *Toplam karışım rasyonlarının (TKR) içeriği ve besin madde kompozisyonları*

Ingredient	Treatment	
	50% Concentrate	70% Concentrate
	% of DM	
Corn silage [1]	43.0	23.0
Alfalfa hay [2]	7.0	7.0
Concentrate [3]		
Corn gluten feed	11.0	15.4
Wheat	11.0	15.4
Sunflower meal	11.0	15.4
Cottonseed meal	6.0	8.4
Barley	5.0	7.0
Wheat bran	3.0	4.2
Limestone	1.5	2.1
Molasses	1.0	1.4
Salt	0.4	0.6
Trace-mineral and Vitamin premix	0.1	0.1
Nutrient	% of DM	
OM	91.7	90.8
CP	16.0	18.3
NDF	49.9	44.8
ADF	35.5	31.1
Ash	8.3	9.2
NE_l (Mcal/kg)	1.50	1.50

[1] Contained 26.1% DM, 10.1% CP, 62.5% NDF, 45.1% ADF, 6.1% ash
[2] Contained 89.5% DM, 15.2% CP, 61.2% NDF, 59.6% ADF, 7.9% ash
[3] Contained 89.1% DM, 21.3% CP, 37.3% NDF, 23.7% ADF, 10.3% ash

refused were recorded daily. Cows were fed *ad-libitum*, and orts were maintained at approximately 10%. Each individual feed, TMR, and orts were analyzed for DM, organic matter (OM), CP [7], ADF, and NDF [8]. Cows were milked a.m. and p.m., and milk yield and milk compositions were measured during the last 3 days of each period. Concentrations of milk fat, protein, lactose, and solid non-fat (SNF) were determined by an ultrasonic milk analyzer (Lactoscan SA®). Mean daily milk composition was an average of a.m. and p.m. samples using the proportion of daily production at each milking as a weighting factor.

Ruminal Measurements

Ruminal pH was measured during the last 2 days of each period with a special filter mounted stomach tube at 0, 3, 6, 9, and 12 h of post-feeding using a hand held pH meter (HI-8314N, Hanna Instruments, UK). The sampled rumen fluid was filtered through two layers of cheesecloth, and 10 ml of duplicate supernatants were mixed with 0.2 ml of 50% H_2SO_4 and then frozen at -20°C until the analyses. Volatile fatty acid (VFA) analyses of the gas chromatography (Agilent Technologies 6890N, Network GC System) conditions were as follows: rumen fluid (2 ml) was transferred into the GC vials after centrifuging at 10.000 rpm; and then 10 µl of concentrated H_2SO_4 was added into each of the vials before analysis with the capillary column (Stabilwax-DA®, Crossbond Carboax-PEG for acidic compounds, 30 meter, 0.25 mm ID, 0.25 µm df, maximum program temperature of 260°C). The column temperature program was started at 100°C for 5 min, then increased by 10°C/min to 160°C for 2 min, and finally maintained at 80°C for 5 min.

Statistical Analysis

Intake, milk production, and composition data were analyzed by PROC GLM; ruminal pH and VFA data were analyzed by PROC MIXED procedure for repeated measures of SAS [9]. For ruminal pH and VFA, period and hour were used as repeated measurements. Treatment mean differences were tested using the least significant difference method after a significant F-test ($P<0.05$).

RESULTS

Effect of the LY supplementation and dietary concentrate level on post-feeding ruminal pH is presented in *Fig. 1*. The LY supplementation in the 50% concentrate diet numerically increased the ruminal pH of cows 3 h of post-feeding compared to NLY supplementation (6.35 vs. 5.97). A similar trend was observed after 9 h of post-feeding, where the LY supplementation in the 70% concentrate diet significantly increased the ruminal pH of cows compared to NLY supplementation (6.40 vs. 5.81; $P<0.05$). In addition, supplementation of the LY alone compared to NLY increased the ruminal pH numerically after 3 (6.17 vs. 5.91), 9 (6.23 vs. 5.92), and 12 h (6.45 vs. 6.23) of post-feeding (data not presented). The level of concentrate had no effect on ruminal pH, averaging 6.04, 5.99, 6.08, and 6.34 at 3, 6, 9, and 12 h of post-feeding.

Effects of the LY supplementation and dietary concentrate level on ruminal VFA concentrations are presented in *Table 2*. Total VFA concentration was not affected by either the LY supplementation or dietary concentrate level, averaging 102.3 m*M*. Neither acetate nor propionate concentrations were affected with the LY supplementation in both 50 and 70% concentrates. However ruminal acetate and propionate concentrations were decreased and increased, respectively in 70% concentrate (58.7 and 26.6 mol/100 mol; $P<0.01$) compared to 50% concentrate diet (61.0 and 24.0 mol/100 mol; $P<0.01$). The LY supplementation decreased the concentrations of other VFAs (isobutyrate + isovalerate + valerate) in both dietary concentrate levels (4.5 vs. 4.7 mol/100 mol; $P= 0.02$). Although the ratio of acetate to propionate was not affected by the LY supplementation, it was decreased in the 70% concentrate diet compared to the 50% concentrate diet (2.3 vs. 2.6; $P<0.01$).

Live yeast supplementation alone had no significant effect on the performance of lactating dairy cows *(Table 3)*. The LY supplementation increased DMI numerically by 0.9 kg/d only in the 50% concentrate diet. Milk yield was increased by the LY supplementation numerically across all diets by an

Fig 1. Effects of live yeast (LY) supplementation and concentrate level on post-feeding ruminal pH

Şekil 1. Canlı maya (CM) ilavesi ve konsantre yem düzeyinin yemleme sonrası ruminal pH üzerine etkileri

** Effects of LY supplementation (P=0.05) and LY supplementation-concentrate level interaction (P<0.05)*

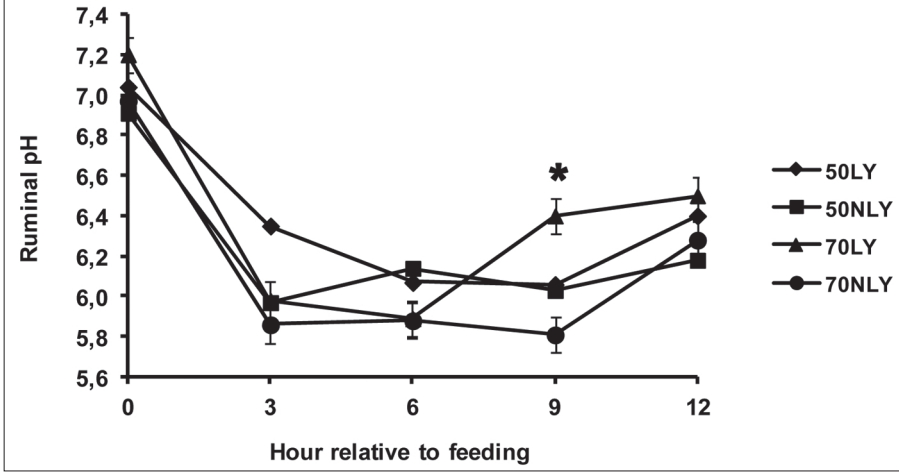

Table 2. *Treatment effects on ruminal volatile fatty acid (VFA) concentrations of dairy cows*
Tablo 2. *Muamelelerin süt ineklerinde ruminal uçucu yağ asitleri (UYA) konsantrasyonu üzerine etkileri*

Item	Treatment[1]				SEM[2]	Effect (P-value)[3]		
	50LY	50NLY	70LY	70NLY		LYS	CL	LYS*CL
Total VFA, m*M*	100.2	102.5	101.0	105.5	2.1	NS[4]	NS	NS
Acetate	61.3	62.2	59.4	61.4	1.3	NS	NS	NS
Propionate	23.8	24.7	26.3	28.2	0.6	=0.09	<0.01	NS
Butyrate	10.7	10.8	10.6	10.8	0.4	NS	NS	NS
Others[5]	4.4	4.8	4.7	5.0	0.1	=0.06	NS	NS
A:P[6]	2.6	2.6	2.3	2.2	0.1	NS	<0.01	NS
VFA, mol/100 mol								
Acetate	61.2	60.8	58.9	58.4	0.3	NS	<0.01	NS
Propionate	23.9	24.1	26.3	26.9	0.4	NS	<0.01	NS
Butyrate	10.6	10.6	10.2	10.0	0.3	NS	NS	NS
Others	4.3	4.6	4.7	4.8	0.1	=0.02	<0.01	NS

*[1] 50LY: 50% concentrate + 10 g/cow/d live yeast, 50NLY: 50% concentrate + no live yeast, 70LY: 70% concentrate + 10 g/cow/d live yeast, 70NLY: 70% concentrate + no live yeast, [2] standart error of mean, [3] **LYS:** live yeast supplementation, **CL:** concentrate level, [4] **NS:** P>0.10, [5] sum of isobutyrate, isovalerate, and valerate, [6] acetate:propionate ratio*

Table 3. *Treatment effects on intake, milk yield, and milk composition of dairy cows*
Tablo 3. *Muamelelerin süt ineklerinde yem tüketimi, süt verimi ve süt kompozisyonu üzerine etkileri*

Item	Treatment[1]				SEM[2]	Effect (P-value)[3]		
	50LY	50NLY	70LY	70NLY		LYS	CL	LYS*CL
DMI, kg/d	17.0	16.1	19.1	19.0	0.7	NS[4]	=0.01	NS
Milk, kg/d	18.6	17.2	21.7	21.1	1.0	NS	<0.05	NS
FCM [5], kg/d	19.1	17.9	19.8	19.8	0.7	NS	NS	NS
ECM [6], kg/d	19.4	18.0	20.6	20.5	0.8	NS	<0.05	NS
Protein, kg/d	0.61	0.54	0.73	0.72	0.04	NS	<0.01	NS
Fat, kg/d	0.66	0.62	0.66	0.67	0.02	NS	NS	=0.06
Lactose, kg/d	0.83	0.73	1.00	0.99	0.05	NS	=0.01	NS
SNF, kg/d	1.80	1.60	2.18	2.14	0.11	NS	<0.01	NS
Protein, %	3.33	3.26	3.32	3.36	0.02	NS	NS	=0.09
Fat, %	3.64	3.84	3.02	3.12	0.17	NS	<0.05	NS
Lactose, %	4.52	4.41	4.55	4.60	0.04	NS	<0.05	=0.07
SNF, %	9.83	9.63	9.85	9.95	0.07	NS	=0.08	=0.08

*[1] 50LY: 50% concentrate + 10 g/cow/d live yeast, 50NLY: 50% concentrate + no live yeast, 70LY: 70% concentrate + 10 g/cow/d live yeast, 70NLY: 70% concentrate + no live yeast, [2] standart error of mean, [3] **LYS:** live yeast supplementation, **CL:** concentrate level, [4] **NS:** P>0.10, [5] 3.5% fat corrected milk= (0.4324 x milk yield, kg) + (16.216 x milk fat yield, kg), [6] energy corrected milk= (0.323 x milk yield, kg) + (12.82 x milk fat yield, kg) + (7.13 x milk protein yield, kg)*

average of 1.0 kg/d. This was more pronounced in the 50% concentrate diet, where milk yield was 1.4 kg/d higher with the LY supplementation. Similarly, FCM and ECM yields were not affected statistically by the LY supplementation across all diets. However, FCM, ECM, protein, fat, lactose, and SNF yields were numerically higher in the LY supplemented diets in the 50% concentrate diet by 1.2, 1.4, 0.07, 0.04, 0.1, and 0.2 kg/d, respectively. Percentages of milk protein (3.32 vs. 3.36%), lactose (4.52 vs. 4.41%), and SNF (9.83 vs. 9.63%) tended to be higher with the LY supplementation in the 50% concentrate diet (P<0.1). The DMI, milk yield, ECM, milk protein and lactose, and SNF increased significantly in the 70% concentrate diet compared to the 50% concentrate diet (P<0.05). Percentage of milk fat was decreased significantly in the 70% concentrate diet without the LY supplementation (3.07 vs. 3.74; P<0.05).

DISCUSSION

Variable DMI responses to LY supplementation have also been demonstrated in previous studies. In dairy cows starting from 4 wk pre-partum to 18 wk post-partum, Wohlt et al.[10] found that LY supplementation (10 g/cow/d *S. cerevisiae,*

$5x10^9$ cfu/g) compared to NLY supplementation increased the DMI significantly only during the first 6 wk of lactation (14.4 vs. 13.8 kg/d), but not during the entire experimental period (18.5 vs. 19.2 kg/d). Similar to our findings, Yalcin et al.[11] found no DMI differences for dairy cows supplemented with LY (50 g/cow/d; $1.3x10^8$ cfu/g) compared to NLY during mid-lactation of 25 d. In addition, Swartz et al.[12] tested the effects of LY supplementation ($5x10^{10}$ cfu/cow/d) on DMI of lactating dairy cows (n= 306) from 7 different commercial farms and found no response. Although the LY supplementation in the present study caused a numerical increase on milk yield (averaging 1.0 kg/d) in both concentrate levels, no statistical significance was detected. Similar to DMI, previous studies of dairy cows supplemented with LY also indicated variable milk yield and milk composition results. Wohlt et al.[13] found a significant difference on milk yield, FCM, and milk fat of cows between the 5th and 8th wk of post-partum periods when supplemented with either 10 or 20 g of LY ($5x10^9$ cfu/g) compared to NLY. Yalcin et al.[11] also found a tendency for higher milk fat yield of cows supplemented with 50 g/d LY. Similarly, milk fat yield tended to be 0.04 kg/d higher with the LY compared to NLY supplementation in the 50% concentrate diet in our experiment. Shaver and Garrett [14] also found 0.9 kg/d more milk yield response in LY supplemented (57 g/cow/d) cows (n= 585) for 30 d of mid-lactation period from 11 commercial farms. Cows on those farms were offered a TMR with 18.8% CP, 18.6% ADF, and 28.2% NDF. Nutrient content of the diet also appears to have an effect on the productivity of cows supplemented with LY. Soder and Holden [15] tested the effects of LY supplementation (15 g/cow/d; $5x10^9$ cfu/g) on the lactation performance of primi- and multiparous cows starting from 4 wk pre-partum to 13 wk post-partum. They found that LY supplementation had no effect on lactation performance and concluded that the positive response should not be related to LY itself alone. Other factors, such as lactation stage, nutrient composition of diet, type of forage fed, feeding practices, and forage to concentrate ratio may also have an effect on this response. Previous research [10,13,16] indicated that a high proportion of dietary forage and NDF could cause a lack of response on the performance of dairy cows supplemented with LY. In the present study, the NDF and ADF contents of the diets were above the NRC [17] minimum requirements (25-33% NDF, 17-21% ADF) for lactating cows, which might have precluded the positive lactation response to the LY supplementation. Although previous research showed that the percentage of milk protein was either decreased [14] or unchanged [11,18] with LY supplementation, we found a tendency for higher milk protein percentage of cows supplemented with the LY in the 50% concentrate diet. Similarly, percentage of milk lactose and SNF also tended to increase in cows supplemented with the LY in the 50% concentrate diet. Moallem et al.[19] found a significant increase for milk lactose with LY supplementation (10^{10} cfu/cow/4 kg DM consumed) in a diet having 60% concentrate and 16.5% CP during the hot season. In our experiment, higher percentages of lactose and SNF with the LY supplementation should be the result of greater digestible nutrient intake in the 50% concentrate diet.

Although NLY supplementation did not cause a drastic pH reduction within 9 h of post-feeding (averaging 5.95) compared to the LY supplementation (averaging 6.13), the presence of lower ruminal pH in a sub-clinic manner over many weeks may cause significant production losses. Previous studies have found that LY supplementation is capable of enhancing ruminal pH in dairy cows due to its nature [20,21]. Jouany [22] proposed that LY may act as a balancer for the ruminal fluid redox potential and thereby maintain optimal fermenting condition for ruminal microflora, which need a high pH. Bach et al.[21] found a significant ruminal pH enhancement (6.05 vs. 5.49) in a continuing measurement of dairy cows supplemented with 5 g/cow/d LY (10^{10} cfu/g). They also found that cows supplemented with LY compared to NLY had a lesser meal interval (3.3 vs. 4.0 h). In the present study, cows fed a high concentrate diet (70%) responded better to the LY compared to NLY supplementation throughout 12 h of post-feeding, averaging 6.39 and 6.16 of ruminal pH, respectively. Koul et al.[23] found that LY supplementation on ruminal pH is most effective after 4 h of post-feeding, and that its efficacy was equal to that of $NaHCO_3$. Similarly, Marden et al.[24] tested the efficacy of both LY (5 g/cow/d; 10^{10} cfu/g) and $NaHCO_3$ (150 g/cow/d) supplementation on ruminal pH, and found that LY (6.14) was as effective as $NaHCO_3$ (6.21) on enhancement of ruminal pH compared to control treatment (5.94).

Results for VFA response to LY supplementation in dairy cows has also varied in previous studies as seen for production variables. Contrary to our findings in this study, Sullivan and Martin [25] found a higher ruminal propionate concentration in LY added to *in vitro* medium. In addition, Dolezal et al.[26] detected a linear increase between the amount of LY supplemented and the total ruminal VFA concentration. Similar to our findings, Longuski et al.[27] found that LY supplementation (56 g/cow/d) had no effect on total ruminal VFA or acetate concentrations in dairy cows. In the present study, individual ruminal VFA concentrations were affected independently by the LY supplementation and concentrate level. This may indicate that the LY supplementation alone had the potential for changing ruminal VFA production pattern without diet nutrient composition. Although we did not measure ruminal lactate concentration in the present study, the pH data supported the fact that the 70% concentrate diet supplemented with the LY could have had a lower ruminal lactate concentration. Desnoyers et al.[6] found in the meta-analysis that LY supplementation compared to no LY in ruminant species (cattle, goats, sheep, and buffaloes) tended to decrease rumen lactic acid concentration (-0.9 m*M* on average).

In conclusion, 10 g/cow/d LY supplementation with 50% dietary concentrate in the present study increased DMI, milk yield, and milk fat by 0.9, 1.4, and 0.04 kg/d, respectively. Furthermore, percentages of milk protein, lactose, and SNF

were increased with the LY supplementation in the 50% concentrate diet by 0.07, 0.11, and 0.20, respectively. It was also obvious that the LY supplementation in the 70% concentrate diet possibly controlled the ruminal pH decrease. In addition to these benefits, chemical composition of rations, stage of lactation, DMI and milk production potentials of animal's, and viability (cfu) of the LY should be considered before determining its supplementation in dairy cow rations.

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REFERENCES

1. Fuller R: Probiotics in man and animals. *J Appl Bact*, 66, 365-378, 1989.

2. Dawson KA, Neuman KE, Boling JA: Effects of microbial supplements containing yeast and lactobacilli on roughage-fed ruminal microbial activities. *J Anim Sci*, 68, 3392-3398, 1990.

3. Nisbet DJ, Martin SA: Effect of *Saccharomyces cerevisiae* culture on lactate utilization by the ruminal bacterium *Selenomonas ruminantium*. *J Anim Sci*, 69, 4628-4633, 1991.

4. Newbold CJ, Wallace RJ, McIntosh FM: Mode of action of the yeast *Saccharomyces cerevisiae* as a feed additive for ruminants. *Brit J Nutr*, 76, 249-261, 1996.

5. Robinson PH, Erasmus LJ: Effects of analyzable diet components on responses of lactating dairy cows to *Saccharomyces cerevisiae* based yeast products: A systematic review of the literature. *Anim Feed Sci Technol*, 149, 185-198, 2009.

6. Desnoyers M, Giger-Reverdin S, Bertin G, Duvaux-Ponter C, Sauvant D: Meta-analysis of the influence of *Saccharomyces cerevisiae* supplementation on ruminal parameters and milk production of ruminants. *J Dairy Sci*, 92, 1620-1632, 2009.

7. AOAC: Official Methods of Analysis. 15th ed., Association of Official Analytical Chemists, Washington, DC. 1990.

8. Van Soest PJ, Robertson JB, Lewis BA: Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci*, 74, 3583-3597, 1991.

9. SAS: SAS User's Guide: Statistics, Version 8.2 Edition. SAS Inst., Inc., Cary, NC. 1999.

10. Wohlt JE, Finkelstein AD, Chung CH: Yeast culture to improve intake, nutrient digestibility, and performance by dairy cattle during early lactation. *J Dairy Sci,* 74, 1395-1400, 1991.

11. Yalcin S, Yalcin S, Can P, Gurdal AO, Bagci C, Eltan O: The nutritive value of live yeast culture (*Saccharomyces cerevisiae*) and its effect on milk yield, milk composition and some blood parameters of dairy cows. *Asian-Aust J Anim Sci,* 24, 1377-1385, 2011.

12. Swartz DL, Muller LD, Rogers GW, Varga GA: Effect of yeast cultures on performance of lactating dairy cows: A field study. *J Dairy Sci*, 77, 3073-3080, 1994.

13. Wohlt JE, Corcione TT, Zajac PK: Effect of yeast on feed intake and performance of cows fed diets based on corn silage during early lactation. *J Dairy Sci*, 81, 1345-1352, 1998.

14. Shaver RD, Garrett JE: Effect of dietary yeast culture on milk yield, composition and component yields at commercial dairies. *The Prof Anim Sci*, 13, 204-207, 1997.

15. Soder KJ, Holden LA: Dry matter intake and milk yield and composition of cows fed yeast prepartum and postpartum. *J Dairy Sci,* 82, 605-610, 1999.

16. Carro MD, Lebzien P, Rohr K: Effects of yeast culture on rumen fermentation, digestibility, and duodenal flow in dairy cows fed a silage based diet. *Livest Prod Sci*, 32, 219-229, 1992.

17. National Research Council: Nutrient Requirements of Dairy Cattle. 7th rev. edn., Natl. Acad. Sci., Washington, DC. 2001.

18. Bruno RGS, Rutigliano HM, Cerri RL, Robinson PH, Santos JEP: Effect of feeding *Saccharomyces cerevisiae* on performance of dairy cows during summer heat stress. *Anim Feed Sci Technol*, 150, 175-186, 2009.

19. Moallem U, Lehrer H, Livshitz M, Zachut M, Yakoby S: The effects of live yeast supplementation to dairy cows during the hot season on production, feed efficiency, and digestibility. *J Dairy Sci,* 92, 343-351, 2009.

20. Williams PEV, Tait CAG, Innes GM, Newbold CJ: Effects of the inclusion of yeast culture (*Saccharomyces cerevisiae plus growth medium*) in the diet of dairy cows on milk yield and forage degradation and fermentation patterns in the rumen of steers. *J Anim Sci*, 69, 3016-3026, 1991.

21. Bach A, Iglesias C, Devant M: Daily rumen pH pattern of loose-housed dairy cattle as affected by feeding pattern and live yeast supplementation. *Anim Feed Sci Technol*, 136, 146-153, 2007.

22. Jouany JP: Optimizing rumen functions in the close-up transition period and early lactation to drive dry matter intake and energy balance in cows. *Anim Reprod Sci*, 96, 250-264, 2006.

23. Koul V, Kumar U, Sareen VK, Singh S: Mode of action of yeast culture (YEA-SACC 1026) for stimulation of rumen fermentation in buffalo calves. *J Sci Food Agric*, 77, 407-413, 1998.

24. Marden JP, Julien C, Monteils V, Auclair E, Moncoulon R, Bayourthe C: How does live yeast differ from sodium bicarbonate to stabilize ruminal pH in high-yielding dairy cows? *J Dairy Sci*, 91, 3528-3535, 2008.

25. Sullivan HM, Martin SA: Effects of a *Saccharomyces cerevisiae* culture on *in vitro* mixed ruminal microorganism fermentation. *J Dairy Sci*, 82, 2011-2016, 1999.

26. Dolezal P, Dolezal J, Trinacty J: The effect of *Saccharomyces cerevisiae* on ruminal fermentation in dairy cows. *Czech J Anim Sci*, 50, 503-510, 2005.

27. Longuski RA, Ying Y, Allen MS: Yeast culture supplementation prevented milk fat depression by a short-term dietary challenge with fermentable starch. *J Dairy Sci*, 92, 160-167, 2009.

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RESEARCH ARTICLE

Reproductive Performance of Ewes and Growth Characteristics of Lambs in Zom Sheep Reared in Karacadağ District [1]

Seyrani KONCAGÜL * Mehmet Emin VURAL ** Ahmet KARATAŞ **
Nalan AKÇA ** Mehmet BİNGÖL ***

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* Department of Animal Science, Faculty of Agriculture, University of Harran, TR-63200 Şanlıurfa - TURKEY
** GAP International Agricultural Research and Training Center, TR-21120 Diyarbakır - TURKEY
*** Department of Animal Science, Faculty of Agriculture, University of Yüzüncü Yıl, TR-65100 Van - Turkey

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Summary

This is the first study and report in which some ewe reproductive and lamb growth performances of Zom sheep were investigated and described in Turkey. The research was carried out on three different flocks randomly chosen among the Zom sheep flocks in the district of Karacadağ surrounded by Diyarbakır, Şanlıurfa and Mardin provinces, and lasted for two years, 2010 and 2011. The data consisted of 242 and 254 heads of lamb born from 226 and 214 heads of ewe in 2010 and 2011, respectively. Lambing rate (LR), twinning rate (TW), fecundity (FEC) and litter size (LITS) were found as 94%, 17%, 1.09 and 1.17 on average, respectively. There was no significant difference among flocks within years or among years within flocks in terms of ewe reproductive performance, except for the flock 2 in terms of TW. Average means of the birth weight (BWT), weaning weight (WWT) at 90th day and 6th month live weight (SMLW) were 4.00 kg and 22.06 kg, 36.11 kg, respectively. The effects of age of dam, type of birth and sex of lambs were significant on BWT, WWT and SMLW ($P<0.05$). Besides the WWT was also significantly affected by the year ($P<0.05$), every 1 kg increase in BWT caused 2.02±0.281 kg increase on average in WWT of lambs ($P<0.01$). In addition, it was observed that the havier lambs at birth were havier in terms of SMLW ($P<0.01$). The average daily weight gain (ADWG1) and survival rate (SR) of lambs at weaning were 200 g/day and 90%, respectively.

Keywords: *Zom sheep, Reproductive performance, Lamb growth, Lamb survival*

Karacadağ Yöresinde Yetiştirilen Zom Koyunlarının Üreme Performansı ve Kuzularının Büyüme Özellikleri

Özet

Bu araştırma, Zom koyunlarında bazı döl verimi ve kuzularda büyüme özelliklerini araştıran ve tanımlayan Türkiye'deki ilk çalışmadır. Araştırma, 2010 ve 2011 yıllarında, Diyarbakır, Şanlıurfa ve Mardin şehirlerinin çevrelediği Karacadağ bölgesinde Zom koyunu yetiştiriciliği yapan işletmeler arasından rastgele belirlenen 3 sürüde yürütülmüştür. Veriler, 2010 ve 2011 yıllarında doğum yapan sırasıyla 226 ve 214 baş koyun ve bunlardan doğan 242 ve 254 baş kuzularadan elde edilmiştir. Kuzulama oranı (KO), ikizlik oranı (İO), koçaltı koyun başına doğan kuzu sayısı (KKKS) ve doğuran koyun başına kuzu sayısı (DKKS) ortalamaları sırasıyla %94, %17, 1.09 ve 1.17 olarak tespit edilmiştir. İkinci sürüdeki İO hariç, koyun üreme performansı bakımından değişik yıllarda sürü içinde ya da aynı yılda sürüler arasında farklılık gözlemlenmemiştir. Ortalama doğum ağırlığı (DA), sütten kesim ağırlığı (SKA) ve altıncı ay canlı ağırlık (AACA) ortalaması sırasıyla 4.00, 22.06 ve 36.11 kg olarak tespit edilmiştir. DA, SKA ve AACA, ana yaşı, doğum tipi ve cinsiyet faktörlerinden önemli derecede etkilenmişlerdir ($P<0.05$). SKA üzerine yıl faktörünün de önemli etkisi tespit edilmiş ($P<0.05$), bunun yanında, DA'da meydana gelen her 1 kg'lık artışa karşılık SKA'da ortalama olarak 2.02±0.281 kg'lık bir artışın meydana geldiği gözlenmiştir ($P<0.01$). Buna ek olarak, doğumda daha ağır olan kuzuların AACA bakımından da daha ağır oldukları tespit edilmiştir ($P<0.01$). Sütten kesime kadar günlük canlı ağırlık artışı (GCAA) ve yaşama gücü sırasıyla 200 g/gün ve %90 olarak belirlenmiştir.

Anahtar sözcükler: *Zom koyunu, Üreme performansı, Kuzu büyüme, Kuzu yaşama gücü*

İletişim (Correspondence)
+90 414 3183713
m_seyrani_n@yahoo.com

INTRODUCTION

During the past 50 years, it has been a loss in livestock genetic resources in Turkey due possibly to reason that the economic, social and environmental developments in animal husbandry have caused to decrease in quantity of native breeds of animals by replacing them with high producing breeds. On the other hand, the native breeds are well adapted to harsh environmental conditions and continue to maintain their presence and to produce milk, meat and wool with little care and inputs [1].

Protection of genetic resources of indigenous breeds of animals is important for the future creation of new types and necessary genetic material for the concerns that may arise in the future due to environmental conditions. In addition, examination and evaluation of the infrastructure of the traditional sheep breeding are important for breeding programs [2].

From this point of view, it is necessary to identify and describe our native genetic resources of the nation, such as fat tailed Zom sheep intensly raised by farmers in Karacadağ district surrounded by Şanlıurfa, Diyarbakır and Mardin provinces. This sheep was first identified and described, and its morphological characteristics were reported [3] based on the results of a project supported by General Directorate of Agricultural Researches and Politics (GDARP). They reported that average live weights, wither heights and body lengths of mature males and females of this sheep were 71.7±2.67 kg and 48.8±0.57 kg, 77.1±2.01 cm and 68.3±0.43 cm, 65.4±1.16 cm and 60.3±0.25 cm, respectively. Given the climate and geographical condition of the region, Zom sheep has an ability of grazing in the stepped, arid and rocky areas.

The GDARP have also started a new project in the year of 2011 for the purpose of genetic improvement of Zom sheep in farmer conditions. Some of the main goals of that project are to improve ewe reproductive performances, lamb survival rate and some growth characteristics of lambs. Reproductive performance of a ewe is determined by several components; such as fertility, litter size and lamb survival [4]. In addition, productivity and profitability of a sheep enterprise are highly influenced by number of lambs weaned per breeding ewe than any other trait [5]. If selective breeding for ewe reproductive performance, lambs' growth and survival rate until weaning are to be implemented into a selection program, then it is necessary to identify the amount and direction of the effects of environmental factors on these traits.

Therefore, the main objectives of the present study are to investigate the reproductive performance of Zom ewes and some growth characteristics and survival rate of Zom lambs, and to explore the effects of environmental factors on these traits in farmer conditions.

MATERIAL and METHODS

Location and Management

Generaly in this region, the flocks consist of 50 to 250 heads of sheep. Matings are started in July and lasted at the end of August and ram/ewe rate is generally 1/25 during this period. The ewes are not provided any additional feed supplemets before or during the matings but the rams are provided crushed barley and lentil hay beside pasture starting 10 days prior to the matings. The rams stay in the flocks during the mating period. During the time this research was carried out, the ewes were also given crushed barley and lentil hay and sometimes mixed feed during 30 days before lambing. The lambings are occured generally at night and in primitive conditions of the farms. As a general practise in this region, ewes and her lamb(s) were transfered to a stall prepared in the sheep barn right after lambing. Then, a little milk from mother at first is discarged and ensured the lamb suckles its mother within 2 h. After ten days the ewes and their lams were kept in this place, they were transferred to a common holding pen in which the other ewes are kept. The lambs are kept with their mothers for about 30 days after lambing, then the ewes and lambs are seperated and gathered together 1 to 2 hours in each morning and evening for suckling for about 2 months. The lambs are weaned around 90 days after lambing and given crushed barley and wheat, lentil hay and wheat straw starting from about 3 weeks after lambing and taken to pasture starting at the end of March.

The animals included in the study were consisted of Zom sheep and lambs born in 2010 and 2011 in three flocks randomly chosen among the Zom sheep flocks in Çınar County in the district of Karacadağ surrounded by Diyarbakır, Şanlıurfa and Mardin provinces. The data were belonged to 242 and 254 Zom lambs born from 226 and 214 ewes in 2010 and 2011, respectively. In the first year, the lambing started on the 27th day of December in 2009 and ended on 28th day of February in 2010, and in the second year the lambing started on the 17th day of November in 2010 and ended on 7th day December in 2010.

Reproductive Traits of Ewes

Reproductive traits of ewes in this study were lambing rate (LR), infertility rate (IR), single lambing rate (SLR), twinning rate (TW), fecundity (FEC) and litter size (LITS) and calculated as:

LR = the number of ewes lambed/the number of ewes in the flock

IR = the number of ewes not lambed/the number of ewes in the flock

SLR = the number of ewes with single live lamb/the number ewes lambed

TR = the number of ewes with twin live lambs/the number ewes lambed

FEC = the number of live lambs/the number of ewes in the flock

LITS = the number of live lambs/the number of ewes lambed

Lambs Growth and Survival Traits

The lambs are weighed using a scale with 50 g sensitivity and ear tagged within 12 h after the lambing and recorded as birth weight (BWT), and their live weights were measured and recorded every 30 days until they were about six months of age. Weaning weights (WWT) and sixth month live weights (SMLW) were adjusted to 90-day and 180-day of age, respectively, and average daily weight gains (ADWG1) from birth to weaning, and from weaning to six months of age (ADWG2) were calculated and used in the analysis. The survival rate (SR) was calculated as the ratio of the number of live lambs at weaning to the number of live lambs at birth.

Statistical Analysis

The analyses were carried out using SAS [6] statistical package program where LSMEANS statement with Tukey-Kramer multiple comparison test option was included in the analysis. The effects of year, sex, age of dam and type of birth of lambs were included in the model. BWT was also included in the analyses of WWT and SMLW as a covariate. Based on the preliminary analysis, the effect of flock was not significant on any growth traits in the study, thus this effect was excluded from the model. Pairwise t-test [7] was used to test the SR of lambs and the significance of differences among the reproductive performences of the ewes between years within flocks and between flocks within years.

RESULTS

The reproductive performance of Zom ewes are given in *Table 1*. There was no significant differences between years within flock in terms of LR, IO, FEC and LITS. The only significant difference was observed between years in TW and/or SLR in the flock 2 due might be to improved management within the flock from 2010 to 2011 (P<0.05). The lambing rate (LR), twining rate (TW), fecundity (FEC) and litter size (LITS) were 94%, 17%, 1.09 and 1.17 lambs on average, respectively.

The least square means and standard errors of BWT, WWT and SMLW are shown in *Table 2*. The overall BWT, WWT and SMLW were 4.00, 22.06 and 36.11 kg, respectively. The significant effects of the all the factors, except year effect, were observed on BWT, WWT and SMLW (P<0.05). Single born and male lambs are havier than twin born lambs and female lambs in terms of BWT, WWT and SMLW (P<0.05), respectively. The ewes at first lambing (2 years old ewes) produced lighter lambs at birth than those at second or more lambing (P<0.05). However, at weaning, the lambs born from modarate age of dam (3, 4 or 5 years old) were havier than those born from young or older dams (2 or 6 years old) (P<0.05) while in terms of SMLW the only significant difference was observed between 2 and 4 years old dams (P<0.05). The effect of year was significant only on WWT (P<0.05) that the lams born in 2010 were havier then those born in 2011. WWT and SMLW were also affected from the variation among the BWT that, on average, every 1 kg increase in BWT caused 2.02±0.281 and 2.26±0.502 kg increase in WWT and SMLW, respectively (P<0.01).

SR, ADWG1 and ADWG2 are presented in *Table 3*. The overall mean of SR was 90% and there was no significant difference between the sexes. However, SR was significantly affected from the type of birth, age of dam and year (P<0.05). The single born lambs have higher (91%) survival rate than twin born lambs (85%) (P<0.05). Lambs born from young or old dams (2 or 6 years old) have higher SR than those born from moderate age of dam (3, 4 and 5 years old) (P<0.05). The significant difference was observed also between the years 2010 (86%) and 2011 (93%) (P<0.05). This might be the result of improved management and feeding conditions in 2011 comparing to the year 2010. According to these results, the Zom lambs have quite high survival rate at farmer condition.

Table 1. *Reproductive performance of Zom sheep*
Tablo 1. *Zom koyunlarının üreme performansı*

Traits	Flock 1		Flock 2		Flock 3		Overall
	2010	2011	2010	2011	2010	2011	
Lambing %	95^{a1}	91^{a1}	96^{a1}	95^{a1}	94^{a1}	91^{a1}	94
Infertility %	5^{a1}	9^{a1}	4^{a1}	5^{a1}	6^{a1}	9^{a1}	6
Single Lambing %	88^{a1}	86^{a1}	90^{a1}	77^{b1}	83^{a1}	75^{a1}	83
Twinning %	12^{a1}	14^{a1}	10^{a1}	23^{b1}	17^{a1}	25^{a1}	17
Fecundity	1.07^{a1}	1.04^{a1}	1.06^{a1}	1.16^{a1}	1.10^{a1}	1.14^{a1}	1.09
Litter Size	1.13^{a1}	1.14^{a1}	1.10^{a1}	1.23^{a1}	1.17^{a1}	1.25^{a1}	1.17

^{a,b}Years with different letter are significantly different within flocks (P<0.05), ^{1,2}Flocks with different number are significantly different within years (P<0.05)

Table 2. *Least square means of birth weight (BWT), weaning weight (WWT), six month live weight (SMLW) and their standart errors in Zom sheep*
Tablo 2. *Zom koyunlarında, doğum ağırlığı (DA), sütten kesim ağırlığı (SKA), altıncı ay canlı ağırlığı (AACA)'nın en küçük kareler ortalamaları ve standart hataları*

Factors	BWT					WWT					SMWT				
Year	**N**	**X±Sx**	**Min**	**Max**	**CV%**	**N**	**X±Sx**	**Min**	**Max**	**CV%**	**N**	**X±Sx**	**Min**	**Max**	**CV%**
2010	242	4.0±0.06	2.1	7.7	19	208	24.1±0.35^{a}	14.1	40.0	20	59	36.3±0.95	22.0	52.4	17
2011	254	4.0±0.05	2.2	6.2	20	237	21.8±0.32^{b}	11.0	39.1	23	236	37.4±0.50	18.4	57.0	20
Type of Birth															
Single	379	4.4±0.04^{a}	2.1	7.7	18	346	24.4±0.25^{a}	12.2	40.0	20	213	38.0±0.60^{a}	18.4	57.0	19
Twin	117	3.6±0.07^{b}	2.2	5.6	17	99	21.5±0.47^{b}	11.0	32.8	23	82	35.7±0.80^{b}	22.6	52.5	18
Age of Dam															
2	57	3.7±0.09^{a}	2.2	5.6	18	55	21.0±0.53^{a}	11.0	33.1	22	48	35.0±0.83^{a}	22.0	48.9	17
3	145	4.0±0.06^{b}	2.1	6.2	20	130	24.0±0.36^{b}	14.2	37.3	19	94	37.6±0.69^{ab}	23.7	54.5	17
4	200	4.0±0.06^{b}	2.3	7.7	20	174	24.8±0.38^{b}	11.7	40.0	20	91	38.7±0.74^{b}	18.4	57.0	20
5	77	4.1±0.08^{b}	2.8	5.9	17	69	23.8±0.47^{b}	12.4	39.1	23	51	37.5±0.87^{ab}	24.2	53.0	18
6≤	17	4.2±0.13^{b}	3.1	5.5	14	17	21.4±0.83^{a}	14.5	29.9	20	11	35.5±1.72^{ab}	30.8	47.3	14
Sex															
Male	236	4.2±0.05^{a}	2.1	7.7	20	211	23.8±0.34^{a}	11.0	40.0	23	150	40.2±0.66^{a}	18.4	57.0	18
Female	260	3.8±0.05^{b}	2.2	6.2	18	234	22.1±0.36^{b}	12.9	39.1	20	145	33.5±0.66^{b}	22.6	51.6	14
BWT	--	--	--	--	--	--	2.02±0.281*	--	--	--	--	2.26±0.502*	--	--	--
Overall	496	4.0±0.04	2.1	7.7	19	445	22.1±0.26	11.0	40.0	22	295	36.1±0.42	18.4	57.0	19

N: *number of lambs;* ***Min:*** *minimum value;* ***Max:*** *maximum value;* ***CV%:*** *coefficient of variataion;* ^{a,b,c}*Means with different letter within traits and factors are significantly different (P<0.05), * P<0.01*

Table 3. *Least square means and standart errors of average daily weight gain from birth to weaning (ADWG1) and from weaning to six month of age (ADWG2) with survival rate of lambs (SR) from birth to weaning in Zom lambs*
Tablo 3. *Zom kuzuların doğumdan sütten kesime kadar (GCAA1) ve sütten kesimden altıncı ay yaşa kadar (GCAA2) günlük canlı ağırlık atışlarının en küçük kareler ortalamaları ve standart hataları ile sütten kesime kadar yaşama gücü (YG)*

Factors/Levels		ADWG1		SR	ADWG2	
		N	**X±Sx**		**N**	**X±Sx**
Overall		445	0.200±0.003	90	295	0.165±0.007
Year	2010	208	0.222±0.004^{a}	86^{a}	59	0.156±0.008^{a}
	2011	237	0.196±0.004^{b}	93^{b}	236	0.177±0.004^{b}
Type of Birth	Single	346	0.225±0.003^{a}	91^{a}	213	0.163±0.005
	Twin	99	0.193±0.005^{b}	85^{b}	82	0.169±0.007
Sex	Male	211	0.217±0.004^{a}	89	150	0.196±0.006^{a}
	Female	234	0.200±0.004^{b}	90	145	0.136±0.006^{b}
Age of Dam	2	55	0.188±0.006^{a}	96^{a}	48	0.167±0.007
	3	130	0.221±0.004^{b}	90^{b}	94	0.162±0.006
	4	174	0.231±0.004^{b}	87^{b}	91	0.160±0.006
	5	69	0.217±0.005^{b}	90^{b}	51	0.166±0.007
	6≤	17	0.188±0.009^{a}	100^{a}	11	0.176±0.015
BWT	---	---	0.012±0.003*	---	---	0.001±0.004^{ns}

^{a,b,c}*Means with different letter within traits and factors are significantly different (P<0.05),* * *P<0.01,* ^{ns} *nonsignificant*

The ADWG1 was affected from the all the factors in the study (P<0.05). The lambs born in 2010, males, single borns and those born from 3 or older dams have higher ADWG1. There was no significant differences among dams older than 2 years old in terms of ADWG1. Weight at birth had also significant effect on ADWG1 that, on average, heavier lambs at birth grew faster until weaning by the amont of 0.012±0.003 kg daily (P<0.01). On the other hand, type of birth, age of dam and weight at birth had no significant effect on ADWG2. The effects of year and sex were significant on ADWG2 (P<0.05) but the type of birth, age of dam and weight at birth had no significant effects.

DISCUSSION

Reproductive Performance of Ewes and Lamb Survival Rate (SR)

The average lambing rate (LR), twinning rate (TW), fecundity (FEC) and litter size (LITS) were 94% (91% to 96%) and 17% (10% to 25%), 1.09 (1.04 to 1.16) and 1.17 (1.10 to 1.25), respectively, according to flocks and years. This results are higher than those reported for TW and LR on Akkaraman sheep [8], and for FEC, LITS and LR [9] on Karakaş sheep and for LR [10,11] on Ramlıç and Tushin sheep.

The SR was estimated as 90% (85% to 100%) according to year, type of birth, sex and age of dam. There was no significant difference between male and female lambs in terms of SR. The effects of year, type of birth and age of dam on SR were significant (P<0.05). SR in the year 2011 was higher than that in 2010. Single born lambs had significantly higher SR than twin born lambs. Similar results were reported for Morkaraman and Kangal-Akkaraman lambs [12] and for various pure or crossbred sheep [13-15] while lower SR was reported on Akkaraman [12]. The variation among the research results in terms of SR of lambs could be related to differences in managemental conditions in the flocks and the care that the lambs have been subjected to until weaning.

Lambs Growth Performance

Birth Weight (BWT): In the present study, the average BWT were found as 4.0±0.04 kg varied between 3.6±0.07 and 4.4±0.04 kg based on type of birth, age of dam and sex of lamb. Our results are similar to reports in previous studies [8,12,16-19]. They reported that BWT were significantly affected by age of dam, type of birth and sex of lamb; females, twin borns and lambs born from young dams were lighter. However, in this study the effect of year was not significant on BWT. Nonsignificant effect of age of dam on BWT was reported in some other researches [20]. This results were similar to the reports in some studies [16,21] while were different from those in some other studies [11,20-23]. The mean BWT was havier in males than females and similar to prior reports [20,22,23]. On the contrary, insignificant effect of sex on BWT was reported on Anatolian Merino sheep [24]. The effect of type of birth on BWT was significant and similar to the findings on Ramlıç, Karacabey Merino, Akkaraman, Morkaraman, Awassi, Konya Merino and Anatolian Merino sheep [11,16,20-23].

Weaning Weight (WWT) and Six Month Live Weight (SMLW): For WWT (22.1 kg), our results are similar to reports [12] in previous studies on Morkaraman and Kangal-Akkaraman lambs. The effect of year was significant and similar to those reported on Konya Merino, Karacabey Merino, Akkaraman and Awassi sheep [25] while insignificant effect of year was reported on WWT [16,20]. This can be explained as that environmental and managemental conditions varies from year to year. The effects of sex on WWT were significant (P<0.05) and males were havier at weaning than females. This result is similar to previous studies [11,19,20,23] but different from some others [8] in which the sex effect was not significant on WWT. The effect of type of birth on WWT was significant (P<0.05) and similar to the results in the studies conducted on Karacabey Merino, Akkaraman, Morkaraman, Ramlıç, Awassi, Konya Merino and Anatolian Merino sheep [11,16,20]. The effect of age of dam on WWT was significant and contradicts with some previous reports [20]. For SMLW (36.11 kg), similar values were reported on Morkaraman and Kangal-Akkaraman lambs [12].

Average Daily Weight Gains (ADWGs): The least square means and standart errors of ADWG1 and ADWG2 are presented in *Table 3*. The all factors in this study were significant on ADWG1 (P<0.05), but only the effects of year and sex were significant on ADWG2 (P<0.05) showing that males grow faster than females between weaning to six month of age. For ADWG1, significant effects of year, sex, and type of birth were reported in previous studies [20,25] and in agreement with our results. However, the effect of age of dam was not significant in this study conratdicting to previous reports [20].

In conclusion, lamb's sex was significant for all traits except SR. For all growth traits in this study, males had higher values then females. Differences in body weight between male and female could be occuring due to the reason that males and females differ in sexual chromosomes, physiological characteristics and endocrinal system [26]. In this study, the effect of type of birth was significant on BWT, WWT, SMLW, ADWG1 and SR. Single borns' body weight in all ages and their average daily weight gain were higher than twins due possibly to reason that the ewes with single lamb give all its care to one lamb comparing to ewes with multiple lambs. Thus, single born lambs receive more care and milk from their mother and ends up with high WWT, ADWG1, consequently high SMLW and ADWG2 due to the reason of high correlation between WWT and SMLW [27,28]. The effect of age of dam was significant on WWT, SMLW, ADWG1. The lambs produced by dams of moderate ages (3, 4, and 5) have more weight than other lambs produced by young or old dams (2 or 6 and older). This difference could be related to lower capacity of milking in association with young or old ewes in comparison to ewes of moderate ages. Environmental factors are significant sources of variation for growth traits including body weight and average daily gains, and play an important role in expression of genetic potential. On the basis of analyses and regarding to high variation observed in the traits in question in this study, genetic improvement of Zom sheep in terms of growth trait and lamb survival rate are highly possible with a planned and continious selection program.

REFERENCES

1. Ertuğrul M, Dellal G, Elmacı C, Akın A O, Pehlivan E, Soysal Mİ, Arat S: Çiftlik Hayvanları Genetik Kaynaklarının Korunması ve Sürdürülebilir Kullanımı. *Türkiye Ziraat Mühendisleri Odası, VII Teknik Kongresi*, Milli Kütüphane Kongre Salonu, 2010.

2. Kaymakçı M: İleri Koyun Yetiştiriciliği. 2006, Bornova - İzmir.

3. Koncagül S, Akça N, Vural M E, Karataş A, Bingöl M: Zom koyunlarının morfolojik özellikleri. *Kafkas Univ Vet Fak Derg*, 18 (5): 829-837, 2012.

4. Wang CT, Dickerson GE: Simulation of life-cycle efficiency of lamb and wool production for genetic levels of competent traits. *J Anim Sci*, 69, 4324-4337, 1991.

5. Matos CAP, Thomas DL, Nash TG, Waldron DF, Stookey JM: Genetic analyses of scrotal circumference size and growth in Rambouillet lambs. *J Anim Sci*, 70, 43-50, 1992.

6. SAS: SAS/STAT. *SAS Inst Inc, Cary, NC, USA*. 2000.

7. Sümbüloğlu K, Sümbüloğlu V: Biyoistatistik. 10th ed., Hatipoğlu Yay. Ankara, 2002.

8. Düzgüneş O, Pekel E: Orta Anadolu Şartlarında Çeşitli Merinos x Akkaraman Melezlerini Verimle İlgili Özellikleri Üzerinde Mukayeseli Araştırmalar. Ankara Üniv. Zir. Fak. Yay.: 312, Bil. Araş. İnc. 194, Ankara, 1968.

9. Aygün T, Karaca O: Karakaş koyunlarında kimi döl verim özellikleri ve tekrarlanma derecesi tahminleri. *Yüzüncü Yıl Üniv Zir Fak Derg*, 6 (4): 177-189, 1996.

10. Kaçar C, Kamiloğlu NN, Gürbulak K, Pancarcm ŞM, Güngör Ö, Güvenç K, Saban H: The effect of administration of testosterone antibody, ß-carotene and vitamin E on multiple pregnancy and MDA (malondialdehyde) in Tuj sheep breed in non-breeding season. *Kafkas Univ Vet Fak Derg*, 14 (1): 51-56, 2008.

11. Ceyhan A, Sezenler T, Yıldırır M, Erdoğan İ: Reproductive performance and lamb growth characteristics of Ramlıç sheep. *Kafkaf Univ Vet Fak Derg*, 16 (2): 213-216, 2010.

12. Akçapınar H, Kadak R:. Morkaraman ve Kangal - Akkaraman kuzularının büyüme ve yaşama kabiliyeti üzerinde karşılaştırmalı araştırmalar. *Fırat Üniv Vet Fak Derg*, 7 (1-2): 203-212, 1982

13. Odabaşıoğlu F, Aslan M, Yertürk M: Morkaraman ve Corriedale x Morkaraman (F_1) kuzularda doğum ağırlığı ve yaşam gücü, Morkaraman koyunlarında gebelik süresine bazı faktörlerin etkisi. *YYÜ Vet Fak Derg*, 7 (1-2): 1-7, 1996.

14. Çolakoğlu N, Özbeyaz C: Akkaraman ve Malya Koyunlarının bazı verim özelliklerinin karsılastırılması. *Turk J Vet Anim Sci*, 23, 351-360, 1999.

15. Ceyhan A, Sezenler T, Erdogan I, Torun O: Improvement studies on mutton sheep for Marmara region conditions: I. Fertility, lamb survival, and growth traits of lambs. *Turk J Vet Anim Sci*, 35 (2): 79-86, 2011.

16. Boztepe S, Öztürk A: İvesi koyunlarında çevre faktörlerinin doğum ve sütten kesim ağırlığına etkileri ve bu karaktere ait kalıtım dereceleri. *Sel Üniv Zir Fak Derg*, 4, 94-100, 1994.

17. Demirulus H, Karaca O: Karakaş kuzularının köylü şartlarında kimi gelişme özellikleri. *Yüzüncü Yıl Üniv Zir Fak Derg*, 4, 75-85. 1994.

18. Özbey O, Akcan A: Akkaraman, Morkaraman ve İvesi koyunlarının yarı-entansif şartlardaki verim performansı. II. Kuzularda büyüme ve yaşama gücü özellikleri. *Vet Bilim Derg*, 17 (1): 57-66, 2001.

19. Daskıran I, Koncagül S, Bingöl M: Growth characteristics of ındigenous Norduz female and male lambs. *Tarım Bilim Derg,* 16, 62-69, 2010.

20. Arslan M, Yılmaz O, Ateş CT: Morkaraman ve Corriedale x Morkaraman (F1) kuzularında büyüme. *Yüzüncü Yıl Üniv Vet Fak Derg,* 14 (1): 46-49, 2003.

21. Öztürk A, Aktaş A H, Gürkan M: Konya Merinosu koyunlarının doğum ağırlığının kalıtım derecesi. *Turk J Vet Anim Sci,* 20, 411-414, 1996.

22. Koyuncu M, Tuncel E, Uzun Ş K: Karacabey Merinosu koyunlarında doğum ağırlığı ve gebelik süresine bazı çevre faktörlerinin etkileri ve genetik parametreler. *Atatürk Üniv Zir Fak Derg,* 32 (2): 163-167, 2001.

23. Öztürk Y, Küçük M, Karslı A: A study on growth, slaughter and carcass traits of Morkaraman and Kivircik x Morkaraman (F1) lambs in semi-intensive condition. Kafkas Univ Vet Fak Derg,18 (1): 1-6, 2012

24. Akkaya V, Eliçin A: Anadolu Merinoslarında karkas özelliklerinin fenotipi ve genetik parametreleri. *Ank Üniv Fen Bil Enst Yay*. 24, Ankara, 1984,

25. Vanlı Y, James J W, Mc Guirk B J, Atkins K: Avusturalya Merinos kuzularında bazı çevre faktörleri ile akrabalı yetiştirmenin sütten kesim özelliklerine etkileri. *Doğa Bilim Derg,* Seri: D. 9 (3): 274-281, 1985.

26. Rashidi A, Mokhtari M S, Jahanshahi A S, Abadi M R M: Genetic parameter estimates of pre-weaning growth traits in Kermani sheep. *Small Rumin Res*, 74, 165-171, 2008.

27. Kalantar M: Evaluation of some environmental effect on growth traits in Zandi sheep. *Agric Res*, 4, 49-58, 2003.

28. Dixit S P, Dhilon J S, Sing G: Genetic and non-genetic parameter estimates for growth traits of Bharat Merino lambs. *Small Rumin Res*, 42, 101-104, 2001.

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RESEARCH ARTICLE

Salam ve Sosislerin Bazı Kimyasal Özelliklerinin İncelenmesi

Çiğdem SEZER * Metin ÖĞÜN ** Aysel GÜVEN ***

* Kafkas Üniversitesi, Veteriner Fakültesi, Gıda Hijyeni ve Teknolojisi Anabilim Dalı, TR-36100 Kars - TÜRKİYE
** Kafkas Üniversitesi, Veteriner Fakültesi, Biyokimya Anabilim Dalı, TR-36100 Kars - TÜRKİYE
*** Kafkas Üniversitesi, Fen Edebiyat Fakültesi, Biyokimya Anabilim Dalı, TR-36100 Kars - TÜRKİYE

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Özet

Bu çalışmada farklı firmalara ait 15 adet salam ve 11 adet sosis örneğinin kalıntı nitrat ve nitrit düzeyleri ile yağ içerikleri araştırılmıştır. Salam ve sosis örneklerinde yağ oranlarının %5-26 arasında değiştiği ve bu değerler ile standartların verdiği limitlere uygun olduğu belirlenmiştir. İncelenen örneklerde en yüksek ve en düşük nitrat değerleri 98-453 mg/kg arasında değişirken nitrit için bu aralık 92–532 mg/kg olmuştur. Araştırma sonucu örneklerin 9 tanesinin (%34.6) Türk Gıda Kodeksi'nde izin verilen limitlerden daha fazla kalıntı nitrat içerdiği tespit edilirken, 25 tanesinin (%96) limitlerden fazla kalıntı nitrit içerdiği belirlenmiştir. Halk sağlığı açısından risk oluşturabilecek bu ürünlerin düzenli ve detaylı incelenmesi oldukça önemlidir.

Anahtar sözcükler: *Salam, Sosis, Nitrat, Nitrit, Yağ*

Determination of Some Chemical Characteristics of Salami and Sausage

Summary

This study was aimed to determine the fat content and levels of residual nitrate and nitrite of 15 pieces salami and 11 pieces sausages in Kars province. Fat content of salami and sausage samples were found to be 5-26%. The levels of fat in these samples were found as below the limit values of TS 980 and TS 979. Nitrate and nitrite levels in the salami and sausage were found to be 98-453 mg/kg; 92-532 mg/kg respectively. The residual nitrate values were detected over limits in nine of samples (34.6%). The residual nitrite values were detected over limits in 25 of samples (96%). In order to protect consumer health, it is mandatory that the salami and sausage is controlled to every stage in the production and that strict inspections be conducted regularly at both small and large-scale operations.

Keywords: *Salami, Sausage, Nitrate, Nitrite, Fat*

GİRİŞ

Sosis, kasaplık büyükbaş ve küçükbaş hayvan gövde etlerinden hazırlanan sosis hamurunun uygun kılıflara doldurulması ve belli aralıklarla boğumlanarak dizi şekline sokulması, yöntemine göre dumanlanması ve haşlanması ile elde edilen et ürünüdür. Salam ise büyükbaş ve küçükbaş hayvan gövde etlerinin veya bunların karışımlarının kemik, yağ, tendo, sinir ve kıkırdaklarından ayrılıp kıyıldıktan sonra, gerekli yardımcı maddelerin katılmasıyla hazırlanan et hamurunun, kılıflara doldurulması ve tiplerine uygun tarzda dumanlanıp, suda pişirilmesiyle yapılan et mamulü olarak tanımlanmaktadır [1,2].

Kür edilmiş tipik et rengi oluşumunda önemli rol oynayan nitrat (NO_3) ve nitrit (NO_2) tumbling ve massaging teknolojisi uygulanarak işlenen ürünlerin vazgeçilmez katkı maddeleridir. Nitrat ve nitritin et ürünlerindeki temel görevleri; nitrat ve nitritin indirgenmesi sonucu oluşan NO gıda zehirlenmesine neden olan *Clostridium botulinum*'un çoğalmasını ve toksin oluşturmasını engeller. Salam ve sosis gibi kür edilmiş, ısıl işlem uygulanmış ürünlerin arzu edilen parlak, pembemsi-kırmızı nitrosohemokrom rengini NO_3 ve NO_2 oluşturur. Tat ve lezzet üzerine etkileri yanında antioksidant özellikleri de dikkat çekmektedir [3-5].

İletişim (Correspondence)
+90 474 2426807
cigdemsezer@hotmail.com

Kürlenmiş et ürünleri üretiminde bu kadar çok görev yüklenen nitrat ve nitritin kullanımları belli bir miktar ile sınırlandırılmıştır. Türk Gıda Kodeksi'nde kürlenmiş et ürünlerinde sodyum nitrit miktarı en çok 150 mg/kg, soyum nitrat miktarı ise en çok 300 mg/kg olarak belirtilmiştir [6].

Et ürünlerine fazla miktarda kullanılan nitratların bakteriyel indirgenmesi veya gıdada bulunan hem sekonder hem de tersiyer aminlerin nitrit ile reaksiyonu sonucu kuvvetli kanserojenik etkili nitrosaminler oluşur [7,8]. Nitrosaminlerin oluşum mekanizmalarını ve tolere edilebilir limitlerin üzerinde vücuda alındıklarında insan sağlığı üzerine etkilerini inceleyen birçok araştırma mevcuttur [9-12].

Üretim yöntemi ve kullanılan hammaddenin yapısı itibariyle sucuk, salam ve sosis hileye oldukça açık olan ürünlerdir. Et ürünlerinin fiyatları arttıkça merdiven altı üretimlerde insan sağlığını hiçe sayarak birçok hile yapılabilmektedir. Tavuk ve hindi etlerinin karıştırılmasıyla elde edilen ürünlerin etiketlerine %100 dana eti yazılabildiği gibi, bozuk ürünlerin tekrar homojenizasyon işlemi ile yeniden tüketime sunulması gibi birçok hile uygulanabilmektedir. Bunlar içerisinde bilinçli veya bilinçsiz olarak yaratılabilecek tehlikelerden biri de nitrat ve nitrit tuzlarının limitlerin üzerinde kullanılmasıdır.

Ülkemizde sevilerek tüketilen özellikle çocuklar tarafından çok sevilen sosis ve salamların üretimden tüketime kadar her aşamada ciddi ve düzenli olarak izlenmesi ve denetlenmesi gerekmektedir. Üretimin teknolojik ve hijyenik şartlarda, eğitimli çalışanlar tarafından yapılması, tüketicilerin bilinçlendirilmesi ile kaliteli ve güvenilir ürünlerin elde edilmesi halk sağlığı açısından çok önemlidir.

Bu çalışma Kars ilinde satışa sunulan salam ve sosislerin kalıntı nitrat ve nitrit içerikleri ile emülsiyondaki yağ oranlarını tespit etmek ve halk sağlığı açısından güvenilirliklerini belirlemek amacıyla yapılmıştır.

MATERYAL ve METOT

Bu araştırmada Kars ilindeki marketlerde satışa sunulan farklı firmalara ait 11 adet sosis ile 15 adet salam alınarak en kısa sürede laboratuara getirilmiş ve analizler süresince 4°C'de muhafaza edilmiştir.

Nitrat ve Nitrit Kalıntı Miktarının Belirlenmesi

Örneklerin nitrit ve nitrat içerikleri Miranda ve ark.'nın [13] bildirdiği spektrofotometrik yönteme göre belirlenmiştir. Yöntem nitratın, vanadyum (III) klorür ile nitrite dönüştürülmesi sonucu nitritle sülfanilamidin asidik ortamda N-(1-Naftil) etilendiamine dihidroklorür ile reaksiyonu sonucu kompleks diazonyum bileşiğinin oluşması esasına dayanır. Örnekler için hazırlanan bu renkli kompleks spektrofotometrede 540 nm'de ölçülerek absorbans değerleri standart eğri ile hesaplanarak kalıntı nitrat ve nitrit miktarları hesaplandı.

Yağ Miktarının Belirlenmesi

Örneklerin yağ miktarı modifiye Babcock metodu [14] ile belirlenmiştir. Bunun için çok ince çekilmiş ve iyi karıştırılmış 9 g örnek poley şişesine tartıldı. Şişe içerisine 93-95°C'lik saf sudan 10 ml koyularak, ince bir baget yardımıyla örnek homojenize edildi. Üzerine 15 ml konsantre H_2SO_4 ilave edilip yavaş yavaş karıştırıldı. Bu aşamada proteinler denatüre edilerek çöktürüldü ve yağın serbest kalması sağlandı. Şişe içerisinde et parçaları tamamen digeste edildi. Şişenin boyun kısmından 93-95°C'lik sudan ilave edilerek yağ fazının tamamının poley şişesinin ölçülü kısmında toplanması sağlandıktan sonra toplanan yağ miktarı okundu. Bu değer direk olarak % yağ miktarı olarak değerlendirildi.

BULGULAR

Nitrat ve Nitrit Miktarı

Türk Gıda Kodeksi'nde kürlenmiş et ürünleri için satış noktalarındaki kalıntı sodyum nitrit miktarı en çok 100 mg/kg, kalıntı soyum nitrat miktarı ise en çok 250 mg/kg olarak belirtilmiştir. İncelenen örneklerde en yüksek ve en düşük nitrat değerleri 98-453 mg/kg arasında değişirken nitrit için bu aralık 92-532 mg/kg olmuştur. Araştırma sonucu incelenen örneklerin 9 tanesinin (%34.6) Türk Gıda Kodeksi'nde izin verilen limitlerden daha fazla kalıntı nitrat içerdiği tespit edilirken, 25 tanesinin (%96) limitlerden fazla kalıntı nitrit içerdiği belirlenmiştir [6]. Sosis ve salam örneklerinin nitrat, nitrit ve yağ oranları *Tablo 1*'de verilmiştir.

Yağ Analizi

TS 980 sosis standardı ve TS 979 salam standardında en

Tablo 1. *Örneklerin analiz sonuçları*
Table 1. *Results of analysis of samples*

Numuneler	Analizler	En Düşük	En Yüksek	Ortalama	Limit Değerler	Standartlara Uymayan Örnek
Sosis (n=11)	Nitrat (mg/kg)	154	453	275.09	<250 mg/kg	% 45
	Nitrit (mg/kg)	92	403	256.90	<100 mg/kg	% 90
	Yağ (%)	10	26	16.18	<%40	% 0
Salam (n=15)	Nitrat (mg/kg)	98	293	193.06	<250 mg/kg	% 36
	Nitrit (mg/kg)	163	532	306.4	<100 mg/kg	% 100
	Yağ (%)	5	24	15.23	<%40	% 0

fazla %40 yağ oranına izin verilmektedir. İncelenen örneklerde en yüksek ve en düşük yağ oranları %5-26 arasında değişmektedir.

TARTIŞMA ve SONUÇ

Salam ve sosis üretiminde istenen ürün renginin sağlanması ve mikrobiyolojik anlamda güvenilirliğin oluşması için kürleme tuzu olarak nitrat ve nitrit kullanılmaktadır. Türk Gıda Kodeksi'nde izin verilen limitlerde kullanıldığı sürece uygun şartlarda herhangi bir sağlık sorunu oluşturmayacağı düşünülen nitrat ve nitritin limitlerin üzerinde kullanımı ciddi sağlık sorunları oluşturabilmektedir. Özellikle gıdalarda oluşturdukları nitrosaminlerin mutajenik ve teratojenik etkileri oldukça yüksektir [9-11].

Salam ve sosislerde bulunan nitrit ve nitrat miktarının araştırıldığı çalışma sonuçları farklılıklar göstermektedir. Yalçın ve ark.[15] 100 adet salam sosis örneklerin tamamında izin verilen limitlerin altında nitrat ve nitrit bulunduğunu belirtirken, Soyutemiz ve Özenir [16] salam örneklerin %60'ının, sosislerin ise %66.6'sının limitlerin üzerinde nitrat ve nitrit içerdiğini tespit etmişlerdir. Şanlı ve Kaya [17] inceledikleri salam ve sosis örneklerinde insan sağlığı için sakıncalı sayılabilecek düzeyde nitrat ve nitrit kullanıldığını bildirmişlerdir. Develi Işıklı [18] piyasadan toplanan sosis örneklerinin %36'sının standartlara uygun olmadığını belirtmiştir. Ülkemizde yapılan bir diğer çalışmada Ankara ilinde satılan salam ve sosislerin nirat ve nitrit içerikleri incelenmiştir. Örneklerin tamamının nitrat ve nitrit yönünden standartlara uygun olduğu belirlenmiştir [19]. Bu çalışmanın aksine aynı ilde yapılan benzer bir araştırmada sosis örneklerinin nitrat içerikleri yönünden uygun olduğu fakat nitrit içerikleri yönünden örneklerin %27.5'inin standartlara uygun olmadığı belirlenmiştir [20]. Elgezdi [21] fermente ve ısıl işlem görmüş et ürünleri üzerine yaptığı araştırmasında salam ve sosis örneklerinin %5'inin nitrit yönünden uygun olmadığını tespit etmiştir.

Kanserojenik etkili nitrosamin bileşikleri yönünden nitrat ve nitritlerin risk oluşturabileceği göz önünde bulundurulduğunda hem üreticilerin hem de tüketicilerin bu konuda bilinçlendirilmesinin zorunlu olduğu görülmektedir.

Salam, sosis ve sucukların etiketleri üzerine maalesef gerçek içeriğin tam olarak yazılmadığı görülmektedir [22]. 410 adet et ve et ürününün analiz edildiği bir araştırmada örneklerin tamamında sığır eti, %20'sinde tavuk eti ve %4.3'ünde at eti tespit edilmiştir. Bu veriler örneklerin %16.3'ünün etiket bilgileri ile uyumlu olmadığını ve %19.2'sinin hileli olduğunu göstermektedir [23]. Üretim prosesleri düşünüldüğünde sucuk, salam sosis gibi hileye oldukça açık et ürünlerinde kullanılan etin orjini oldukça önemlidir. Bu hem toplumsal inançlar hem de ekonomik anlamda önem taşır. Etiket bilgisinde %100 dana ibaresi olup kokteyl ürünler olan salam ve sosiste renk sorunları görülebilmektedir. Kırmızı ete nazaran daha beyaz renge sahip olan tavuk ve hindi eti, salam ve/veya sosis emülsiyonlarında renk açılmasına sebep olabilmektedir [24,25]. Aynı şekilde kokteyl olarak nitelendirilen bu ürünlerde beyaz et ile kırmızı etin raf ömrü arasındaki fark da muhafaza sırasında hem mikrobiyel anlamda hem de renk yönünden sorun oluşturabilmektedir. Bu sorunları gidermek için bazı üreticiler limitlerin üzerinde nitrat ve nitrit kullanabilmektedir. Salam sosis örneklerinde yürütülen bu çalışmada özellikle tavuk ve hindi eti ile karışık olduğu etikette (kokteyl) belirtilen ürünler ile tavuk salamı ve hindi salamı etiketi bulunan ürünlerin daha yüksek konsantrasyonda nitrat ve nitrit içerdiği görülmektedir.

Türk Gıda Kodeksi'nde üretim esnasında kullanılmasına izin verilen limitlerin bile çok üstünde kalıntı nitrat nitrit içeriği oldukça endişe vericidir. Bu araştırma sonuçları kontrollü üretim ve üretim sonrası denetimlerin ne denli önemli olduğunu bir kere daha vurgulamıştır. Üreticilerin gıda güvenliği ve halk sağlığına yönelik eğitim alması da belirlenen bir diğer zorunluluktur. Gıda sanayinde nitrat ve nitrite alternatif olarak kullanılabilecek doğal katkı maddelerine ihtiyaç duyulmaktadır.

Sucuk üzerine yapılan büyük hilelerden biri fazla miktarda yağ ilavesidir [26,27]. Ülkemizde sıkça tüketilen sucukların aksine bu çalışmada salam ve sosis örneklerinde yağ oranları standartların verdiği limitlere uygun olarak bulunmuştur.

KAYNAKLAR

1. Anonim: Sosis, TS 980, Türk Standartları Enstitüsü. Ankara, 2002.

2. Anonim: Salam, TS 979, Türk Standartları Enstitüsü. Ankara, 2002.

3. Arslan A: Et Muayenesi ve Et ürünleri Teknolojisi. Medipres. Elazığ, 2002.

4. Tekinşen OC, Doğruer Y: Her Yönüyle Pastırma. Birinci Basım. Selçuk Üniversitesi Basımevi, Konya, 2000.

5. Gökalp H, Kaya M, Zorba Ö: Et Ürünleri İşleme Mühendisliği. Üçüncü Baskı. Atatürk Üniversitesi Yayın No: 786. Atatürk Üniversitesi Ziraat Fakültesi Ofset Tesisi, Erzurum, 1999.

6. Anonim: Türk Gıda Kodeksi Yönetmeliği, T.C. Resmi Gazete, 16 Kasım 1997, Sayı 23172, s. 44. Ankara, 1997.

7. Varnam AH, Sutherland JP: Meat and Meat Products. Technology, Chemistry and Microbiology. Vol. 3, Food Products Series. Chapman and Hall, London, 1995.

8. Öztan A: Et Bilimi ve Teknolojisi. TMMOB Gıda Mühendisleri Odası Yayınları Kitaplar Serisi Yayın No: 1. Düzeltilmiş ve Genişletilmiş 6. Baskı, Ankara, 2008.

9. Vermeer IT, Pachen DM, Dallinga JW, Kleinjans JC, van Maanen JM: Volatile N-nitrosamine formation after intake of nitrate at the ADI level in combination with an amine-rich diet. *Environ Health Perspect,* 106 (8): 459-463, 1998.

10. Michaud DS, Holick CN, Batchelor TT, Giovannucci E, Hunter DJ: Prospective study of meat intake and dietary nitrates, nitrites, and nitrosamines and risk of adult glioma. *Am J Clin Nutr,* 90 (3): 570-577, 2009.

11. Griesenbeck JS, Steck MD, Huber JC, Sharkey JR, Rene AA, Brender JD: Development of estimates of dietary nitrates, nitrites, and nitrosamines for use with the short willet food frequency questionnaire. *Nutr J,* 6, 8-16. 2009.

12. Gökalp HY: Et ürünlerine katılan nitrat, nitrit miktarlarının azaltılması, N-nitrosamin oluşum reaksiyonlarının engellenmesi ve gıdalarda N-nitrosaminlerin saptanması. *Gıda*, 10 (3): 161-167, 1985.

13. Miranda KM, Espey MG, Wink DA: A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide*, 5, 62-71, 2001.

14. Gökalp HY, Kaya M, Tülek Y, Zorba Ö: Et ve Et Ürünlerinde Kalite Kontrolü ve Laboratuvar Uygulama Klavuzu. IV. Baskı, Atatürk Üniv Yayınları,

Yayın No: 751. Ezurum, 2001.

15. Yalçın H, Pelin CÖ, Türkoğlu M: Mersin ilinde tüketime sunulan salam, sosis ve sucuklardaki kalıntı nitrat ve nitrit düzeylerinin belirlenmesi. *Gıda*, 37 (1): 33-37, 2012.

16. Soyutemiz GE, Özenir A: Bursa'da tüketilen sucuk, salam, sosis ve pastırmalardaki kalıntı nitrat ve nitrit miktarlarının saptanması. *Gıda*, 21, 471-476, 1996.

17. Şanlı Y, Kaya S: Ankara piyasasında satılan bazı işlenmiş et ürünlerinin nitratve nitrit içerikleri üzerine araştırmalar. *Ankara Üniv Vet Fak Derg,* 35 (1): 24-46, 1988.

18. Develi Işıklı N: Et ürünlerinde nitrit tayininde kullanılan yöntemin teşhis limitinin belirlenmesi. *Gıda,* 26 (5): 347-351, 2001.

19. Kuyumcu A: Ankara piyasasında satılan salam, sucuk sosislerin nem, yağ, tuz, kül ve kalıntı nitrat, nitrit miktarlarının tayini üzerine bir araştırma. *Yüksek Lisans Tezi,* Hacettepe Üniv. Sağlık Bil. Esnt., 1999.

20. Arlı V: Ankara piyasasında satılan sosislerde bazı kürleme ajanlarının tesbiti üzerinde çalışmalar. *Yüksek Lisans Tezi,* Ankara Üniv., Sağlık Bil. Enst., 1993.

21. Elgezdi Y: Fermente ve ısı işlemi görmüş et mamüllerinde nitrit düzeyleri üzerine bir araştırma. *Yüksek Lisans Tezi,* İstanbul Üniv. Sağlık Bil. Enst., 1996.

22. Anonim: Gıda Tarım ve Hayvancılık Bakanlığı 13.04.2012 tarihli kamuoyu duyurusu. (http://kamuoyuduyuru.tarim.gov.tr), 2012.

23. Günşen U, Aydın A, Ovalı BB, Coşkun Y: Çiğ et ve ısıl işlem görmüş et ürünlerinde ELISA tekniği ile farklı et türlerinin tespiti. *İstanbul Üniv Vet Fak Derg*, 32 (2): 45-52, 2006.

24. Keleş A, Atasever M, Güner A, Uçar G: Sığır eti ilavesiyle tavuk salamı üretimi. *Vet Bil Derg,* 16 (2): 5-14, 2000.

25. Sarıçoban C, Karakaya M: Sığır etine farklı oranlarda karıştırılan yumurta tavuğu etinin Türk tipi sucuk üretiminde kullanılabilme olanakları üzerine bir araştırma. *Gıda,* 26 (2): 109-113, 2001.

26. Çon AH, Gökalp HY: Türkiye pazarlarındaki sucukların bazı kimyasal ve mikrobiyolojik nitelikleri. *Gıda,* 23, 347-355, 1998.

27. Elibol C: Van piyasasında tüketime sunulan salam ve sosislerin mikrobiyolojik, kimyasal, fiziksel ve duyusal niteliklerinin incelenmesi. *Yüksek Lisans Tezi,* Yüzüncü Yıl Üniv. Sağlık Bil. Enst., 1996.

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RESEARCH ARTICLE

An Immunohistochemical Study of the Insulin-, Glucagon-, Orexin A- and Ghrelin- Immunoreactive Cells in the Endocrine Pancreas of Guinea Pig

Mine YAMAN * Ali BAYRAKDAR *

* Department of Histology and Embryology, Faculty of Veterinary Medicine, Firat University, TR-23119, Elazig - TURKEY

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Summary

The present study was conducted to clarify the regional distribution and relative frequency of endocrine cells secreting insulin, glucagon, orexin A (OXA) and ghrelin in the pancreatic islets of guinea pig, by specific immunohistochemical methods. In the pancreatic islets, most of insulin-IR cells were detected in the central region, partially in mantle region. Glucagon-IR cells were located in the periphery of the pancreatic islets. In addition, they had lower frequency in the central region. OXA and ghrelin- IR cells were identifed in central and peripheral regions of the pancreatic islets. Clearly, OXA-IR cells exhibited a characteristic distribution pattern resemble to those for the glucagon- IR cells. However, ghrelin immunoreactivity exhibited a characteristic distribution pattern different from that of insulin-immunoreactive cells. In conclusion, some species-dependent unique distributions characteristics of endocrine cells in the pancreatic islets were observed in the guinea pig.

Keywords: *Immunohistochemistry, Pancreas, Endocrine cell, Guinea pig*

Kobay Endokrin Pankreasında İnsülin-, Glukagon-, Orexin A- ve Ghrelin- İmmunoreaktif Hücrelerin İmmunohistokimyasal Çalışması

Özet

Bu çalışma, kobay pankreatik adacıklarında immunohistokimyasal metodlar kullanarak insülin, glukagon, orexin A (OXA) ve ghrelin salgılayan endokrin hücrelerin bölgesel dağılımını ve nisbi frekansını ortaya koymak için yapıldı. İnsülin-IR hücrelerin çoğu pankreatik adacıkların merkez bölgesinde, kısmen de manto bölgesinde belirlendi. Glukagon-IR hücreler genellikle pankreatik adacıkların periferinde, çok az yoğunlukta ise merkez bölgede tespit edildi. OXA ve ghrelin-IR hücreler ise pankreatik adacıkların merkezi ve periferal bölgelerine lokalize oldu. Daha açık ifadeyle, OXA-IR hücrelerin lokalizasyonu glukagon-IR hücrelere benzerlik gösterdi. Buna karşın, ghrelin immunoreaktivitesi insülin-IR hücrelerinkinden farklı bir dağılım sergiledi. Sonuç olarak; kobayların pankreatik adacıklarındaki endokrin hücrelerin karekteristik dağılımı türe özgü farklılık göstermektedir.

Anahtar sözcükler: *İmmunohistokimya, Pankreas, Endokrin hücre, Kobay*

INTRODUCTION

The pancreas is formed by two distinct compartments, different in morphology and function: the exocrine portion, which secretes its digestive juice into the duodenum, and the endocrine portion, which produces hormones for the control of the carbohydrate metabolism [1]. The endocrine pancreas, known as islets of langerhans, contains A-, B-, D- and F-cells producing glucagon, insulin, somatostatin and pancreatic polypeptide and probably also neuropeptide Y, respectively [2].

A few years ago, a fifth mammalian islet cell type, the ghrelin-producing epsilon cell, was described [3,4]. However, the cell type that produces ghrelin in the pancreatic islets remains controversial, whether it be the Acells, B cells, the newly identified islet epsilon cells (ε-cell), or a unique novel islet cell type [5-8]. More recently, orexin A was also found in human and rodent pancreas where it is expressed only in a few endocrine cells, subsequently identified as B type insulin-

İletişim (Correspondence)
+90 424 2370000/4037
yaman@firat.edu.tr

IR cells by immunohistochemical techniques [9].

The pancreas is a valuable organ for endocrine studies, with the endocrine pancreas being extensively studied in diabetes [10]. In addition, investigations of gastroentero-pancreatic (GEP) endocrine cells are considered to be an important part of phylogenetic study [11,12].

Many neuropeptides play a significant role in the regulation of both exocrine and endocrine pancreatic secretion in the rat [5,13]. Immunohistochemical localization of pancreatic hormones, several neuropeptides, has been determined in the pancreas of the rodents such as hamster [14], mouse [4], C57BL/6 mouse [15], ddN mouse [16], wood mouse [17], guinea pig [18], gerbil [19] and rat [5,20,21].

It has been classically admitted that insulin immuno-reactive cells are located in the central regions and glucagon immunoreactive cells are located in the peripheral regions [22]. Also, orexin-A has been shown to localize to B cells [23] and ghrelin has been shown to localize to B cells [6] and A cells [5], and to the newly discovered type of cell, that does not stain for any of the known islet hormones, and called the E cell [24]. But, many researches suggested that the regional distribution and relative frequency of immunoreactive (IR) endocrine cells in the pancreatic islets are different in different portions of the pancreas even within the same pancreas of the same animal [17,25] and a species-dependent characteristic distribution of pancreatic endocrine cells originating from feeding habits has also been suggested [22].

The initial focus of many studies was on insulin, somato-statin, glucagon, pancreatic polypeptide in the endocrine portion, and other neuropeptides found in the exocrine portion [16,26,27]. In the present study, the regional distribution and relative frequency of endocrine cells in guinea pig pancreas were examined by immunohistochemical method using specific antisera against insulin, glucagon, orexin A (OXA) and ghrelin.

MATERIAL and METHODS

Animals and Tissue Samples

Ten female guinea pig were used in this study. After the guinea pig were anaesthetized with pentathol (6 ml/kg), the left carotid artery was cannulated at the base of the neck and allowed to exsanguinate. Tissue samples were taken from pancreas and fixed in 10% neutral-buffered formalin for 24 h. They were then dehydrated through graded ethanol and embedded in paraffin. Five µm thick sections, mounted on poly-l-lysine coated glass slides, were obtained and processed for immunohistochemical staining.

Immunohistochemistry

Tissues were incubated in citrate buffer (10 mM citric acid, pH 6.0) for 20 min to retrieve antigenicity. Immuno-histochemical staining was performed according to the manufacturer's protocol the SuperPicture™ 3rd Gen IHC Detection Kit (Invitrogen; Cat. No: 87-8973). Sections were incubated with primary antibodies for 16-20 h at 4°C. The working dilutions and the sources of the primer antibodies were listed in *Table 1*. The primary antibodies were diluted in PBS containing 0.25% sodium azide and 2.5% bovine serum albumin (BSA). Negative control sections were performed by replacing the primary antibodies with PBS. The sections were then incubated with HRP polymer conjugate 10 min at room temperature. Subsequently, the sections treated with DAB chromogen according to the kit instructions, washed in distilled water and counter-stained with Mayer's hematoxylin. Finally, sections were dehydrated and coverslips mounted with mounting medium.

The specificity of each immunohistochemical reaction was determined as recommended by Sternberger [28], including the replacement of specific antiserum by the same antiserum, which had been preincubated with its corresponding antigen. Sections were examined with Olympus BX-51 microscope and photographs were taken.

RESULTS

Guinea pig pancreas was found to be consisted of exocrine and endocrine parts (pancreatic islets). The endocrine parts of the pancreas were scattered singly or in small groups of islets of various shapes and size in the interstitium of the exocrine parts.

The pancreatic islets of this study were distinguished as three distinct regions, central, mantle and peripheral regions, with their composition of immunoreactive (IR) cells. In this study, all four types of the IR endocrine cells were detected

Table 1. *Details of antibodies used in this study*
Tablo 1. *Çalışmada kullanılan antikorlar*

Primer Antibodies	Dilution	Trade Name	Cat. No.
Insulin	1:1000	Abcam	ab63820
Glucagon	1:200	Santa Cruz Biotecnology, Inc. Germany	sc-13091
Orexin A	1:1000	Chemicon (Millipore)	AB3704
Ghrelin	1:100	Santa Cruz Biotecnology, Inc. Germany	sc-50297

All antisera were raised in rabbit (polyclonal)

with the antisera against insulin, glucagon, orexin A and ghrelin in pancreatic islets of the guinea pig.

Insulin-IR Cells (B Cells): Round and/or oval shaped insulin-IR cells were abundant in the whole pancreatic islets. The most of insulin-IR cells were located in the central region of islets except for a small area. A few insulin-IR cells were detected in mantle region. Endocrine cells in islets were stained strongly with insulin *(Fig. 1.A)*. Insulin positive cells were not found in the peripheral regions or in the exocrine portions.

Glucagon- IR Cells (A Cells): Glucagon-IR cells were located in the periphery of the pancreatic islets where the cell population is primarily A cells and a somewhat lower frequency of cells was noticed in the central region intermingled with insulin-IR cells. They were generally shuttle or oval shaped. The intensity of glucagon staining was similar to insulin *(Fig. 1.B)*. In addition, distribution of islets localized endocrine cells were more less than insulin. Glucagon-IR cells were not found in the exocrine portions or centroacinar cells.

Orexin A- IR Cells: Orexin A-IR cells were localized in central and peripheral regions of the pancreatic islets. The IR-cells were generally observed small clusters composed of 2-3 cells and they were in variable shapes. Orexin A-IR cells exhibited a characteristic distribution pattern resemble to those for the glucagon- IR cells. However, the intensity of the staining was not so strong as in glucagon-IR cells *(Fig. 1.C)*. Orexin A-IR cells were not detected in exocrine portion or centroacinar cells.

Ghrelin- IR Cells: Ghrelin-IR cells were usually round or

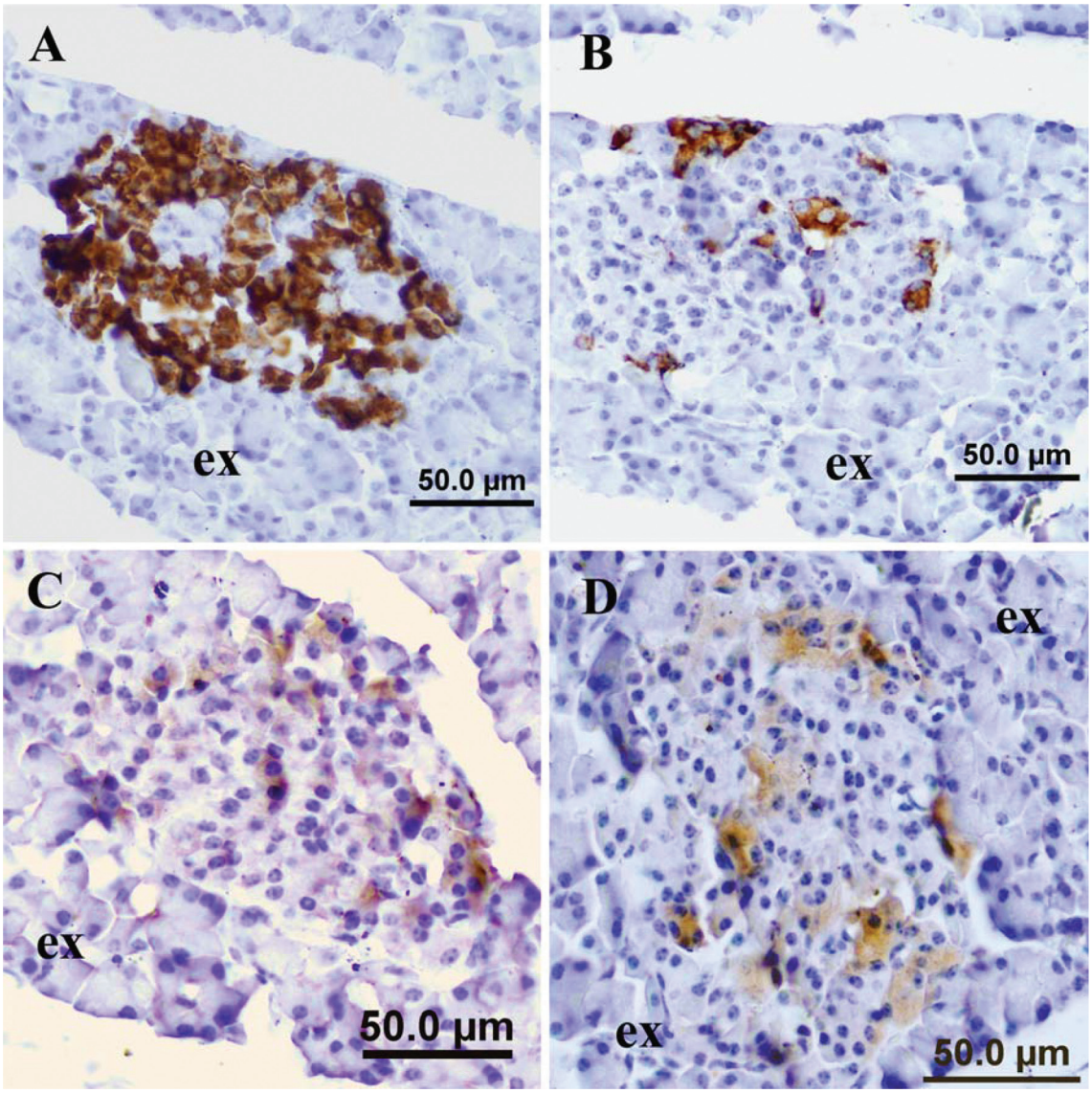

Fig 1. Guinea pig pancreatic islets showing immunoreactive cells (ex: exocrine), **A-** Insulin-IR cells, **B-** Glucagon-IR cells s, **C-** Orexin A-IR cells, **D-** Ghrelin-IR cells

Şekil 1. Kobay pankreatik adacıklarında immunoreaktif hücrelerin görünümü (ex: ekzokrin), **A-** İnsulin-IR hücreler, **B-** Glukagon-IR hücreler, **C-** Oreksin A-IR hücreler, **D-** Ghrelin-IR hücreler

ovoid in shape and were located in both the peripheral and central regions of the islets, either as single cells or small clusters of cells. However these IR cells were more diffuse in peripheral regions of some islets. Cells positive for ghrelin immunoreactivity exhibited a characteristic distribution pattern different from that of insulin-immunoreactive cells. The intensity of ghrelin staining in endocrine cells were more weak than that of insulin- and glucagon-IR *(Fig. 1.D)*. Ghrelin-IR cells were not detected in exocrine portion or centroacinar cells.

DISCUSSION

The function of hormones released from pancreatic endocrine cells is directly related to the regulation of pancreatic digestive enzymes and serum glucose levels [29]. Therefore the different distribution patterns and frequency of these pancreatic endocrine cells are considered to be a result of differences in feeding habits, especially for glucose and proteins. This study was carried to determine the existence and distribution of pancreatic endocrine cells in guinea pig pancreas.

Insulin is synthesized in the B cells of the pancreatic islets and regulates the blood glucose levels [30]. In the mammals, the regional distribution and the relative frequency of pancreatic insulin-IR cells were reported in the hamster [14], rat [27], guinea pig [18], C57BL/6 mouse [15], gerbil [19], wood mouse [17], and various laboratory animals [22]. According to the previous studies, insulin immunoreactive cells were observed in the central regions of the pancreatic islets and other cells, such as glucagon- and somatostatin-IR cells, surrounded them. However, it was described that insulin-IR B cells occupied the majority of the periphery regions of islets in monkey and possum pancreas [31,32]. In the present study, most of the insulin-IR cells were restricted to the central and mantle regions of pancreatic islets in the guinea pig. The results of this study were compatible with the findings reported by previous studies on rodents except for monkey and possum [14,15,18,19,22,27].

Glucagon is synthesized in the A cells of the pancreas and also participates to the regulation of blood glucose concentrations [30]. The distribution of glucagon-IR cells was quite different from species regardless of the same mouse strains. Although most of the glucagon-IR cells were situated in the peripheral regions of the pancreatic islets, they were also demonstrated in the central regions in the both splenic and duodenal lobes of the ddN mouse [16]. In the other examinations performed on mouse strains [25,33], glucagon-IR cells were mainly detected to peripheral regions but some these cells were also present in the central regions of the pancreatic islets. However, Ku et al.[15] reported that these immunoreactive cells were mantle and peripheral located in C57BL/6 mouse. Interestingly, in the monkey, glucagon-IR cells were found in the central regions of pancreatic islets where insulin-IR cells were numerously located in most of the domestic animals [32]. However, some researchers reported that glucagon-IR cells were found in peripheral regions of rat pancreatic islets [27,32]. In the present study, glucagon-IR cells were located in the peripheral regions of pancreatic islets; a somewhat smaller number of these cells were also observed in the central regions intermingled with insulin-IR cells. These results were found to be similar to that of mouse strains [16,25,33] but different from the other species [27,32].

The orexin family is a complex system composed of two neu- ropeptides, orexin-A and orexin-B (OXA and OXB), and two cognate receptors, orexin type 1 and orexin type 2 (OX1R and OX2R). Recently, orexins have been studied extensively to comprehend their complex functional roles. In fact, since the first identification of OXA and OXB in rat hypothalamus [34], the presence of this system has been found in both the central and peripheral nervous systems as well as in several other different tissues and organs [35]. Recently, it has been found in peripheral tissues and, particularly, in endocrine cells and neurons of the enteric nervous system (ENS) of different portions of the gastro-intestinal tract and in the pancreas and salivary glands [9,36].

In the rat, guinea-pig and mice pancreas, OXA immuno-reactivity was detected in B cells of pancreatic islets. Furthermore, insulin-IR islet cells also displayed OX1R-like immuno-reactivity, and OX1R mRNA was detected in the rat pancreas [37]. In double immunostaining in the human pancreas, a great majority of insulin-IR cells was simultaneously positive for orexin-A [38]. Conspicuously, OXA-immunoreactivity was detected in almost 60% of insulin positive cells in pancreatic islets of domestic animals [9]. In contrast, OXA and OX1 immuno-reactivity were found in glucagon-IR cells in rat pancreatic islets [20]. Interestingly, OX1R-positive cells were observed in the periphery of pancreatic islets of normal and in both the peripheral and central regions of the islet of diabetic rats [39,40]. Similar to the findings reported for rats, OXA-IR cells were found peripherally and more less centrally located in pancreatic islets in the present study.

Ghrelin, a 28-amino acid peptide which was isolated from rat and human stomach, acts as an endogenous ligand for GHS-R [41]. Pancreas ghrelin is emerging as a key player in the regulation of insulin secretion by the B cell, suggesting that it may play an important role in glucose metabolism [42,43].

Recently, ghrelin was identified to be present in pancreatic islet cells by immunostaining and immunofluorescence methods. Immunostaining for ghrelin was observed in B-cells of human pancreatic islets [6]; however, it was detected in the A-cells of rat and human pancreas [5]. Furthermore, ghrelin-IR cells correspond to glucagon-IR cells, and GHS-R-like (ghrelin receptor) immunoreactivities were located in glucagon- and insulin-IR cells of the rat pancreas [21,44]. In a study performed on rats, were detected ghrelin immuno-staining either independently of glucagon staining or together with glucagon immunostaining in a minority of A-cells while not detect ghrelin immunostaining in B-cells [24].

Interestingly, in the examinations performed on rodent, ghrelin was observed in a newly discovered type of cell, that does not stain for any of the known islet hormones, and called the epsilon cell (ε-cell) [4,8,45]. Thereafter, the presence of these cells was confirmed in fetal and adult human pancreas. Epsilon cells were usually round or ovoid in shape and were often located at the periphery of the islets, either as single cells or small clusters of cells [46]. In the present study, ghrelin-IR cells were detected peripheral and central regions of the islets. Conspicuously, it was demonstrated that significantly less B-cell area (central region) and markedly larger A- cell areas (peripheral region) of islets. The distributions of these cells were resemble with the results of rats [21,44] but different from mammalian [4,8,24,45,46]. These differences might be due to different antisera, methods and/or species used in each study.

The present study was revealed the existence and distribution of endocrine cells in the pancreas. In this study; isulin, glucagon, OXA and ghrelin were detected to exist in the pancreatic islets of the guinea pig. The general distribution patterns of pancreatic endocrine cells of the guinea pig was similar to those of some rodent and other species. However, some species-dependent unique distributions characteristics of endocrine cells in the pancreatic islets were also observed in the present study. The characteristic existence may be variety in feeding habits (between an animal species) and/or caused by this species specific differences among species.

REFERENCES

1. Motta PM, Macchiarelli G, Nottola SA, Correr S: Histology of the exocrine pancreas. *Microsc Res Tech*, 37, 384-398, 1997.

2. Konturek SJ, Zabielski R, Konturek JW, Czarnecki J: Neuro-endocrinology of the pancreas; role of brain-gut axis in pancreatic secretion. *Eur J Pharmacol*, 481, 1-14, 2003.

3. Wierup N, Svensson H, Mulder H, Sundler F: The ghrelin cell: A novel developmentally regulated islet cell in the human pancreas. *Regul Pept*, 107, 63-69, 2002.

4. Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L: Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proc Natl Acad Sci USA*, 101, 2924-2929, 2004.

5. Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, Kangawa K, Arima T, Matsuo H, Yada T, Matsukura S: Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. *Diabetes*, 51, 124-129, 2002.

6. Volante M, Allia E, Gugliotta P, Funaro A, Broglio F, Deghenghi R: Expression of ghrelin and of the GH secretagogue receptor by pancreatic islet cells and related endocrine tumors. *J Clin Endocrinol Metab*, 87, 1300-1308, 2002.

7. Wierup N, Yang S, McEvilly RJ, Mulder H, Sundler F: Ghrelin is expressed in a novel endocrine cell type in developing rat islets and inhibits insulin secretion from INS-1 (832/13) cells. *J Histochem Cytochem,* 52, 301-310, 2004.

8. Wierup N, Sundler F: Ultrastructure of islet ghrelin cells in the human fetus. *Cell Tissue Res*, 319, 423-428, 2005.

9. Dall'Aglio C, Pedini V, Scocco P, Boiti C, Ceccarelli P: Immuno-histochemical evidence of Orexin-A in the pancreatic beta cells of domestic animals. *Res Veter Sci* 89, 147-149, 2010.

10. Jansson L, Sandler S: The influence of cyclosporin A on the vascular permeability of the pancreatic islets and on diabetes induced by multiple low dose of streptozotocin in the mouse. Virchows Archiv A Pathol. *Anat Histopathol*, 412, 225-230, 1988.

11. Perez-Tomas R, Ballesta J, Pastor LM, Madrid JF, Polak JM: Comparative immunohistochemical study of the gastroenteropancreatic endocrine system of three reptiles. *Gen Comp Endocrinol*, 76, 171-191, 1989.

12. D'Este, L, Buffa R, Pelagi M, Siccardi AG, Renda T: Immuno-histochemical localization of chromogranin A and B in the endocrine cells of the alimentary tract of the gren frog, *Rana esculanta*. *Cell Tissue Res*, 277, 341-349, 1994.

13. Persson-Sjögren S, Forsgren S, Taljedal IB: Peptides and other neuronal markers in transplanted pancreatic islets. *Peptides*, 21, 741-52, 2000.

14. Camihort G, Del Zotto H, Gomez-Dumm CL, Gagliardino JJ: Quantitative ultrastructural changes induced by sucrose administration in the pancreatic B cells of normal hamsters. *Biocell*, 24, 31-37, 2000.

15. Ku SK, Lee HS, Lee JH: An immunohistochemical study on the pancreatic endocrine cells of the C57BL/6 mouse. *J Vet Sci*, 3, 327-33, 2002.

16. Lee HS, Chang JH, Ku SK: An immunohistochemical study of the pancreatic endocrine cells of the ddN Mouse. *Folia Histochem Cytobiol*, 48, 387-393, 2010.

17. Yukawa M, Takeuchi T, Watanabe T, Kitamura S: Proportions of various endocrine cells in the pancreatic islets of wood mice *(Apodemus speciosus)*. *Anat Histol Embryol*, 28, 13-16, 1999.

18. Reedy SN, Bibby NJ, Elliott RB: Cellular distribution of insulin, glucagon, pancreatic polypeptide hormone and somatostatin in the fetal and adult pancreas of guinea pig: A comparative immunohistochemical study. *Eur J Cell Biol*, 38, 301-305, 1985.

19. Ku SK, Lee HS, Park KD, Lee JH: An immunohistochemical study on the pancreatic islets cells of the Mongolian gerbils, *Meriones unguiculatus*. *J Vet Sci*, 2, 9-14, 2001.

20. Ouedraogo R, Naslund E, Kirchgessner AL: Glucose regulates the release of orexin-A from the endocrine pancreas. *Diabetes* 52, 111-117, 2003.

21. Kageyama H, Funahashi H, Hirayama M, Takenoya F, Kita T, Kato S, Sakurai J, Lee EY, Inoue S, Date Y, Nakazato M, Kangawa K, Shioda S: Morphological analysis of ghrelin and its receptor distribution in the rat pancreas. *Regul Pept*, 126, 67-71, 2005.

22. Wieczorek G, Pospischil A, Perentes EA: A comparative immuno-histochemical study of pancreatic islets in laboratory animals (rats, dogs, minipigs, nonhuman primates). *Exp Toxicol Pathol*, 50, 151-172, 1998.

23. Kirchgessner AL: Orexins in the brain-gut axis. *Endocr Rev,* 23, 1-15, 2002.

24. Walia P, Asadi A, Kieffer TJ, Johnson JD, Chanoine JP: Ontogeny of ghrelin, obestatin, preproghrelin, and prohormone convertases in rat pancreas and stomach *Pediatric Res*, 65, 39-44, 2009.

25. Ku SK, Lee HS: An immunohistochemical study of the pancreatic endocrine cells of the nude mouse, Balb/c-nu/nu. *Eur J Histochem* 50, 61-68, 2006.

26. Bayrakdar A, Yaman M, Atalar O, Tarakci Gencer B, Ceribasi S: Distribution of neuropeptides in endocrine and exocrine pancreas of long-legged buzzard *(Buteo rufinus)*: An immunohistochemical study. *Regul Peptides*, 166, 121-127, 2011.

27. Kocamıs H, Sarı EK, Nazlı M, Gulmez N, Aslan S, Deprem T: Immuno-histochemical distribution of ınsulin-, glucagon-, and somatostatin-containing cells in the pancreas of the rat *(Wistar albino)*. *Kafkas Univ Vet Fak Derg*, 15, 611-614, 2009.

28. Sternberger LA: The unlabelled antibody peroxidase-antiperoxidase (PAP) method, **In,** Sternberger LA (Ed): Immunocytochemistry. pp. 104-169, J. Wiley, Sons, New York, 1979.

29. Hsu WH, Crump MH: The endocrine pancreas. **In,** McDonald LE, Pineda MH (Eds): Veterinary Endocrinology and Reproduction. pp. 186-201, Lea and Febiger, Philadelphia, 1989.

30. Hsu SM, Raine L, Fanger H: Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) prosedures. *J Histochem Cytochem*, 29, 577-580, 1981.

31. Reedy SN, Bibby NJ, Fisher SL, Elliott RB: Immunolocalization

of insulin, glucagon, pancreatic polypeptide and somatostatin in the pancreatic islets of the possum, *Trichosorus vulpecula*. *Gen Comp Endocrinol*, 64, 157-162, 1986.

32. Sujatha SR, Pulimood A, Gunasekaran S: Comparative immunocytochemistry of isolated rat and monkey pancreatic islets cell types. *Indian J Med Res*, 119, 38-44, 2004.

33. Ku SK, Lee HS: Distribution and frequency of endocrine cells in the pancreas of the ddY mouse: An immunohistochemical study. *Eur J Histochem*, 49, 125-130, 2005.

34. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR: Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell*, 92, 573-585, 1998.

35. Heinonen MV, Purhonen AK, Makela KA, Herzig KH: Functions of orexins in peripheral tissues. *Acta Physiol (Oxf)*, 192, 471-85, 2008.

36. Dall'Aglio C, Pascucci L, Boiti C, Ceccarelli P: Immunohistochemical localization of the orexinic system in the sheep mandibular gland. **In,** *Abstracts of the LXIV Annual Meeting of the Italian Society for Veterinary Sciences.* 36, 2010.

37. Kirchgessner AL, Liu ML: Orexin synthesis and response in the gut. *Neuron*, 24, 941-951, 1999.

38. Nakabayashi M, Suzuki T, Takahashi K, Totsune K, Muramatsu Y, Kaneko C, Date F, Takeyama J, Darnel AD, Mariya T, Sasano H: Orexin-A expression in human peripheral tissues. *Mol Cell Endocrinol*, 205, 43-50, 2003.

39. Adeghate E, Hameed RS: Immunolocalization of orexin-1 receptor the pancreas of normal and diabetic rats. *Biogenic Amines*, 19, 347-355, 2005.

40. Adeghate E, Fernandez-Cabezudo M, Hameed R, El-Hasasna H, El Wasila M, Abbas T, al-Ramadi B: Orexin-1 receptor co-localizes with pancreatic hormones in islet cells and modulates the outcome of streptozotocin-induced diabetes mellitus. *PLoS One,* 5, e8587, 2010.

41. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, 402, 656-660,1999.

42. Dezaki K, Sone H, Yada T: Ghrelin is a physiological regulator of insulin release in pancreatic islets and glucose homeostasis. *Pharmacol Ther*, 118, 239-249, 2008.

43. Reimer MK, Pacini G, Ahren B: Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. *Endocrinology*, 144, 916-921, 2003.

44. Dezaki K, Hosoda H, Kakei M, Hashiguchi S, Watanabe M, Kangawa K, Yada T: Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca^{2+} signaling in β-cells. Implications in the glycemic control in rodents. *Diabetes*, 53, 3142-3151, 2004.

45. Heller RS, Jenny M, Collombat P, Mansouri A, Tomasetto C, Madsen OD, Mellitzer G, Gradwohl G, Serup P: Genetic determinants of pancreatic epsilon-cell development. *Dev Biol*, 286, 217-224, 2005.

46. Andralojc KM, Mercalli A, Nowak KW, Albarello L, Calcagno R, Luzi L, Bonifacio E, Piemonti L: Ghrelin-producing epsilon cells in the developing and adult human pancreas. *Diabetologia*, 52, 486-493, 2009.

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RESEARCH ARTICLE

Prevalence of Bovine Hypodermosis in Water Buffalo *(Bubalus bubalis)* from Jhelum District, Pakistan

Haroon AHMED [1] Mobushir Riaz KHAN [2] Rosario Panadero-FONTAN [3]
Ceferino López SANDEZ [3] Irfan MUSTAFA [4] Abdul GHANI [4] Munib HUSSAIN [5]
Saira ASIF [6] Aftab AHMAD [6] Syed Muhammad Saqlan NAQVI [6] Mazhar QAYYUM [6]

[1] Department of Biosciences,COMSATS Institute of Information Technology, Park Road, 46300, Islamabad, PAKISTAN
[2] Department of Meterology,COMSATS Institute of Information Technology, Park Road, 46300, Islamabad, PAKISTAN
[3] Departamento de Patología Animal: Sanidad Animal, Facultad de Veterinaria de Lugo, Universidad de Santiago de Compostela, 27071 Lugo, SPAIN
[4] Department of Biological Sciences, University of Sargodha, Sargodha, PAKISTAN
[5] Animal Health Institute, Animal Health Program, NARC, Islamabad, 46300, PAKISTAN
[6] PMAS Arid Agriculture University, Rawalpindi, 46300, PAKISTAN

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Summary

Hypodermosis is an endemic disease in semi-hilly and mountainous areas of Pakistan. Keeping in view the importance of buffaloes an epidemiological survey was conducted to determine the prevalence of hypodermosis in district Jhelum Punjab, Pakistan, during the year 2010-2011. Out of 1000 buffaloes examined clinically in the study area, 32 (3.2%) found to be positive for the warble fly infestation. The number of nodules in the infested animals ranged from 1-5 (2.7±1.1). There were significant differences ($P<0.05$) in the prevalence of *Hypoderma* spp. when the sex, age and different geographic areas were considered. The Prevalence was higher in males and young animals and also in those animals grazing in hilly and semi-hilly areas. The climatic conditions (temperature, humidity, sunshine and wind speed) favoured the warble fly activity and contributed in the onset of disease.

Keywords: *Hypodermosis, Prevalence, Water Buffalo, Jhelum, Pakistan*

Pakistan'ın Jhelum Bölgesindeki Su Mandalarında *(Bulbous bulbous)* Sığır Hypodermozis'inin Dağılımı

Özet

Hipodermozis Pakistanın dağlık ve tepelik bölgelerinde rastlanan endemik bir hastalıktır. Bu epidemiyolojik çalışma, Bufaloların önemi göz önünde bulundurularak, bu hayvanlarda hipodermozisin dağılımını ortaya koymak amacıyla 2010-2011 yıllarında Pakistan'ın Jhelum Punjab bölgesinde gerçekleştirilmiştir. Bu amaçla çalışma bölgesinde 1000'in üzerinde buffalo klinik olarak nokra sineği enfestasyonu yönünden kontrol edilmiş ve 32 (%3.2)'si pozitif bulunmuştur. Enfestasyon bulunan hayvanlardaki nodül sayısı 1-5 (2.7±1.1) arasında değişkenlik göstermiştir. Cinsiyet, yaş ve farklı coğrafik bölgeler yönünden değerlendirildiğinde Hypoderma dağılımında önemli farklılıklar belirlenmiştir. Hastalığın dağılımı erkek ve genç hayvanlarda ve aynı zamanda tepelik ve yarı-tepelik bölgelerde otlayan hayvanlarda daha yüksek bulunmuştur. İklimle ilgili faktörler (sıcaklık, nem, güneş, rüzgar hızı) nokra sineği aktivitesini artırmakta ve hastalığın başlamasında rol oynamaktadır.

Anahtar sözcükler: *Hipodermosis, Dağılım, Su Mandası, Jhelum, Pakistan*

INTRODUCTION

Pakistan is an agricultural country with semi arid landscape and subtropical climate. Most of the people earn their livelihood from selling agro-livestock products and rearing of livestock (cattle, sheep, goats and buffaloes). The productivity in the livestock sector is low due to several abiotic and biotic factors influencing productive potential of domesticated

İletişim (Correspondence)
+09251 9290267
haroonahmad12@yahoo.com

animals. One of these factors is the prevalence of parasitic infections, which plays a vital role in low livestock productivity.

Hypodermosis is a parasitic disease caused by a parasite commonly known as Warble Fly, belongs to genus *Hypoderma* (Diptera: Oestridae). Each species of this genus is strictly a parasite of a ruminant species. *H. diana* is specific to deer, *H. tarandi* to reindeer, *H. bovis* and *H. lineatum* to cattle, Buffalo [1].

Hypodermosis is widely distributed in all over the Northern hemisphere from Europe [2] to Eastern China [3]. The prevalence of hypodermosis was 41.9% in eastern region and 47.8% in southern region of Turkey [4]. Hypodermosis is a notorious and common disease of cattle, buffaloes and goats in Pakistan [5]. The prevalence of hypodermosis was 22-24% in cattle of different endemic areas due to the *Hypoderma* spp [6]. This myiasis is endemic in the semi-hilly, mountainous areas of the country [7]. The fly's egg laying season in different areas occurs from February-June. Warbles on the back of infested animals are generally recorded from November to January. Third instars (L_3) complete their development and fall to the ground by mid January.

Hypodermosis can be the cause of economic losses due to meat trim at slaughter, and the effect on hides is well established [8].The Prevalence of warble fly infestation has been 18.40% reported from four districts of Northern Punjab, Pakistan [9] and was 31.9% in kars province, Turkey [10]. The cattle and buffalo were equally exposed to hypodermosis [11]. The prevalence of hypodermosis in buffalo of Chakwal district was 5.20% [12].

Keeping in view the importance of buffaloes an epidemiological survey was conducted to find the prevalence of hypodermosis in different areas of district Jhelum (Pakistan). Individual factors (age, sex) affecting the infection by *Hypoderma* spp. in those hosts have been also studied.

MATERIAL and METHODS

Location and Sample Size

The present study was conducted in Jhelum district (32° 56'23"N 73°43'11"E) of Punjab, Pakistan *(Fig. 1)*. Jhelum is a city on the right bank of the Jhelum River, in the district of the same name in the north of Punjab province. The agriculture activities in the district Jhelum depends mainly on rainfall. The average rainfall of the area varies from 508-1.016 mm. The maximum mean temperature in summer (June to September) is recorded as 45.7°C (June), where in winter (October to February) the minimum temperature as recorded is 1.8°C (January). Average annual rainfall is about 900 mm [13].

From September 2010 to February 2011, a total of one thousand buffalo belonging to 16 herds from four different villages of Jhelum district were examined for the presence of hypodermosis. All the animals of Purana Metha, Deena Bypass, Hadali and Stadpur were examined on monthly basis. The prevalence was determined by the hand palpation method (by examining the nodules on all parts of body) *(Fig. 2)*.

Factors Considered in Risk Analysis

Sex and age of the animals were recorded. Three age groups were created: (1) including calves and yearlings (< 37 months), (2) integrated by sub-adults (37-72 months) and adults (> 72 months). In village Purana Metha, Hadali, Stad pur and Deena Bypass the number of examined female were 242, 235, 256 and 176, respectively. Similarly, the male were 40, 16, 13 and 22, respectively.

Since geographical variations could affect the development of pupae to adult, fly activity and the subsequent infection levels in the host population, the animals were also grouped by the village of precedence *(Table 1)*: Purana Metha, Hadali,

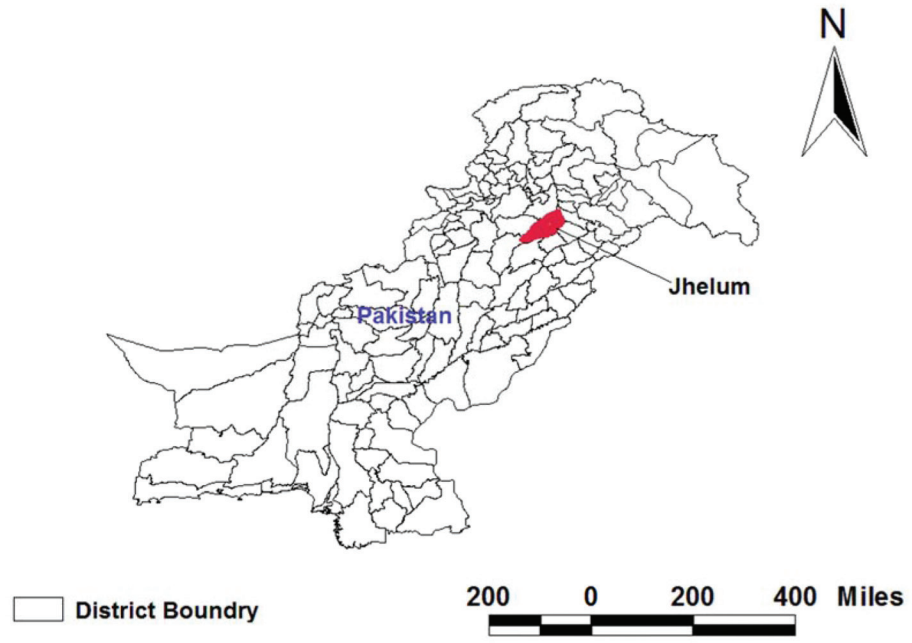

Fig 1. Map of Pakistan showing the location of the district Jehlum in the Northern Punjab, Pakistan

Şekil 1. Pakistanın kuzey Punjab (Jhelum) bölgesinin haritadaki yeri

Table 1. *Age and village wise prevalence of Hypodermosis in water buffalo in Northern Punjab (District Jhelum), Pakistan*
Tablo 1. *Pakistan'ın Kuzey Punjab (Jhelum) bölgesindeki su mandalarında Hipodermozisin yaşa ve köylere göre dağılımı*

Village	Age						Total No of Observed Animals	No of Infested Animals	Overall Prevalence (% age)
	<1-3		4-6		7-9<				
	Nº	Inf	Nº	Inf	Nº	Inf			
Purana Metha	55	17	23	6	204	-	282	23	8.15%
Hadali	50	2	14	7	187	-	251	9	3.58%
Stad por	36	-	18	-	215	-	269	0	0%
Deena Road (Bypas)	41	-	22	-	135	-	198	0	0%
Total	182	19	77	13	741	-	1000	32	3.2%

Nº *= Non Infested,* ***Inf*** *= Infested*

Fig 2. Nodules were detected by manual palpation
Şekil 2. Palpasyon ile tespit edilen nodüller

Fig 3. Warble on the back of a water buffalo from Northern Punjab, Pakistan
Şekil 3. Pakistanın kuzey Punjab bölgesindeki bir su mandasının sırt kısmında belirlenen nokra

Stad por and Deena Road (Bypas).

The information on the treatment procedure (Anti-parasitic) of animals against hypodermosis in the studied herds was determined in this study.

Statistical Analysis

The risk of being infected by *Hypoderma* larvae was evaluated with a data mining classification tree [14], taking into account the factors previously indicated. Particularly, an exhaustive Chi-squared automatic interaction detector (exhaustive CHAID) as described in Lopez [15] was applied. Buffaloes were classified as positive (larvae detected in palpation) or negative (no larvae detected) and CHAID algorithm identified variables that divide buffaloes in subgroups (tree nodes) with different positive/negative ratio. CHAID provided a way to identify major factors using as criteria the significance of a Chi-squared test and successively splitting data in increasingly homogeneous nodes in relation to dependent variable (larvae detection) until the classification tree was fully grown.

Statistical analyses were performed with statistical package SPSS for Windows 18.0 and SPSS answer Tree 3.1 (SPSS Inc., Chicago, IL USA).

RESULTS

Out of one thousand buffalo, 32 (3.2%; 95% CI 2.2-4.5) were found to be infested by *Hypoderma* spp. The number of nodules in the infested animals ranged from 1-5 (2.7±1.1). The nodules were observed on the back, hump and flank *(Fig. 3)*.

The warble started to appear by the end of September and skin perforation started from end of October to December. The larvae collected from infested buffaloes were identified as *Hypoderma lineatum* according to Zumpt [16]. This is the first report of *Hypoderma lineatum* in the buffalo of Jhelum district.

The CHAID analysis indicates that age was the most determining factor in hypodermosis prevalence *(Table 1; Fig. 4)*. The age of infested buffalo ranged from 1-6 years, whereas, 7-9 yr-old buffaloes were not infested in the present study. In the node 1, comprised by 1-6-yr-old buffaloes, village of precedence was detected as an influencing factor in hypodermosis prevalence. In Purana Metha village (node 3), 23 out of 81 buffaloes with less than 73 months of age were infested with hypodermosis (28.4% prevalence). In village Hadali, (node 4) nine were infested with hypodermosis (14.3%

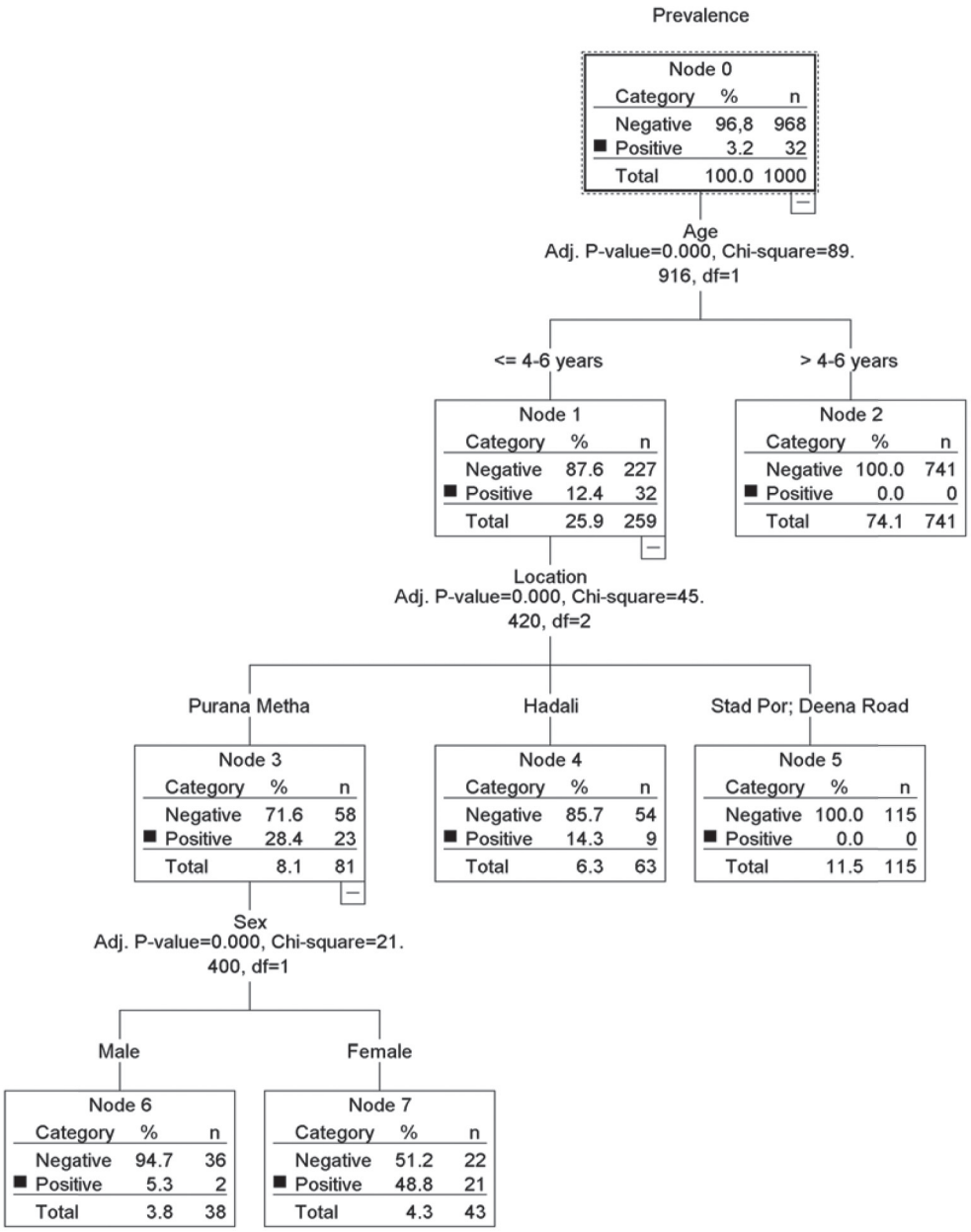

Fig 4. Statistical analysis

Şekil 4. İstatistiksel analizler

Prevalence in young buffalos). In village Stadpor and Deena Road (Bypass) no animals were infested by *Hypoderma* (Node 5). Finally, hypodermosis prevalence in buffaloes from Purana Metha buffaloes was influenced by sex. Female showed higher prevalence (48.8%, node 7) than male (5.3%, node 6).

In this study a total of sixteen herds were examined from different villages of district Jhelum and the WFI was only found in six herds *(Table 2)*.

In the present study, the 3.08% (28/881) female were infested with hypodermosis, while in male the prevalence was 4.39% (4/87), which shows higher prevalence in male than female *(Table 3)*.

Taramira oil *(Eruca sativa)* is commonly known as jamba oil. The oil is extracted from seeds. The taramera oil was used in non- infested buffalo herds on monthly basis as an anti-parasitic that might be very useful for control of hypodermosis in the study area. The opinion of these buffalo farmers is to use this oil as a local control. Ivermectin were also used in some herds along this local treatment.

DISCUSSION

In the present study the prevalence of hypodermosis in water buffalo from Northern Punjab was low (3.2%). A similar percentage (5.2%) of buffaloes was infested with hypodermosis from field in Chakwal [12]. However, the prevalence was much lower as eleven buffalo were infested out of thirty thousand examined buffalos from Dera Ghazi Khan [11], 10.04% in Jhelum district (Pakistan) [9], 31.9% in Kars province (Turkey) [10] and 41.9% in eastern Region (Turkey) [4].

Table 2. *Herd-level prevalence by Hypoderma in water buffalo from different villages of Northern Punjab (District Jehlum), Pakistan*
Tablo 2. *Pakistan'ın Kuzey Punjab (Jhelum) bölgesindeki su mandalarında Hypodermozisin farklı köylerde sürü bazındaki dağılımı*

Village	Grazing Pattern	Herd Name	No of Individual Examined/Herd	No of Individual Infested/Herd	Herd-level Prevalence (%)
Purana Metha	Field & Hilly	A	95	10	10.52%
		B	51	3	5.79%
		C	67	5	7.42%
		D	39	-	0%
		E	30	5	16.66%
Hadali	Field & Hilly	F	104	7	6.73%
		G	76	2	2.63%
		H	71	-	0%
Stad por	Non Hilly	I	121	-	0%
		J	47	-	0%
		K	24	-	0%
		L	77	-	0%
Deena Road(Bypas)	Non Hilly	M	51	-	0%
		N	36	-	0%
		O	89	-	0%
		P	22	-	0%
Total		**16**	**1000**	**32**	**3.2%**

Table 3. *Sex based prevalence by Hypoderma in Water Buffalo from different villages of Northern Punjab (District Jehlum), Pakistan*
Tablo 3. *Pakistan'ın Kuzey Punjab (Jhelum) bölgesindeki su mandalarında Hypodermozisin farklı köylerde cinsiyete göre dağılımı*

S. No	Village Name	Sex					
		Female			Male		
		Total No of Observed Animals	No of Infested Animals	Prevalence (%)	Total No of Observed Animals	No of Infested Animals	Prevalence (%)
1	Purana Metha	242	21	8.67%	40	2	5%
2	Hadali	235	7	2.97%	16	2	12.5%
3	Stadpur	256	0	0%	13	0	0%
4	Deena Bypass	176	0	0%	22	0	0%
Grand Total	**4**	**909**	**28**	**3.08%**	**91**	**4**	**4.39%**

The statistical analysis showed that there is a significant difference in the prevalence of hypodermosis between the different age groups, sex and village basis. The animals having age between 1-6 years were more infested with hypodermosis as compared to the animals having 7-9 age groups. While there is no difference age is found between the animals having 1-3 and 4-6 years age groups *(Fig. 4)*.

Those results agree with those of higher prevalence of WFI in young animals could be due to their softer skin, which facilitates the penetration of first instars of *Hypoderma* as reported earlier [17]. The prevalence of WFI was higher in the field young vs old animals in both the districts [18]. Intrinsic host determinants, however, may also contribute towards lower prevalence of WFI in older animals. It may be due to thicker skin of aged animals not allowing penetration of larvae, suppression of the developing larvae by internal regulatory systems of the host and development of resistance by continuous exposure of animals to larvae. Similar trends of age-wise prevalence of WFI have also been reported by Pruett and Kunz [19] and Papadopoulos [20].

When the village wise prevalence was considered, we found that the animals in village Purana Metha (8.15%) were significantly more infested followed by village Hadali (3.58%) as compared to all other two villages due to the suitable conditions. So in the present study the statistical analysis shows that there is a significant difference in the prevalence of buffalo hypodermosis in different villages of jhelum district. Similarly among all the villages, the village Purana Metha has more risks for hypodermosis due to the different biotic and abiotic factors (Hilly location) and animals grazing practices.

In the present study, the male animals were more infested with hypodermosis as compared to female due to the grazing practices and male were kept tied at home *(Table 3)*. Our results correlates as high prevalence due to the grazing pattern [21] as under grazing system are male were more proned to infestation, because they were kept tied [22]. Similarly, the prevalence of hypodermosis in male buffalo was higher then female [12]. The prevalence of hypodermosis was higher in male (26.50%) than in females (20.50 %) in Chakwal district, Pakistan [18].

The prevalence of WFI is higher in males then females due to the physiological differences between the two genders and the management practices in the area. The females are grazed in the study area and the males are kept tied. Hence, males are more prone to infestation than females which can run away from the attacking flies [7,22]. Herd-wise prevalence in the village Purana Metha is higher due to the suitable conditions (Temperature, Humidity, and Wind-speed) and grazing pattern.

It is concluded from the present study that the prevalence of hypodermosis is low, but in future it may increase and cause the economic losses in Pakistan. It has as important impact on animal health, behaviour, milk and leather industry. So it is very important to explore this disease in other agro-ecological areas of Pakistan. This study is very useful in determining the prevalence of hypodermosis in buffalo of Jhelum district, Pakistan.

REFRENCES

1. Boulard C: Durably controlling bovine hypodermosis. *Vet Res*, 33, 455-464, 2002.

2. Brien DJ: 1998. Warble fly prevalence in Europe, COST 811, 1997.

3. Yin H, Miling H, Gailing Y, Haung S, Zheijie L, Jiaxutn L, Guiquan G: Hypodermosis in China. *Proceedings of INCO Meeting Held in Lanzhou* (China), 2001.

4. Sayin F, Kalkan A, Karaer Z: Epidemiological studies on cattle hypodermosis in Turkey. *F.U. Sağlık Bil Derg,* 14, 115-127, 2000.

5. Khan MQ: Cattle hypodermosis in Pakistan. *Chin J Vet Parasitol*, 12, 31-37, 2004.

6. Ayaz MM: Prevalence and treatment of goat warbles in Fort Munro and akni, Pakistan. *Pak Vet J*, 18, 162-164, 1979.

7. Khan MQ, Afzal M, Khan MI: Efficacy of ivermectin against cattle warbles in Pakistan. *Acta Parasitol*, 42, 173-175, 1997.

8. Hall M, Wall R: Myiasis of humans and domestic animals. *Advan in Parasitol,* 35, 257-334, 1995.

9. Ahmed H, Khan MR, Fontan RP, Sandez CL, Iqbal MF, Naqvi SMS, Qayyum M: Geographical distribution of Hypodermosis (*Hypoderma* sp.) in Northern Punjab, Pakistan. *Kafkas Univ Vet Fak Derg*, 18 (Suppl-A): A215-A219, 2012.

10. Kara M, Arsalan MO, Gicik Y: The prevalence of bovine hypodermosis in kars province. *Trop Anim Health Prod*, 37, 617-622, 2005.

11. Ayaz MM, Anwer M: Hypodermatosis in Buffaloes of Pakistan. *On line J Biol Sci*, 2, 204, 2002, http://198.170.104.138/jbs/2002/204.pdf, 2002.

12. Hasan M, Khan MN, Mushtaq M, Iqbal Z, Farooq AA, Sajid MS, Waheed HM: Prevalence of warble fly infestation in Buffalo in Chakwal, Punjab. *Ital J Anim Sci*, 6 (2): 942-944, 2007.

13. Annonymous: Government of Pakistan. Economic survey of Pakistan, Economic Advisor s Wing, Finance Division, Islamabad, 2009-2010.

14. Kass G: An exploratory technique for investigating large quantities of categorical data. *Appl Stat*, 29, 119-127, 1980.

15. Lopez C, Fernandez M, Cienfuegos M, Panadero R, Vázquez L, Díaz P, Pato J, Lago N, Dacal V, Díez-Baños P, Morrondo P: Protostrongylid infection in meat sheep from Northwestern Spain: prevalence and risk factors. *Vet Parasitol,* 31, 108-114, 2011.

16. Zumpt F: Myasis in Man and Animals in the Old World. Butterworth & Co, 266, 1965.

17. Scholl PJ, Schwinghammer KA, Chamberlain WF: Technique for age-grading late third-instar *Hypoderma lineatum* (Diptera: Oestridae). *J O Econom Entomol,* 26, 230-233, 1989.

18. Khan MN, Iqbal Z, Sajid MS, Anwar M, Needham GR, Hassan M: Bovine hypodermosis: Prevalence and economic significance in southern Punjab, Pakistan. *Vet Parasitol*, 141, 386-390, 2006.

19. Pruett JH, Kunz SE: Thermal requirements for *Hypoderma lineatum* (Diptera: Oestridae) egg development. *J O Med Entomol*, 33, 976-978, 1996.

20. Papadopoulos E: Hypodermosis in Greece. *Chin J Vet Parasitol*, 12, 20-23. 2004.

21. Scholl PJ, Weintraub J: Gonadotropin development in *Hypoderma lineatum* and *H. bovis* with notes on reproductive capacity. *Ann Entomol Soci O Ammer*, 81, 318-324, 1988.

22. Scholl PJ, Schwinghammer KA, Chamberlain WF: Technique for age-grading late third-instar *Hypoderma lineatum* (Diptera: Oestridae). *J O Eco Entomol,* 26, 230-233, 1989.

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RESEARCH ARTICLE

The Effect of Canine Ovariohysterectomy on HSP70 and Anti-HSP70 Antibodies [1]

Murat YÜKSEL * Nevzat SAAT ** Ali RIŞVANLI **

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* Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, University of Cumhuriyet, TR-58100 Sivas - TURKEY

** Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, University of Firat, TR-23119 Elazig - TURKEY

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Summary

The present study aimed to determine the effects of ovariohysterectomy on blood serum concentrations of Heat Shock Protein (HSP) 70 and anti-HSP70 antibodies in dogs. For this purpose, 87 female stray dogs were used as the materials. Ten milliliters of blood was taken from the animals preoperatively and on the first day postoperatively. The concentration of extracellular HSP70 and anti-HSP70 antibodies in blood serums were measured by using commercial ELISA kits. Subsequently, the preoperatively and postoperatively obtained data were compared. As a result, the mean +/- SD concentration of HSP70 (4.86±0.99 ng/mL) and anti-HSP70 (109.77±16.64 ng/mL) antibodies in the dogs' blood samples taken after ovariohysterectomy were found to be significantly lower when compared to the preoperative amounts.

Keywords: *HSP70, Anti-HSP70, Ovariohysterectomy, Dog*

Köpeklerde Ovaryohisterektomi'nin HSP70 ve Anti-HSP70 Antikorları Üzerine Etkisi

Özet

Bu çalışmada köpeklerde kan serumu Heat Shock Protein (HSP) 70 ve anti-HSP 70 antikorlarının düzeyleri üzerine ovaryohisterektominin etkilerinin belirlenmesi amaçlanmıştır. Bu amaçla, 87 adet dişi sokak köpeği materyal olarak kullanıldı. Hayvanlardan operasyon öncesi ve sonrası 1. gün 10 ml kan örnekleri alındı. Kan serumlarındaki hücre dışı HSP70 ve anti-HSP70 antikorlarının miktarları ticari ELISA kitleri kullanılarak ölçüldü. Daha sonra operasyon öncesi ve sonrası elde edilen verilerin istatistikî analizleri yapıldı. Sonuç olarak; köpeklerde ovaryohisterektomi sonrası alınan kan numunelerindeki HSP70 (4.86±0.99 ng/ml) ve anti-HSP70 (109.77±16.64 ng/ml) antikorlarının miktarlarının operasyon öncesine göre daha düşük olduğu tespit edildi.

Anahtar sözcükler: *HSP70, Anti-HSP70, Ovaryohisterektomi, Köpek*

INTRODUCTION

Ovariohysterectomy is a surgical operation frequently used to control reproduction dogs. Following the operation, some complications may develop in dogs such as obesity, cardiac stress, urinary incontinence, hair loss, and change of temper, infection of the operation site, peritonitis, colonic and urethral obstructions developing due to peritoneal adhesions, stump pyometra, and ovarian remnant syndrome [1]. Although one can take preoperative and postoperative precautions to prevent such complications, there is always a possibility that they may develop. Therefore, there is a need for novel research about the causes of and solutions for such complications and postoperative development of infections in particular. At this point, correct assessment of the state of a dog's immune system prior to and following ovariohysterectomy is important to prevent complications or at least, to identify their causes.

İletişim (Correspondence)
+90 424 2370000
arisvanli@firat.edu.tr

Recent years have witnessed an increase in the number of studies on HSPs in dogs as well as in other living organisms. Various publications have shed light on the relationship of these proteins with the immune system [2,3].

Heat Shock Proteins enhance a cell's resistance to stress. In cases of cell damage occurring under stressful conditions, there is an increase in the amount of these proteins, which serve in the process of renewing damaged cells [4,5]. The amount of HSPs increases due to high temperature, fever, inflammation, ischemia, hypertrophy, cellular damage, diseases like cancer, and cellular interactions [4,6].

Heat Shock Proteins are molecules that help to many antigens induce an immune response in the host. Moreover, these proteins are also believed to assist the formation of molecules that allow the immune system to identify diseased or normal cells. Appearing as a result of immune responses against these proteins, anti-HSP70 antibodies may also cause the development of a reaction against the cell itself. These antibodies may also cause autoimmune diseases [7,8].

Heat Shock Proteins are grouped in five according to their molecular weight, structure, and functions. These groups include HSP100, HSP90, HSP70, HSP60, and small heat shock proteins [4]. Heat Shock Protein 70 functions in intracellular protein delivery. It protects stressed proteins, prevents the aggregation of unfolded proteins, and binds polypeptides. This molecule is found at tissue level as well as in serum [4,9].

Intracellular HSP70 has a cell protective effect and serves in the development of ischemic tolerance in tissues like the brain [10,11]. It has been shown that in cases which induce sublethal cellular stress such as hypothermia or ischemia, intracellular HSP70 plays a significant role in rats, rabbits, mice, and dogs [12].

In this study was aimed to determine the effects of ovariohysterectomy on blood serum levels of HSP 70 and anti-HSP 70 antibodies in dogs.

MATERIAL and METHODS

The materials used in this project included 87 healthy female stray animals of various breeds and at varying ages (8 months - 6 years, nonpregnant) brought for ovario-hysterectomy to the Small Animals Clinic in the Animal Hospital at the Veterinary Faculty of Firat University. An Ethical Board Report was obtained from the Ethical Board for Experimental Animals at Firat University to conduct the study (2011/3-51).

Xylazine (0.1-0.8 mg/kg IV)/Ketamin HCl (7-10 mg/kg, IM) anesthesia was performed for ovariohysterectomy. Following a mid-line incision, the ovaries and uterus were removed. As a preventive measure against following surgery complications, a parenteral antibiotic (Enrofloxacin, 2.5 mg/kg IM) was administered. The analgesic drug (Meloxicam, 0.2 mg/kg IM) was used postoperative.

10 ml of blood was taken from the animals preoperatively and on the first day postoperatively (after 24 h). The blood samples used in the study has been not taken especially for this study. Also, the blood samples were used from the examples that routinely taken before and after operations. This application has to reveal for the hematological status of animals. The blood samples were taken from anesthetized animals before the operation. Following routine procedures, the blood samples were separated into serums, which were kept at -80°C until the measurements.

Commercial ELISA kits were used to measure the amounts of extracellular HSP70 (Uscn Life Sciences, PRC) and anti-HSP70 (Enzo Life Sciences, USA) antibodies in the blood serums [13,14]. Next, the pre- and postoperatively-obtained data were subjected to statistical analyses for comparison.

The statistical analyses were performed by using the Wilcoxon test in SPSS 11.5 software.

RESULTS

No complications occurred in animals after operations. *Table 1* summarizes the data obtained from blood the samples collected pre- and postoperatively. Thus, the amounts of HSP70 (4.86±0.99 ng/mL) and anti-HSP70 (109.77±16.64 ng/mL) in the blood serums after the ovariohysterectomy were found to be lower when compared to preoperative amounts (7.22±1.30 ng/mL, 143.67±20.81 ng/mL) ($P<0.01$).

Table 1. *Anti-HSP70 and HSP70 concentrations before and after operation.*
Tablo 1. *Operasyon öncesi ve sonrası Anti-HSP70 ve HSP70 konsantrasyonları*

Parameter	Preoperation (n=87)	Postoperation (n=87)	P
HSP70 (ng/mL)	7.22±1.30[a]	4.86±0.99[b]	*
Anti-HSP70 (ng/mL)	143.67±20.81[a]	109.77±16.64[b]	*

** $P<0.01$, [ab] The difference between different letter carrying averages is significant*

DISCUSSION

Increased HSP concentrations in cells exposed to stress are observed as being protective against possible future damages [15]. Under physiologic conditions, HSPs play roles in important activities for cell functions. Among them, delivery and configuration of newly-synthesized proteins occupy a significant place. HSPs have crucial roles in ensuring the survival of cells and their protection against harmful effects under hyperthermia or other (oxidative, toxic, osmotic, hypothermic, etc.) stress types and HSP70 is an important HSP that is increased under the abovementioned stress conditions [16,17]. Heat Shock Protein 70 has a direct role in protection against ischemia-reperfusion damages [18,19]. The amounts of HSP70-related proteins and mRNA are known to

increase in dogs following ischemias developing in organs like the heart. In such cases, HSP70 has been proposed to provide protection against ischemic damages [19,20].

Canine HSP70 gene has a 90-95 percent of sequence similarity to bovine, human, and mouse HSP70 proteins [21]. Accordingly, in the present study, concentrations of canine HSP70 and anti-HSP70 antibodies were measured by using human ELISA kits.

Heat Shock Proteins are immunogenic [22]. Therefore, antibodies developing against HSPs were identified in the blood serums of normal individuals [23,24]. Anti-HSP70 antibodies are known to be autoantibodies naturally found in the blood circulation of healthy pregnant women. However, it is also suggested that further research should be conducted to investigate the relationship of normal pregnancy and the immunobiology of preeclampsia with the antibodies against HSPs [25].

Oxidative stress is increased during and following surgical operations depending on reactive oxygen species and related cytokines, leading to the activation of Heat Shock reactions, which results in an increase in HSPs depending on the intensity of stimulation [26]. Yet, following such interventions, different cases may also occur due to the negative feedback responses of Heat Shock reactions, regardless of systemic inflammatory response. Accordingly, a significant postoperative decrease in HSP70 has been reported in humans following serious surgical interventions. It is also reported that after such operations, HSP70 concentration rapidly declines to the lowest levels during the postoperative period; therefore, HSP70 can be used as a postoperative prognostic marker. Similarly, a decreased amount of postoperative intracellular HSP70 is in parallel to the decrease in the amount of autoantibodies in blood circulation [27]. In this study, we also found that the dogs had lower amounts of HSP70 and anti-HSP70 in their blood serums following ovariohysterectomy when compared to the preoperative amounts.

Following acute ischemias, the amounts of anti-HSP70 antibodies also increase due to increased amounts of HSP70. Greater amounts of Heat Shock Protein 70 proteins in blood circulation may also cause an increase in the amounts anti-HSP70 antibodies [28].

Leng et al.[29] reported that the rates of anti-HSP70 antibodies increased in atherosclerosis-induced rats. Quijada et al.[30] argued that the amount of anti-HSP70 antibodies does not increase during canine Leishmania infections.

It has been reported that ovariectomy in rats significantly elevated HSP70 concentrations in left ventricular cell lysates [31]. However, this view has been rejected in some studies. For instance, the researchers in a study observed a significant down-regulation of HSP72 in the heart muscles of ovariectomized rats [32]. Similarly, ovariectomy in aged mice has been claimed to lower the level of HSP70 proteins in the brain, when compared to younger animals. This study reported that estrogen deficiency due to aging down-regulates brain HSP70 expression and that this hormone has significant effects upon the regulation of HSP release in the brain [33]. In the present study, we also found that the amounts of HSP70 (4.86±0.99 ng/ml) and anti-HSP70 (109.77±16.64 ng/ml) in canine blood serum following ovariohysterectomy were lower than the preoperative levels *(Table 1)*.

In the light of the study's results, the amounts of HSP70 and thus anti-HSP70 antibodies in canine blood serum following ovariohysterectomy were found to be lower than the preoperative amounts. The relationship of HSPs with immune system has been shown in dogs as well as other species. This has led to the hypothesis that ovariohysterectomy reduces HSP70 concentrations in dogs, which weakens the immune system and precipitates the development of various postoperative complications. Nevertheless, this conclusion needs to be confirmed by further studies.

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None of the authors have any conflict of interest to declare. All listed authors have made substantial contributions to the research design, analysis and interpretation of data; and to drafting the paper and revising it critically.

REFERENCES

1. Stone EA: Ovary and uterus. **In,** Slatter DH (Ed): Small Animal Surgery. 3rd ed., pp. 1487-1502, WB Saunders, Philadelphia, 2002.

2. Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H: HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *J Biol Chem*, 277, 15107-15112, 2002.

3. van Eden W, van der Zee R, Prakken B: Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol,* 5, 318-330, 2005.

4. Askar TK, Ergun N, Turunc V: Heat shock proteins and their physiological roles. *Kafkas Univ Vet Fak Derg,* 13, 109-114, 2007.

5. Dillmann WH: Heat shock proteins and protection against ischemic injury. *Infect Dis Obstet Gynecol,* 7, 55-71, 1999.

6. Kregel K: Molecular biology of thermoregulation: Invited review: Heat shock proteins: Modifying factors in physiological stress responses and acquired thermotolerance. *J Appl Physiol,* 92, 2177-2186, 2002.

7. Kimura F, Itoh H, Ambiru S, Shimizu H, Togawa A, Yoshidome H, Ohtsuka M, Shimamura F, Kati A, Nukui Y, Miyazaki, M: Circulating heat-shock protein 70 is associated with postoperative infection and organ dysfunction after liver resection. *Am J Surg,* 187, 777-784, 2004.

8. Lancaster G, Febbraio M: Mechanisms of stress-induced cellular HSP72 release: Implications for exercise-induced increases in extracellular HSP72. Exerc *Immunol Rev,* 11, 46-52, 2005.

9. Pockley AG, Wu R, Lemne C, Kiessling R, de Faire U, Frostegård J: Circulating heat shock protein 60 is associated with early cardiovascular disease. *Hypertension,* 36, 303-307, 2000.

10. Kelly S, Bieneman A, Horsburgh K, Hughes D, Sofroniew MV, McCulloch J, Uney JB: Targeting expression of hsp70i to discrete neuronal populations using the Lmo-1 promoter: Assessment of the neuroprotective effects of hsp70i *in vivo* and *in vitro. J Cereb Blood Flow Metab,* 21, 972-981, 2001.

11. Calderwood SK, Theriault JR, Gong J: Message in a bottle: Role of the 70-kDa heat shock protein family in anti-tumor immunity. *Eur J Immunol* 35, 2518-2527, 2005.

12. Cizkova D, Carmel JB, Yamamoto K, Kakinohana O, Sun D, Hart RP, Marsala M: Characterization of spinal HSP72 induction and development of ischemic tolerance after spinal ischemia in rats. *Exp Neurol,* 185, 97-108 2004.

13. Oglesbee MJ, Herdman AV, Passmore GG, Hoffman WH: Diabetic ketoacidosis increases extracellular levels of the major inducible 70-kDa heat shock protein. *Clin Biochem,* 38, 900-904, 2005.

14. Awad H, Suntres Z, Heijmans J, Smeak D, Bergdall-Costell V, Christofi FL, Magro C, Oglesbee M: Intracellular and extracellular expression of the major inducible 70kDa heat shock protein in experimental ischemia-reperfusion injury of the spinal cord. *Exp Neurol,* 212, 275-284, 2008.

15. Kapron-Bras CM, Hales BF: Heat-shock induced tolerance to the embryotoxic effects of hyperthermia and cadmium in mouse embryos in vitro. *Teratology,* 43, 83-94, 1991.

16. Levy R: Genetic regulation of preimplantation embryo survival. *Int Rev Cytol,* 210, 1-37, 2001.

17. Soti C, Nagy E, Giricz Z, Vigh L, Csermely P, Ferdinandy P: Heat shock proteins as emerging therapeutic targets. *Br J Pharmacol,* 146, 769-780, 2005.

18. Marber MS, Mestril R, Chi SH, Sayen MR, Yellon DM, et al: Overexpression of the rat inducible 70 kDa heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J Clin Invest,* 95, 1446-1456, 1995.

19. Dillmann WH, Mehta HB, Barrieux A, Guth BD, Neelcy WE, Ross J: Ischemia of the dog heart induces the appearance of a cardiac mRNA coding for a protein with migration characteristics similar to heat shock/stress protein 71. *Circ Res,* 59, 110-114, 1986.

20. Mehta HB, Popovich BK, Dillmann WH: Ischcmia induces changes in the level of mRNAs coding for stres protein 71 and crcatinc kinases M. *Circ Res,* 63, 512-517, 1988.

21. Kano R, Abe K, Hasegawa A: cDNA of canine heat shock protein 70 (HSP70). *Vet Res Commun,* 28, 395-405, 2004.

22. Singh-Jasuja H, Hilf N, Arnold-Schild D, Schild H: The role of heat shock proteins and their receptors in the activation of the immune system. *Biol Chem,* 382, 629-636, 2001.

23. Pockley AG, Shepherd J, Corton JM: Detection of heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal individuals. *Immunol Invest,* 27, 367-377, 1998.

24. Rea IM, McNerlan S, Pockley AG. Serum heat shock protein and anti-heat shock protein antibody levels in aging. *Exp Gerontol,* 36, 341-352, 2001.

25. Molvarec A, Derzsy Z, Kocsis J, Boze T, Nagy B, Balogh K, Makó V, Cervenak L, Mézes M, Karádi I, Prohászka Z, Rigó J: Circulating anti-heat-shock-protein antibodies in normal pregnancy and preeclampsia. *Cell Stress Chaperon,* 4, 491-498, 2009.

26. Ramos R, Dulin E, Sánchez MJ, Guisasola MC: Heat shock proteins 70 in surgical stress: Thoracotomy vs. herniorrhaphy. *Cytokine,* 48, 46-90, 2009.

27. Fernández RR, Zulueta MCG: 70 kDa heat-shock proteins in surgical stress: thoracotomy vs. herniorrhaphy. *Cir Esp,* 88, 23-29, 2010.

28. Vogt S, Portig I, Kusch B, Pankuweit S, Sirat AS, Troitzsch D, Maisch B, Moosdorf R: Detection of anti-hsp70 immunoglobulin G antibodies indicates better outcome in coronary artery bypass grafting patients suffering from severe preoperative angina. *Ann Thorac Surg,* 78, 883-889, 2004.

29. Leng X, Zhan R, Wang XX, Lu XH, Wang LQ, Gao XJ, Qian LJ: Level and effect of anti-HSP70 antibody changed in the procession of rat atherosclerosis. *Zhongguo Ying Yong Sheng Li Xue Za Zhi,* 25, 450-454, 2009.

30. Quijada L, Requena JM, Soto M, Alonso C: During canine viscerocutaneous leishmaniasis the anti-Hsp70 antibodies are specifically elicited by the parasite protein. *Parasitol,* 112, 277-284, 1996.

31. Bhuiyan S, Shioda N, Fukunaga K: Ovariectomy augments pressure overload-induced hypertrophy associated with changes in Akt and nitric oxide synthase signaling pathways in female rats. *Am J Physiol Endocrinol Metab,* 293, 1606-16014, 2007.

32. Bupha-Intr T, Wattanapermpool J: Cardioprotective effects of exercise training on myofilament calcium activation in ovariectomized rats. *J Appl Physiol,* 96, 1755-1760, 2004.

33. Hou Y, Wei H, Luo Y, Liu G: Modulating expression of brain heat shock proteins by estrogen in ovariectomized mice model of aging. *Exp Gerontol,* 45, 323-330, 2010.

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RESEARCH ARTICLE

Some Physico-chemical Properties and Organic Acid Profiles of Herby Cheeses

Arzu KAVAZ * İhsan BAKIRCI ** Güzin KABAN **

* Department of Food Engineering, Engineering Faculty, Adıyaman University, TR-02040 Adıyaman - TURKEY
** Department of Food Engineering, Agricultural Faculty, Atatürk University, TR-25240 Erzurum - TURKEY

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Summary

The aim of this study was to determine the effects of different herbs on the physicochemical properties and organic acid profiles of Herby cheeses. Five batches of cheese were manufactured: CC, cheese without herb; CH, cheese containing Helis (*Ferule* sp.); CK, cheese containing Kekik (*Thymus* sp.); CS, cheese containing Sirmo (*Allium* sp.) and CM, cheese containing Mendo (*Anhriscus* sp.). Analyses were performed for total solids, ash, fat, salt and protein contents on the first day of production while titratable acidity, pH and organic acid profiles were carried out on days 1, 7 and 14, 21 and 28 of storage. The results indicated that the herbs influenced significantly (P<0.01) all parameters investigated of the Herby cheeses during storage. Lactic, acetic and butyric acid contents increased during storage, while orotic, citric, pyruvic and propionic acid values decreased in all samples including control.

Keywords: *Herby cheese, Organic acid, Storage period*

Otlu Peynirlerin Bazı Fizikokimyasal Özellikleri ve Organik Asit Profilleri

Özet

Bu çalışmanın amacı, Otlu peynirin fizikokimyasal özellikleri ve organik asit profili üzerine farklı otların meydana getirdiği etkiyi saptamaktır. Peynir, beş grup halinde üretilmiştir: (Otsuz üretilen peynir; CH, Helis içeren peynir (*Ferule* sp.); CK, Kekik içeren peynir (*Thymus* sp.); CS, Sirmo içeren peynir (*Allium* sp.) ve CM, Mendo içeren peynir (*Anhriscus* sp.)). Örneklerin toplam kuru madde, kül, yağ ve protein içerikleri depolamanın 1. gününde belirlenmiş, titrasyon asitliği, pH ve organik asit profili ise depolamanın 1, 7, 14, 21 ve 28. günlerinde gerçekleştirilmiştir. Elde edilen sonuçlar, kullanılan otların depolama süresince Otlu peynirin tüm parametreleri üzerinde önemli ölçüde (P<0.01) etkili olduğunu göstermiştir. Örneklerin laktik, asetik ve bütirik asit içerikleri depolama süresince artarken orotik, sitrik, pürivik ve propiyonik asit değerleri ise kontrol dahil tüm örneklerde düşüş göstermiştir.

Anahtar sözcükler: *Otlu peynir, Organik asit, Depolama*

INTRODUCTION

Herby cheese, a traditional Turkish cheese type, is generally produced by adding some aromatic herbs, which are well known for many years in Turkey [1]. Recent years, it has been not only produced in small-scale businesses with traditional methods but also in well-equipped factories. Even though more than 20 kinds of herbs have been used for the production, the most used kinds are Sirmo (*Allium* sp.), Kekik (*Thymus* sp.), Helis (*Ferule* sp.), Mendo (*Anhriscus* sp.), Cünk (*Rannunculus* sp.), Dereotu (*Anethum* sp.), Nane (*Mentha* sp.) [2]. The herbs have usually the flavors characteristics in the spices. They are harvested in their vegetative period of the springtime and usually added into the cheeses with two ways. In the first way, the herbs are used freshly after washed and sliced. The second way is to make pickle. For later way, after harvesting, herbs are washed and sliced, then put into the brine with 16% salt. After about 20 days they are ready to add into the cheese. They can also be stored for long time and can be used whenever needed [3]. Some authors [4-6] reported that the herbs have the flavoring and preservative functions during storage. Numerous researches have already been carried out

İletişim (Correspondence)
+90 416 2232727
arzu-kavaz23@hotmail.com; akavaz@adiyaman.edu.tr

on Herby white pickled cheese [5-9] and Herby cacik [10,11], but there is no any study about the overall composition and the changes of organic acids during the storage time of Herby cheese. The aim of this study was to determine the compositional properties and the changes of organic acids of Herby cheese during storage period.

MATERIAL and METHODS

Materials

The milk used in this study was obtained from the pilot milk-processing plant of the Agricultural Collage of Atatürk University. Jars with 100 g capacity for packaged of Herby cheeses were purchased from local markets of Erzurum, Turkey. Sirmo (*Allium* sp.), Mendo (*Anhriscus* sp.), Helis (*Ferule* sp.) and Kekik (*Thymus* sp.) purchased from a cheese shopping center of Van City. While Sirmo and Mendo herbs were obtained in pickled form, Kekik and Helis were taken dry form from local markets in Van, Turkey.

Methods

To produce Herby cheese, a total amount of 75 kg whole-fat milk was used. Firstly, milk was left over a day for turn to sour at room temperature. Afterwards sour milk was heated to boiling point (90-95°C, 30 min). Then it was gently and continuously stirred and avoided from the boiling rapidly. Milk coagulation occurred gradually at 85-90°C then curd was cooled to 25-30°C. Then curds were collected in handle colander and transferred to cloth bag. The mouth of the cloth bag was tied and the bag was hung up to drain excess water. After the whey removed, cheeses were divided into 5 equal parts (CC, cheese without herb; CH, cheese containing Helis (*Ferule* sp.); CK, cheese containing Kekik (*Thymus* sp.); CS, cheese containing Sirmo (*Allium* sp.) and CM, cheese containing Mendo (*Anhriscus* sp.) and each herb was added to the curds at a ratio of 2% except control group and salted with high microbiologically and chemically qualified salt. For analyses, control and herby cheeses groups were filled into the sterile jars of approximately 200 g capacity and stored at 4±1°C for 28 days. The analyses were performed after 1, 7, 14, 21 and 28 days of storage. Two experiments were carried out, and results were given as the means of these experiments.

Physico-chemical Analysis

Dry matter and ash contents of cheeses were determined by the gravimetric method and total nitrogen by Kjeldahl method as described by IDF [12,13]. Fat content was analyzed by the Gerber method as described by Kurt *et al.*[14]. For pH measurement, 10 g cheese sample was weighted and diluted with 20 mL of distilled water and was measured using a digital pH meter (WTW 340-1) [15]. Titratable acidity was determined as lactic acid percentage by titrating with 0.1 N NaOH. For salt analysis, 5 g sample was weighted and diluted with distilled water at 80°C and filtered from filter paper. Then 25 mL filtrate was titrated with 0.1 N $AgNO_3$ using potassium chromate as indicator [14].

Organic Acid Concentrations

The quantification of organic acids of Herby cheeses were analyzed by high-performance liquid chromatography (Agilent HPLC 1100 series G 1322 A, Germany) according to the modified methods described by Fernandez-Garcia and McGregor [16] and Akalin *et al.*[17]. For the extraction of organic acids, 4 g of Herby cheese sample was diluted with 25 mL 0.001 N H_2SO_4 and centrifuged at the 5.000xg for 10 min. The supernatant was filtered through Whatman No.1 filter paper and through a 0.45 µm membrane filter (PALL, USA), and 2 mL aliquots were stored in HPLC vials at -20°C until HPLC analysis. The degassed mobile phase of 0.001 N H_2SO_4 was used at a flow rate of 0.6 mL/min. The wavelength of detection was optimized at 210 nm for quantification of organic acids. Duplicate injections (about10 µl) were performed for all samples. The standard solutions of orotic, pyruvic, citric, lactic, acetic, butyric and hippuyric acids were prepared in distilled water to establish the elution times and calibration curves.

Statistical Analysis

The study was designed according to randomized complete block design by 5 (herbs) x 5 (storage period) factorial experiments. All statistical analyses were performed with SPSS 13.0 for Windows [18]. Differences between means were compared by Duncan's multiple range tests ($P<0.05$, $P<0.01$). The data analyzed are presented as the mean ± standard deviation (mean ± SD).

RESULTS

General Composition

The mean values of total solids, ash, fat, salt and protein contents of Herby cheeses are showed in *Table 1*. When considering the general composition of the experimental cheeses, the obtained results were as expected.

The highest mean value of titratable acidity (0.28±0.06%) was found in CS and the lowest mean value (0.25±0.03%) in CK *(Table 2)* and the differences among the samples were determined statistically significant ($P<0.01$).

During storage period, titratable acidity values of Herby cheeses were lower than that of the control at the beginning of storage period. After 7 days, a higher increase was observed in the herby samples than the control in terms of titratable acidity. Additionally, titratable acidity contents of all samples including control reached the highest value on 14th day of storage in all groups. Then, a decrease was observed in varying rates between 14th and 21th days of storage. At the end of storage, a rise was again determined *(Fig. 1)*.

The highest mean value of pH (5.82±0.07) were found in CC and CK and the lowest mean value was (5.79±0.07) in

Table 1. *Some physicochemical properties of herby cheeses (mean±SD)*
Tablo 1. *Otlu peynirlerin bazı fizikokimyasal özellikleri (ortalama±SD)*

Group	Total Solids (%)	Ash (%)	Fat (%)	Salt (%)	Protein (%)
CC (Control)	58.15±0.28	5.25±0.57	21±1.15	3.63±0.14	35.48±3.52
CH	57.76±0.63	4.92±0.17	21.50±0.41	2.98±0.22	29.16±1.01
CK	58.10±0.43	4.42±0.44	21.75±1.26	2.75±0.35	29.53±0.48
CS	55.03±0.46	3.61±0.10	21.50±0.58	1.81±0.22	27.10±0.45
CM	53.12±1.92	3.87±0.29	21.00±1.15	2.57±0.19	21.72±0.77

CC: *Cheese with no added herb (Control),* ***CH:*** *Cheese with Helis (Ferule sp.),* ***CK:*** *Cheese with Kekik (Thymus sp.),* ***CS:*** *Cheese with Sirmo (Allium sp.),* ***CM:*** *Cheese with Mendo (Anhriscus sp.)*

Table 2. *Titratable acidity and pH values of herby cheeses (mean±SD)*
Tablo 2. *Otlu peynirlerin titrasyon asitliği ve pH değerleri (ortalama±SD)*

Group	Titratable Acidity (LA%)	pH
CC (Control)	0.27±0.02^{b*}	5.82±0.07^{a}
CH	0.26±0.02^{bc}	5.81±0.06^{a}
CK	0.25±0.03^{c}	5.82±0.07^{a}
CS	0.28±0.06^{a}	5.79±0.07^{b}
CM	0.26±0.04^{bc}	5.81±0.11^{a}

CC: *Cheese with no added herb (Control),* ***CH:*** *Cheese with Helis (Ferule sp.),* ***CK:*** *Cheese with Kekik (Thymus sp.),* ***CS:*** *Cheese with Sirmo (Allium sp.),* ***CM:*** *Cheese with Mendo (Anhriscus sp.),* ******* *Different letters in the same row represent significant differences at P<0.01*

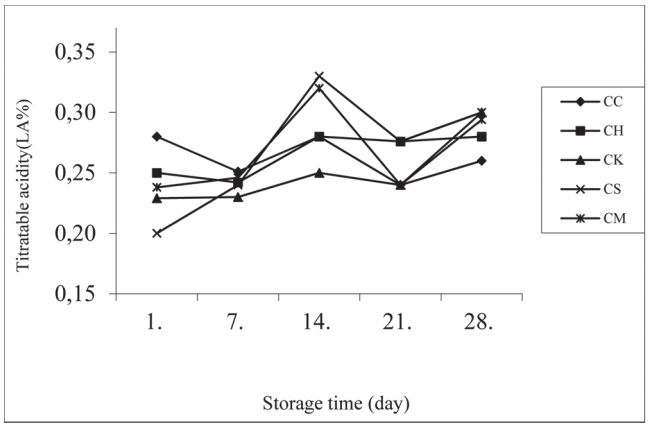

Fig 1. Titratable acidity values of the herby cheeses during storage
Şekil 1. Otlu peynirlere ait titrasyon asitliği değerlerinin depolama süresince değişimi

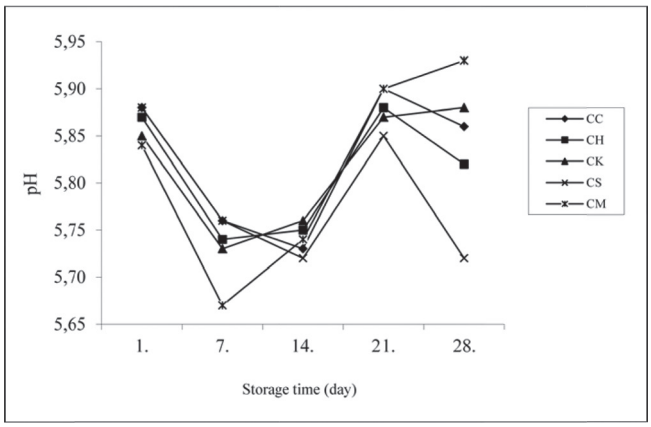

Fig 2. pH values of the herby cheeses during storage
Şekil 2. Otlu peynirlere ait pH değerlerinin depolama süresince değişimi

CS. There were no significant (P>0.05) differences between the groups except cheese with Sirmo (*Allium* sp.) in terms of pH value *(Table 2)*. The highest value was observed in the CM while the lowest value was found in the CS at the end of storage *(Fig. 2)*.

Organic Acid Concentrations

In this research, seven different organic acids were determined in the cheese samples during storage period.

The highest mean value of orotic acid (1.93±0.097) was found in CC and the lowest mean value (0.77±0.32) was in CM. Differences among the samples were significant (P<0.01) statistically *(Table 3)*.

Table 3. *Organic acid profiles of Herby cheeses (mean±SD)*
Tablo 3. *Otlu peynirlerin organik asit düzeyleri (ortalama±SD)*

Group	Orotic Acid	Citric Acid	Pyruvic Acid	Lactic Acid	Acetic Acid	Propionic Acid	Butyric Acid
CC (Control)	1.93±0.097^{ab*}	77.49±5.23^{a}	10.45±0.52^{a}	91.54±23.58^{c}	15.08±4.76^{d}	47.05±25.00^{d}	30.65±4.15^{b}
CH	1.25±0.32^{a}	74.29±11.30^{a}	9.18±0.99^{b}	145.76±33.86^{b}	115.13±57.67^{a}	87.94±15.95^{a}	30.15±5.85^{b}
CK	1.07±0.19^{ab}	68.07±18.39^{b}	8.06±1.68^{c}	227.27±152.13^{a}	65.55±74.24^{b}	77.17±27.88^{b}	56.05±25.77^{a}
CS	1.03±0.09^{b}	55.68±17.40^{b}	9.44±0.85^{b}	196.12±130.72^{a}	41.55±19.77^{c}	17.58±11.09^{e}	45.19±22.59^{a}
CM	0.77±0.32^{c}	50.55±17.56^{c}	8.55±1.35b^{c}	142.36±30.99^{b}	18.92±8.76^{d}	60.22±29.13^{c}	58.88±36.47^{a}

CC: *Cheese with no added herb (Control),* ***CH:*** *Cheese with Helis (Ferule sp.),* ***CK:*** *Cheese with Kekik (Thymus sp.),* ***CS:*** *Cheese with Sirmo (Allium sp.),* ***CM:*** *Cheese with Mendo (Anhriscus sp.),* ******* *Different letters in the same row represent significant differences at P<0.01*

Changes of orotic acid concentrations in samples during storage are shown in *Fig. 3*. In this experiment, the orotic acid value decreased after 14th day of storage until not detectable level. This could be attributed to the storage time.

The mean values of citric acid in the cheese samples changed between 77.49±5.23 and 50.55±17.56 µg/g. When the herby samples compared to the control, differences between the experiments were statistically significant (P<0.01) except for CH sample in terms of citric acid level *(Table 3)*. As seen from *Fig. 4*, citric acid contents in all samples decreased after 14th day of storage.

In groups, the mean concentrations of pyruvic acid changed between 10.45±0.52 and 8.06±1.68 µg/g. Differences between the control and herby samples were found to be statistically significant (P<0.01) *(Table 3)*. On the other hand, pyruvic acid values generally showed an irregular change during storage period *(Fig. 5)*.

The mean lactic acid values of cheeses ranged from

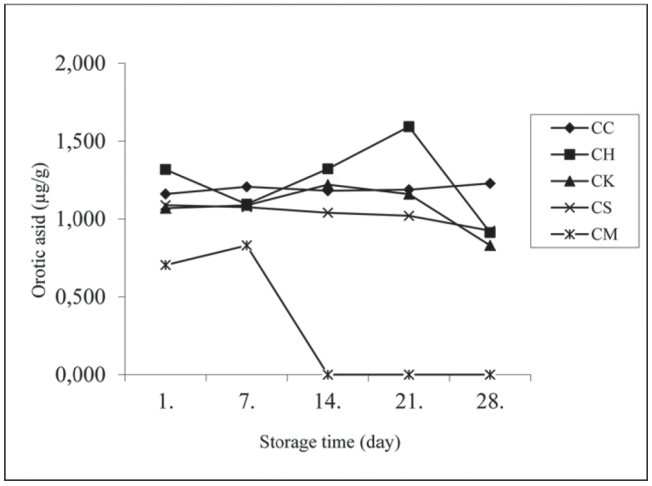

Fig 3. Orotic acid levels of the herby cheeses during storage

Şekil 3. Otlu peynirlere ait orotik asit düzeylerinin depolama süresince değişimi

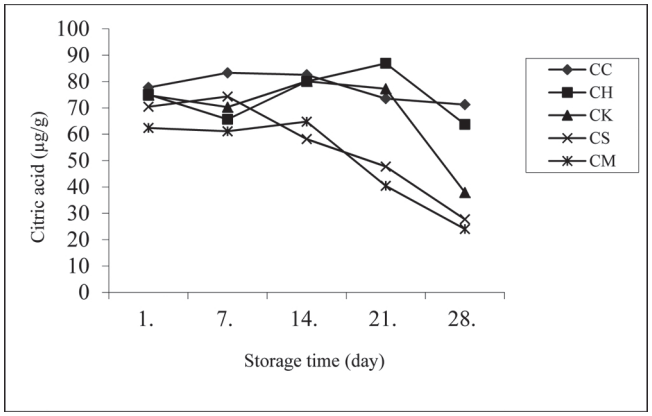

Fig 4. Citric acid levels of the herby cheeses during storage

Şekil 4. Otlu peynirlere ait sitrik asit düzeylerinin depolama süresince değişimi

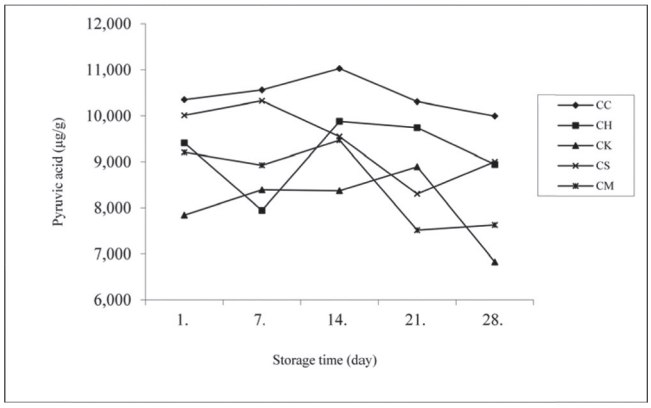

Fig 5. Pyruvic acid levels of the cheeses during storage

Şekil 5. Otlu peynirlere ait pirüvik asit düzeylerinin depolama süresince değişimi

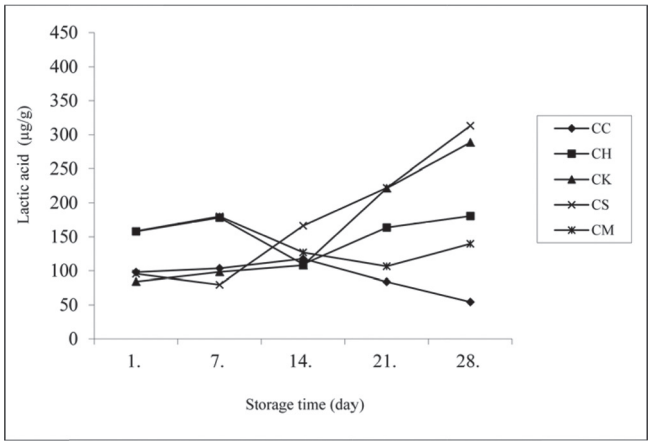

Fig 6. Lactic acid levels of the herby cheeses during storage

Şekil 6. Otlu peynirlere ait laktik asit düzeylerinin depolama süresince değişimi

227.27±152.13 to 91.54±23.58 µg/g. According to statistical evaluations, CM and CH cheeses were similar to each other, and CS and CK cheeses showed a similar trend with respect of statistical evaluations. In contrary, control cheese differed from the herby samples (P<0.01) *(Table 3)*. On the other hand, lactic acid contents of all samples increased during storage period except for control *(Fig. 6)*.

The mean acetic acid values of the groups ranged from 15.08±4.76 to 115.13±57.67 µg/g. The levels of acetic acid were higher in the herby cheeses compared with the control sample and this was found to be significant statistically (P<0.01) *(Table 3)*. Acetic acid concentrations of cheeses were shown in *Fig. 7*.

The amounts of propionic acid in cheeses changed between 17.58±11.09 and 87.94±15.95 µg/g, and a variation was observed among the experiments in terms of propionic acid levels in herby samples and control, these differences were significant (P<0.01) statistically *(Table 3)*. Propionic acid contents of cheeses were shown in *Fig. 8*.

The lowest mean value (30.15±5.85 µg/g) of butyric acid

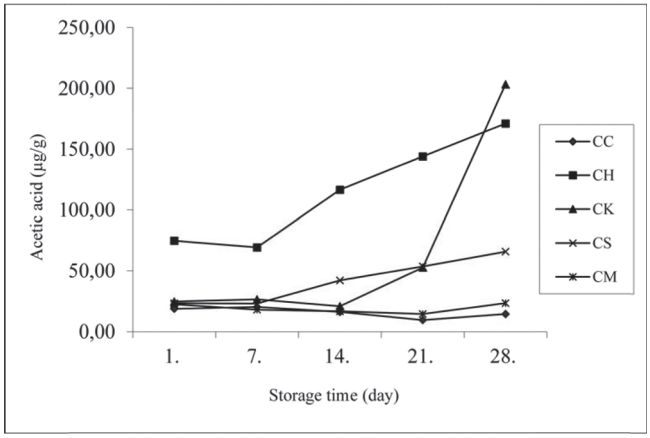

Fig 7. Acetic acid levels of the herby cheeses during storage

Şekil 7. Otlu peynirlere ait asetik asit düzeylerinin depolama süresince değişimi

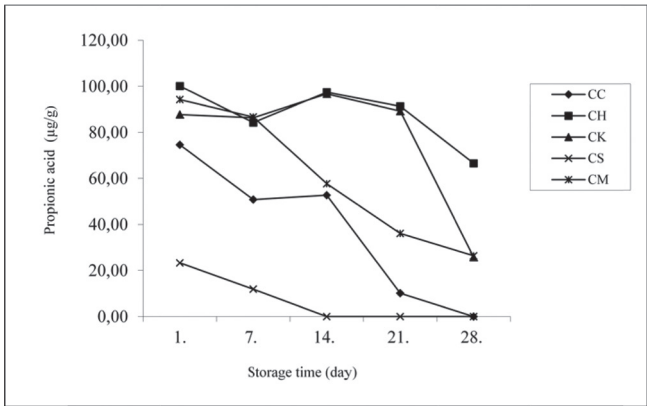

Fig 8. Propionic acid levels of the herby cheeses during storage

Şekil 8. Otlu peynirlere ait propiyonik asit düzeylerinin depolama süresince değişimi

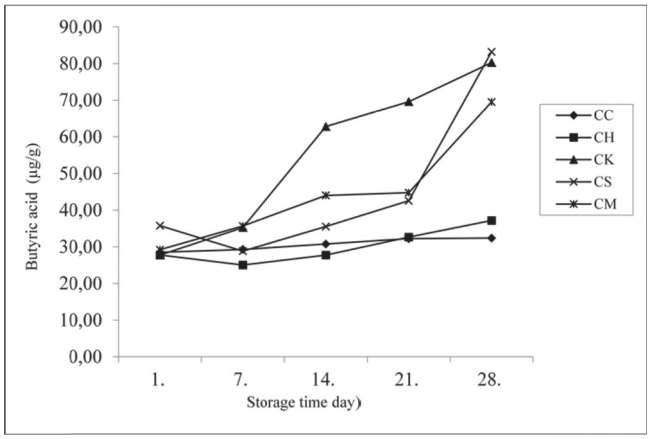

Fig 9. Butyric acid levels of the herby cheeses during storage

Şekil 9. Otlu peynirlere ait bütirik asit düzeylerinin depolama süresince değişimi

was found in cheese with helis (CH) and the highest mean value (58.88±36.47 µg/g) was found in cheese with mendo (CM). On the other hand, differences among the samples were found statistically significant (P<0.01) *(Table 3)*. Changes of butyric acid concentrations in samples during storage were shown in *Fig. 9*.

DISCUSSION

Titratable Acidity and pH

The titratable acid values of cheese samples were affected significantly (P<0.01). This could be attributed to the characteristics of herbs used. Zaika and Kissinger [19] reported that some herbs and spices influence the growth and activities of lactic acid bacteria at different levels.

Differences between titratable acidity values of the experimental cheeses during storage can be explained by the stimulating effects of the herbs on lactic acid bacteria. Bakırcı [20] suggested that nitrogenous compounds and carbohydrates in herbs might serve as additional source of carbon and nitrogen for nonstarter lactic acid bacteria (NSLAB) and contribute to the activity of the cultures. A similar titratable acidity pattern was also reported by Tarakci [8] and Şenel [21] for herby cheese.

The effects of herbs on pH values were similar to control (CC) during storage. This situation can be explained with alkaline compounds forming as a result of proteolytic degradation, yeast and moulds activities and compositional characteristics of herbs [22-25].

Organic Acid Concentrations of Herby Cheeses

Organic acids are the major products of carbohydrate catabolism of lactic acid bacteria. They contribute to cheese quality, playing an integral role in flavor [26] and affect the flavors of most mature cheeses. They are formed as a result of hydrolysis of milk fat during lipolysis, bacterial growth, normal ruminant metabolic processes or addition of acidulants in cheese making [16,27-30]. Quantitative determination of organic acids is an important tool for flavor and nutritional quality as well as being an indicator of bacterial activity in mature cheeses, as the total aroma intensity is correlated with organic acid levels in grading cheeses [31,32].

Differences among the samples were found statistically significant (P<0.01). This might be explained by the properties of herbs used. The effects of herbs on the orotic acid were similar except CM.

Larson and Hegarty [33] indicated that the orotic acid levels in cheese products showed wide variations. The authors stated that washing of curd and fermentation degree effect the orotic acid level in cheese and suggested that the mature cheeses contain the lowest level of orotic acid. Also, Fernandez-Garcia and McGregor [16]; Tormo and Izco [30]; Güzel-Seydim *et al.*[34]; Kristo *et al.*[35] suggested that orotic acid concentrations of milk and milk products reduced to the levels of 45-48% during fermentation and storage.

Citric acid is the most abundant organic acid present in

raw milk and is available at the level of 0.2% concentration averagely [30,36]. Lactic acid bacteria produce diacetyl from citric acid in milk by using the pyruvate that occurred as an intermediate product during fermentation [37,38]. Citric acid is not the first energy source of bacteria, but can be metabolized very rapidly by *Lactococcus lactis* subsp. *diacetylactis* or *Leuconostoc* spp. in cheese [30]. In all samples, citric acid concentrations decreased after 14th day of storage. This can be explained by the fermentation of citrate to some organic acids including acetic acid, propionic acid and volatile compounds [39,40]. Similar results were reported by Ong and Shah [41] for cheddar cheese.

Pyruvic acid occurs as an intermediate product of protein and carbohydrate metabolism. Firstly, pyruvic acid is formed from lactose by bacterial fermentation, then this product is converted to the lactic acid and other metabolites by an enzyme series [34,36]. Pyruvic acid concentrations of samples showed an increases and decreases during storage. Because pyruvate is readily formed through the glycolytic pathway. Also, it acts as a substrate of several metabolic reactions such as the formation of formic acid, ethanol, diacetyl, acetoin and 2,3-butylene glycol [42].

Formation of lactic acid is essential for flavor development and keeping quality of cheeses. Generally lactic acid concentrations of samples showed an increase during storage period except for control sample. Control cheese showed statistically important differences ($P<0.01$) from herby cheese samples. This was probably due to the effects of herbs, which are known a stimulating effect towards the lactic acid production [19]. Lactic acid concentrations of samples increased except for control during storage and the highest value was observed in the cheese with sirmo (CS), the lowest value was found in control sample (CC) at end of the storage.

Acetic acid is one of the important flavor compounds in many cheeses [26]. Acetic acid can be produced from citrate, lactose and amino acids [43]. In general, it gives vinegar taste and aroma to the product. Therefore, the acceptability of the products shows a very rapid decline during storage [44]. The acetic acid contents of CK, CH and CS samples increased during storage time, while CC and CM samples remained relatively constant during this period *(Fig. 7)*. A similar result was reported by Bouzas *et al.*[39] for acetic acid content of Cheddar cheese.

Propionic acid bacteria are used in dairy industry during Emmental type cheese maturation to produce CO_2, volatile fatty acids and diacetyl that represent characteristic flavours of this cheese [45]. In addition, it is well known that the propionic acid fermentation leads to characteristic eyes and nutty flavour to the cheese [46]. Among samples, significant differences were observed in terms of propionic acid concentrations ($P<0.01$). This was probably due to the inhibitory effect of herbs on propionic acid-producing bacteria [47]. It was observed a decline in all samples in terms of propionic acid contents during storage, but the sharply decrease was seen in the cheese containing sirmo *(Fig. 8)*. This situation can be explained by the result of the lipolytic and proteolytic activities of non-starter bacteria [31].

Butyric acid appears in cheeses as a result of, either lipolysis or deamination of amino acids [36,37]. The butyric acid contents of herby samples were higher than that of control sample. This was presumably due to the stimulating effect of herbs used. In addition, the butyric acid contents of samples progressively increased during storage, but the increase in control and CH samples became a slower rate during the storage *(Fig. 9)*.

This study suggested that the use of some herbs in herby cheese affected the physicochemical properties and organic acid composition of cheeses at different levels. It was seemed that titratable acidity values of Herby cheeses were higher than that of control depending on the herb types. Titratable acidity values of all cheeses increased until 14 day of storage, then an irregularly change was determined. PH values of the cheeses showed a similar trend in all experiments including control during storage. Orotic, citric, pyruvic and propionic acid values of herby samples were lower than that of control, and these values decreased after 14th day of storage in all cheeses. On the contrary, lactic, acetic and butyric acid values generally increased after 14th day of storage in herby cheeses, but decreased in control and some herby samples. It was concluded that further studies are essential to evaluate the effects of herbs on the properties of this type cheeses.

REFERENCES

1. Coskun H, Bakirci I: A study on the determination of herb-addition rate in Van Herby Cheese. *Yüzüncü Yıl Üniv Ziraat Fak Derg*, 6, 97-103,1996.

2. Özçelik H: Van ve yöresinde süt mamullerinin hazırlanmasında yararlanılan bitkilerin kullanışları üzerinde bir araştırma. *TUBITAK, Doğa Türk Tarım ve Ormancılık Derg,* 13 (2): 356-360,1989.

3. Kurt A: Van otlu peynir üzerinde araştırmalar. *A.Ü.Z.F. Zirai Araştırmalar Enstitüsü Bülteni* 33, 1-29, 1968.

4. Zaika LL, Zell TE, Palumbo SA, Smith JL: Effect of spices and salt on fermentation of Lebanon Bologna-type sausage. *J Food Sci*, 43, 186-189, 1978.

5. Coskun H, Tuncturk Y: The effect of *Allium* sp. on the extension of lipolysis and proteolysis in Van Herby Cheese during maturation. *Nahrung*, 44, 52-55, 2000.

6. Tarakci Z, Akyüz N: Effects of packaging materials and filing mehtods on selected characteristics of Otlu (Herby) cheese. *Int J Food Prop,* 12, 496-511, 2009.

7. Zaika LL, Kissinger JC, Wasserman AE: Inhibition of lactic acid bacteria by herbs. *J Food Sci,* 48, 1455-1459, 1983.

8. Tarakci Z: The influence of helis (*Prangos* sp.) on characteristics of vacuum-packed Van Herby Cheese during ripening. *Milchwissenschaft,* 59, 619-623, 2004.

9. Tarakçı Z, Coşkun H, Tuncturk Y: Some properties of fresh and ripened herby cheese, a traditional variety produced in Turkey. *Food Technol Biotechnol,* 42 (1): 47-50, 2004.

10. Küçüköner E, Tarakçı Z: Van ve yöresinde üretilen cacığın (otlu çökelek) bazı özelliklerinin araştırılması. *V. Süt ve Süt Ürünleri Sempozyumu,* 21-22 Mayıs, Tekirdağ, pp.175-185, 1998.

11. Kucukoner E, Tarakci Z, Sagdic O: Physicochemical and microbiological characteristics and mineral content of herby cacik, a traditional Turkish dairy product. *J Sci Food Agr,* 86, 333-338, 2006.

12. International Dairy Federation: Cheese and Processed Cheese: Determination of the Total Solids Contents. Reference Method 4A, Brussels: *International Dairy Federation*, 1982.

13. International Dairy Federation. Standard Method 20B: Milk. Determination of Nitrogen Content. IDF, Brussels, Belgium: *International Dairy Federation*, 1993.

14. Kurt A, Çakmakçı S, Çaglar A: A Guide Book of Analysis Methods of Milk and Milk Products. *Atatürk University, Agricultural Faculty Pub,* No.254/D Erzurum, Turkey, 2006.

15. Savello PA, Ernstrom CA, Kalab M: Microstructure and meltability of model process cheese made with rennet and acid casein. *J Dairy Sci,* 72, 11, 1989.

16. Fernandez-Garcia E, McGregor JU: Determination of organic acids during the fermentation and cold siorage of yogurt. *J Dairy Sci,* 11, 2934-2939, 1994.

17. Akalin AS, Fenderya S, Akbulut N: Viability and activity of bifidobacteria in yoghurt containing fructooligosaccharide during refrigerated storage. *Int J Food Sci Technol,* 39, 613-621, 2004.

18. SPSS Inc: Statistical package for the social sciences SPSS ver. 13.0 for *Windows. Chiago, IL,* 2004.

19. Zaika LL, Kissinger JC: Effects of some species on acid production by starter cultures. *J Food Protect,* 47, 572-576, 1979.

20. Bakırcı İ: The effects of some herbs on the activities of thermophilic dairy cultures. *Nahrung*, 43, 333-335, 1999.

21. Şenel E, Yıldız F, Yetişemeyen A, Durlu-Özkaya F, Öztekin FŞ, Şanlı E: Evaluation of the biogenic amin content and some chemical and microbiological properties of Urfa and Van herby cheeses. *Kafkas Univ Vet Fak Derg,* 18 (4): 537-544, 2012.

22. Fox PF, Law J, Mc Sweeney PLH, Wallace J: Biochemistry of cheese ripening. **In,** Fox PF (Ed): Cheese: Chemistry, Physics and Microbiology. pp. 389-438, Chapman and Hall, London, 1993.

23. Guinee TP, Fox PF: General Aspects. **In,** Fox PF (Ed): Cheese: Chemistry, Physics and Microbiology. pp. 257-302, Chapman and Hall, London, 1993.

24. Coşkun, H: Microbiological and biochemical changes in herby cheese during ripening. *Nahrung*, 42, 309-313, 1998.

25. Alemdar S, Ağaoğlu S: Survival of *Salmonella typhimurium* during the ripening of herby cheese (Otlu peynir). *J Food Safety,* 30, 526-536, 2010.

26. Buffa M, Guamis B, Saldo J, Trujillo AJ: Changes in organic acids during ripening of cheeses made from raw, pasteurized or high-pressure-treated goats' milk. *Lebensm.-Wiss. u.-Technol,* 37, 247-253, 2004.

27. Marsili RT, Ostapenko H, Simmons RE, Green DE: High performance liquid chromatographic determination of organic acids in dairy products. *J Food Sci,* 46, 52-57, 1981.

28. Adda J, Gripon JC, Vassal I: The chemistry of flavor and texture generation in cheese. *Food Chem,* 9, 115-129, 1982.

29. Adhikari K, Grün IU, Mustapha A, Fernando LN: Changes in the profile of organic acids in plain set and stirred yogurts during manufacture and refrigerated storage. *J Food Quality*, 25, 435-451, 2002.

30. Tormo M, Izco JM: Alternative reversed-phase high performance liquid chromatography method to analyse organic acids in dairy products. *J Chomatography Anal,* 1033, 305-310, 2004.

31. Akalin AS, Gonc S, Akbas Y: Variation in organic acids content during ripening of pickled white cheese. *J Dairy Sci,* 85, 1670-1676, 2002.

32. Ong L, Henriksson A, Shah NP: Proteolytic pattern and organic acid profiles of probiotic Cheddar cheese as influenced by probiotic strains of Lactobacillus acidophilus, *Lb. paracasei, Lb. casei* or *Bifidobacterium* sp. *Int Dairy J* 17, 67-78, 2007.

33. Larson BL, Hegarty M: Orotic acid in milks of various species and commercial dairy products. *J Dairy Sci,* 62, 1641-1644, 1979.

34. Güzel-Seydim ZB, Seydim AC, Grene AK: Organic acids and volatile flavour components evolved during refrigerated storage of kefir. *J Dairy Sci,* 83, 275-277, 2000.

35. Kristo E, Biliaderis CG, Tzanetakis N: Modelling of rheological, microbiological and acidification properties of a fermented milk product containing a probiotic strain of Lactobacillus paracasei. *Int Dairy J,* 13, 517-528, 2003.

36. Metin M: Süt Teknolojisi. Sütün Bileşimi ve İşlenmesi. 6. Baskı, s. 802, Ege Üniversitesi Basımevi, Bornova, İzmir, 2005.

37. Daeschel MA: Antimicrobial substances from lactic acid bacteria for use as food preservatives. *Food Technol,* 43, 164-167, 1989.

38. Tekinşen OC, Atasever M: Süt Ürünleri Üretiminde Starter Kültür. *Selçuk Üniversitesi Veteriner Fakültesi Yayını,* Konya, 150s, 1994.

39. Bouzas J, Kantt CA, Bodyfelt F, Torres JA: Time and temperature influence on chemical aging indicators for a commercial Cheddar cheese. *J Food Sci*, 58, 1307-1312, 1993.

40. McSweeney PLH, Sousa MJ: Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Lait,* 80, 293-324, 2000.

41. Ong L, Shah NP: Influence of probiotic *Lactobacillus acidophilus* and *L. helveticus* on proteolysis, organic acid profiles, and ACE-Inhibitory activity of cheddar Cheeses ripened at 4, 8, and 12°C. *J Food Sci,* 73, 111-120, 2008.

42. Marth EH: Fermentaions. **In,** Webb B, Johnson AH, Alford JA (Eds): Fundamentals of Dairy Chemistry. AVI Publishing Co., Westport, Conn., pp. 772-872, 1974.

43. Aston JW, Dulley JR: Cheddar cheese flavour. *Aust J Dairy Technol,* 37, 59-64, 1982.

44. Gaafar AM: Volatile flavour compounds of yoghurt. *Int J Food Sci Technol,* 27, 87-91, 1992.

45. Cavin JF, Saint C, Divies C: Continuous production of emmental cheese flavours and propionic acid starters by immobilized cells of a propionic acid bacterium. *Biotechnol Lett,* 7, 821-826, 1985.

46. Fröhlich-Wyder MT, Bachmann HP: Cheeses with propionic acid fermantation. **In,** Fox PF, McSweeney PLH, Coagan TM, Guinee T (Eds): Cheese: Chemistry, Physics and Microbiology. Vol. 2, Third ed., pp. 141-157, Major Cheese Groups, *Vol. 2, Major Cheese Groups, Oxford, Elsevier*, 2004.

47. Fisher K, Phillips C: Potential antimicrobial uses of essential oils in food: Is citrus the answer. *Trends Food Sci Technol,* 19, 156-164, 2008.

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RESEARCH ARTICLE

Effects of Platelet-activating Factor Receptor Antagonist (PAFRA) on Selected Inflammatory and Biochemical Parameters in Lipopolysaccharide-Induced Rat Endotoxemia Model [1]

Ramazan ÇÖL * Ercan KESKİN *

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* Department of Physiology, Faculty of Veterinary Medicine, University of Seçuk, Campus, TR-42031 Selçuklu, Konya - TURKEY

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Summary

Platelet-activating factor (PAF) is a significant phospholipid mediator of the immune system produced by a veriety of cells involved in inflammatory reactions in sepsis. In this experimental study, our aim was to investigate the role of PAF receptor antagonist (PAFRA) on biochemical and inflammatory disturbances in lipopolysaccharide (LPS)-treated rats. A total of 32 adult male Wistar rats were divided into four equal groups: Group 1 (control group, C) was treated with 0.9% saline. Group 2: LPS was injected intravenously (1.6 mg/100 g), Group 3 received PAFRA treatment (10 mg/kg) 2 min before 0.9% saline injection, Group 4 received PAFRA treatment 2 min before endotoxin treatment. Blood samples were collected 6 h after treatment. LPS (Group-II) caused statistically significant increases in serum TNF-α, IL-6 and IL1β levels, CRP, LDH, AST, ALT, creatinine, BUN, cholesterol, triglyceride concentration, and caused statistically significant decreases in platelet count, glucose, total protein and albumin levels. Also, when compared to control group leukopenia and significant changes in the leukocyte differential were evident. In group 4, PAFRA inhibited serum TNF-α and IL1β levels, leukopenia compared with the group 2 ($P<0.05$). However, there were no significant differences in the other parameters between the two groups. The results demonstrate that at the administered dose and route, PAFRA has a slight effect in the pathogenesis of endotoxemia.

Keywords: *Endotoxin, Cytokines, Biochemical parameters, Platelet-activating factor Receptor antagonist, Rat*

Lipopolisakkarit ile İndüklenen Rat Endotoksemi Modelinde Bazı Yangısel ve Biyokimyasal Parametreler Üzerine Platelet Aktive Edici Faktör Reseptör Antagonisti (PAFRA)'nin Etkileri

Özet

Platelet Aktive edici Faktör (PAF), sepsisde yangısel reaksiyonlara karışan birçok hücre tarafından üretilen immun sistemin önemli bir fosfolipid mediyatörüdür. Bu deneysel çalışmada amacımız, lipopolisakkarit (LPS) uygulanan sıçanlarda biyokimyasal ve yangısel bozukluklar üzerine PAF reseptör antagonisti (PAFRA)'nın rolünü araştırmaktı. Total 32 adet yetişkin erkek sıçan dört eşit gruba ayrıldı: grup 1 kontrol (C) olarak hizmet etti. Grup 2'deki hayvanlara intravenöz LPS (1.6 mg/100 g, *Escherichia Coli*, 0.111:B4) verildi. Grup 3'de 0.9% serum fizyolojik enjeksiyonundan 2 dak. önce PAFRA (10mg/kg) intraperitoneal olarak enjekte edildi. Grup 4'de, LPS uygulamasından 2 dak. önce PAFRA uygulandı. Kan örnekleri uygulamadan sonraki 6.saatte toplandı. LPS (grup 2), serum TNF-α, IL-6 ve IL1β seviyesi, CRP, LDH, AST, ALT, kreatinin, BUN, kolesterol, trigliserit konsantrasyonunu önemli düzeyde artırdı, platelet sayısı, glikoz, total protein ve albumin seviyesini önemli oranda düşürdü. Ayrıca kontrol grupla karşılaştırıldığında LPS grupta lökopeni ve diferensiyal lökosit sayısında önemli değişiklikler mevcuttu ($P<0.05$). Grup 2 ile karşılaştırıldığında grup 4'de PAFRA, TNF-α ve IL1β seviyelerini ve lökopeniyi inhibe etti ($P<0.05$). Buna rağmen iki grup arasındaki diğer parametrelerde önemli değişiklikler yoktu. Mevcut sonuçlar; uygulanan doz ve yolda PAFRA'nın endotokseminin patogenezinde hafif bir etkiye sahip olduğunu göstermektedir.

Anahtar sözcükler: *Endotoksin, Sitokin, Biyokimyasal parametreler, Platelet aktive edici faktör reseptör antagonisti, Sıçan*

INTRODUCTION

Sepsis from gram-negative bacterial infections such as some enteric disease, septisemia, metritis, mastitis, and pneumonia may be complicated by a variety of conditions characterized by fever, tachycardia, tachypnea, hypotension,

İletişim (Correspondence)
+90 332 2232635
rcol@selcuk.edu.tr

disseminated intravascular coagulation (DIC), multiple organ failure, and even death [1,2] . Despite the potent antimicrobial treatments, improved levels of monitoring and intensive supportive care in the last decade, sepsis increasingly remains one of major causes of death, and the mortality rate (60%) in animals [3,4]. Sepsis causes a generalized inflammatory reaction including the concurrent activation of several endogenous mediator systems such as immune system, endothelium, and coagulation system [5]. Endotoxin (LPS), a cell wall constituent of gram-negative bacteria, is involved in the patogenesis of endotoxic shock, coagulopathy. Administration of LPS to experimental animals leads to the production of the pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 from monocytes, macrophages and endothelium [6]. In recent years, it has become apparent that the mediators of inflammation have critical roles in sepsis. After intravenous endotoxin challenge, rapid production and release of proinflammatory cytokines (e.g. TNF-α, IL-1β, IL-6) from monocytes, macrophages and endothelium were detected [7]. Release of these pro-inflammatory cytokines determines the development and incidence of tissue damage, multi organ failure (MOF) or even death [8]. In recent years, some therapeutic strategies for human and animal septic shock have been designed to neutralize the inflammatory mediators. Especially, anti-cytokine strategies such as anti-inflammatory cytokines (IL-10, IL-13), IL-1 receptor antagonist (IL-1Ra), knock-out of TNFR (p55), and anticytokine antibodies has gained increasing importance endotoxemia studies [2,6,9].

Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glyceryl - 3-phosphonocholine) is a natural phospholipid synthesized by several different cells including basophils, macrophages, neutrophils and platelets, in response to various stimuli including lipopolysaccharide (LPS), and tissue factors released after endothelial disruption [10]. The administration of PAF to experimental animals causes diverse pathophysiological changes very similar to those observed during endotoxaemia such as hypotension, increased vascular permeability, thrombocytopenia and gastrointestinal damage [10,11]. LPS affects the expression of both PAF and its receptor [12]. The effects of PAF are mediated through specific PAF receptors (PAF-R) [13]. PAF-R is a G-protein coupled receptor and it exists in various cells such as platelet, neutrophil. Engagement of the PAFR by PAF activate a variety of intracellular signaling cascades and, induces functional responses of PAFR-bearing cells that then initiate or amplify inflammatory and thrombotic events [14,15]. Early observations indicated that additive or synergistic activities of PAF and cytokines may have key pathologic effects in the pathogenesis of lethal septicemia, and showed that interactions between PAF, tumor necrosis factor a (TNF-α), and IL-1 signaling cascades are particularly important [14]. PAF is an important mediator in experimental models. The effects of PAF can be inhibited both *in vivo* and *in vitro* with PAF receptor antagonists in LPS-induced sepsis [16]. Multiple studies have shown that complete protection against LPS-induced sepsis can be achieved if the agent is administered prior to the onset of the experimental intervention causing sepsis [12]. Ginkgolide B (BN52021) is a specific PAF-R antagonist and It is able to antagonize binding of PAF and its receptor (PAF-R) competitively, and thus PAF is unable to activate effector enzyme through G-protein transduction to block signal transduction of PAF-R. PAFRA may inhibit platelet aggregation, antagonize inflammation and shock, and protect blood vessels of heart and brain [15].

The present study was planned to determine whether administration of PAFRA attenuates the cytokine response and biochemical disturbances due to LPS-induced inflammation in rat endotoxemia model.

MATERIAL and METHODS

In our study, thirty two healthy adult male wistar rats (weight range: 200-250 g, Kobay experimental animal laboratory, Ankara) were acclimated at a constant temperature of 20ºC for at least a week. The animals were fed a standard pellet diet, and tap water was available *ad libitum*. All rats were in excellent physical condition prior to the experiments. This study was conducted according to the guidelines approved by the local ethics committee of the Faculty of Veterinary Medicine (University of Selcuk, Konya, Turkey, report no. 2011/005). Lipopolysaccharide (*Escherichia Coli*, 0.111:B4, SIGMA Cat.no: L4130) was dissolved in physiological saline immediately before use.

A total of 32 adult male Wistar rats were randomly divided into four equal groups: Group 1, Control group (C) was treated with 0.9% saline (0.2 ml iv). Group 2 (LPS): lipopolysaccharide (LPS) was dissolved in physiological saline immediately before use. LPS (*Escherichia coli* lipopolysaccharide, 0.111:B4 serotype, Sigma L4130) was injected intravenously (1.6 mg/100 g, into the tail vein). Group 3 (PAFRA): the rats in this group received PAFRA treatment alone (10mg/kg, Sigma G6910) 2 min prior to a single injection of saline solution (0.2 ml, iv.) instead of LPS. Group 4 (LPS + PAFRA): these rats received 10mg/kg IP PAFRA 2 min before endotoxin challenge (1.6 mg/100 g). Blood samples (2-3 ml) were collected by cardiac puncture 6 h after treatment. At the end of experiment, rats were sacrificed under deep anesthesia with high doses of thiopental sodium (Pental® sodium inj., IE Ulagay Ilac Sanayi, Istanbul, Turkey).

The levels of serum tumor necrosis factor-*α* (TNF-*α*) (eBioscience International, Inc. rat TNFα kit, Nivelles, Belgium), interleukin-1β (IL-1β) (eBioscience International, Inc. rat IL-1β kit), interleukin-6 (IL-6) (eBioscience International, Inc. rat IL-6 kit) and, C-reactive protein (CRP) (Alpha Diagnostic International Rat CRP kit) were determined by enzyme-linked immunosorbent assay (ELISA) using an ELISA reader (Anthos Labtec Instruments, A5022, Salzburg). For biochemical analyses, serum concentrations of cholesterol, triglycerides,

creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), glucose, total protein (TP), albumin (Alb) were determined by an autoanalyser (Siemens Dimension RxL Max otoanalizatör) using commercial kits (Dade Behring). The leukocyte count and platelet count (PLT) were determined by a haemocytometer method using Turk and Rees-Ecker solution, respectively. Selected blood smears were stained with May-Grünwald and Giemsa solution, and then used to determine the percentage values of different leukocytes.

Values are reported as mean ± standard error and were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's test, in the SPSS-15.0. In all cases, probability of error of less than 0.05 was selected as the criterion for statistical significance. To calculate the true concentration, raw data from ELISA array were multiplied by the appropriate dilution factor (x2 for cytokines and x 20K for CRP).

RESULTS

The effects of PAFRA on inflammatory and biochemical parameters of groups including control, LPS, PAFRA and PAFRA+LPS-treated rats are presented in *Table 1*.

When compared with the control group, there were no significant changes in any of the measured parameters in only PAFRA-treated rats (group 3) (P>0.05).

As compared to the control group, LPS injection displayed statistically significant increases in serum TNF-a, IL-6 and IL-1β levels, CRP, AST, ALT, LDH, creatinine, BUN, cholesterol, triglyceride concentration, and caused statistically significant decreases in platelet count, glucose, total protein and albumin levels. LPS administration (group 2) caused a decrease in leukocyte count with a significant neutrophilia and lymphopenia. In group 4, PAFRA inhibited serum TNF-α and IL1β levels compared with the group 2 (P<0.05). Additionally, the diminution observed in leukocyte count, changes in the percentage of neutrophils and lymphocytes following endotoxin administration was suppressed by PAFRA (P<0.05). However, the other parameters were not suppressed by the administration of PAFRA.

Table 1. *Effect of PAFRA on selected serum cytokine levels in a rat endotoxemic model (mean±SE)*

Tablo 1. *Rat endotoksemi modelinde belirli serum sitokin düzeyleri üzerine PAFRA'nın etkileri (mean±SE)*

Investigated Parameters	Control (n=8)	LPS (n=8)	PAFRA (n=8)	PAFRA+LPS (n=8)
TNF-a (pg/ml)	BDL	2404±333^{a}	BDL	1683±253^{b}
IL-6 (pg/ml)	BDL	4158±514^{a}	BDL	3727±415^{a}
IL-1β (pg/ml)	BDL	2781±334^{a}	BDL	2080±195^{b}

a,b,c,d: *Differences in the same row are statistically significant when the values are marked with different letters (P<0.05),* ***LPS;*** *Lipopolysaccharide,* ***PAFRA;*** *Platelet-activating factor receptor antagonist,* ***BDL;*** *below the detection limit*

Table 2. *Effect of PAFRA on some haematological parameters in endotoxaemic rats (mean ± SE)*

Tablo 2. *Rat endotoksemi modelinde bazı hematolojik parametreler üzerine PAFRA'nın etkileri (mean±SE)*

Investigated Parameters	Control (n=8)	LPS (n=8)	PAFRA (n=8)	PAFRA+LPS (n=8)
CRP (µg/ml)	214±50^{b}	2506±497^{a}	208±36^{b}	2371±392^{a}
PLT (x10^{9} / L)	629±45^{a}	120±12^{b}	659±26^{a}	175±38^{b}
Leukocyte (mm^{3})	6452±890^{a}	1512±195^{c}	6129±682^{a}	3988±391^{b}
Neutrophil (%)	24.4±3.2^{c}	71.5±3.9^{a}	27.5±3.6^{c}	45.4±4.3^{b}
Lymphocyte (%)	67.3±3.9^{a}	24.6±3.0^{c}	64.1±3.6^{a}	49.8±4.8^{b}

a,b,c,d; *Differences in the same row are statistically significant when the values are marked with different letters (P<0.05).* ***LPS;*** *Lipopolysaccharide,* ***PAFRA;*** *Platelet-activating factor receptor antagonist*

Table 3. *Effects of PAFRA on some biochemical parameters in endotoxaemic rats (mean ± SE)*

Tablo 3. *Rat endotoksemi modelinde bazı biyokimyasal parametreler üzerine PAFRA'nın etkileri (mean ± SE)*

Investigated Parameters	Control (n=8)	LPS (n=8)	PAFRA (n=8)	PAFRA+LPS (n=8)
AST U/L	132±22^{b}	795±162^{a}	112±13^{b}	728±124^{a}
ALT U/L	69.9±7.6^{b}	249.4±33.3^{a}	62.6±6.5^{b}	213.0±20.4^{a}
LDH (U/L)	349±31^{b}	1321±227^{a}	299±35^{b}	1129±192^{a}
Creatinine (mg/dL)	0.26±0.03^{b}	0.75±0.12^{a}	0.30±0.03^{b}	0.66±0.11^{a}
BUN (mg/dL)	13.9±0.9^{b}	40.9±3.1^{a}	14.4±1.4^{b}	38.6±3.7^{a}
T. Protein (g/dL)	5.28±0.21^{a}	3.94±0.10^{b}	5.44±0.20^{a}	4.11±0.29^{b}
Albumin (g/dL)	2.94±0.17^{a}	2.24±0.15^{b}	3.09±0.13^{a}	2.38±0.20^{b}
Triglyceride (mg/dL)	76.1±9.4^{bc}	123.5±16.9^{a}	70.4±6.3^{c}	107.8±10.7^{ab}
Cholesterol (mg/dL)	51.9±5.3^{b}	87.0±7.7^{a}	57.0±5.2^{b}	91.3±8.5^{a}
Glucose (mg/dL)	126.6±9.5^{b}	92.1±6.9^{a}	118.3±5.4^{b}	97.0±6.1^{a}

a,b,c,d; *Differences in the same row are statistically significant when the values are marked with different letters (P<0.05).* ***LPS;*** *Lipopolysaccharide,* ***PAFRA;*** *Platelet-activating factor receptor antagonist*

DISCUSSION

In experimental studies on laboratory animals, LPS-induced endotoxemia are well used to mimic the clinical features observed in animals with sepsis [18]. In endotoxemia, cytokines such as TNF-a, IL-1β and IL-6 are central mediators of pathological processes. LPS stimulates cytokine secretion from macrophages and induces endothelial cell damage. In earlier experimental and clinical trials with sepsis, PAFRA effectively exhibited potent protective effect on LPS-induced antioxidant and antiinflammatory disturbances [14,19,20], but PAFRA administration on the levels of serum proinflammatory cytokines and biochemical parameters in endotoxemia is as yet unclear.

In our study, PAFRA (10mg/kg IP, ginkgolide B Sigma Cat No G6910) and LPS (1.6 mg/100g IV) were administrated simultaneously. The dose of PAFRA used in this study was chosen from those previously reported [10,21].

In our work the selected LPS dose (*Escherichia Coli*, 0.111:B4 1.6 mg/100 g) is a sufficient dose to reach a high concentration of plasma cytokines during endotoxemi in rat [4]. Various researchers have reported the release of LPS-induced proinflammatory cytokines in rat endotoxemic models [3,4,22,23]. Mathiak et al.[24] have determined that LPS-induced IL-6 has the highest plasma concentration peak around 4-6 h. Earlier investigation reported that the increase of IL-6 concen-tration correlates with the severity of septic patients [6]. In this study, serum TNF-α, IL-1β and IL-6 were undetectable in control group (C), there were a marked elevation of serum TNF-α, IL 1β and IL-6 levels at 6 h after LPS administration (group II) ($P<0.05$) *(Table 1)*. In group IV PAFRA significantly inhibited LPS-induced increases in the levels of serum TNF-α, IL-1β when compared with LPS- group II ($P<0.05$) *(Table 1)*. In a study the over expression of the PAFR increases lethality in response to LPS administration in mice [25]. Moreover, during lethal CLP sepsis, there was a dysregulated elevation of systemic TNF-α and IL-6 levels and that PAFR blockade significantly reduced the levels of these cytokines [20]. PAFRA has been shown to reduce TNF-α production by 40% compared to that in placebo-treated animals in studies of endotoxin-induced sepsis [26]. On the other hand, in a stud carried out by Suputtamongkol et al.[13], levels in blood of the proinflammatory cytokines TNF-α, IL-6 and IL-8 were very high on admission and remained elevated in patients who developed multi organ failure with sepsis, but PAFRA (lexipafant) did not lower the levels of any of these cytokines significantly compared to the placebo treatment. Han et al.[19] have investigated the molecular mechanisms underlying the biphasic activation of NF-$_{\kappa}$B in response to LPS. They have showed that PAF, which is released in response to LPS injection, activates the early phase of NF-$_{\kappa}$B activation. This NF-$_{\kappa}$B activity leads to induction of proinflammatory cytokines (TNF and IL-1) expression, which leads to another stimulus for the synthesis of PAF, resulting in the second phase of NF-$_{\kappa}$B activation. Additionally, pretreatment with the PAF antagonist BN50739 or CV 6209 prior to LPS injection resulted in abrogation of the early peak of NF-$_{\kappa}$B. Ogata et al.[27] postulate that PAFRA block the biological effects of endo- genous PAF induced by bacteria or bacterial toxins. Therefore, PAFRA may attenuate the synergism between endogenous PAF and bacterial toxins, ultimately inhibiting inflammatory cytokine signal transduction. In a study, PAFRA inhibited LPS-induced TNF mRNA expression [28]. Also, Ishii et al.[29] reported that that the PAF receptor is not an LPS receptor but plays an important role in LPS-induced transcriptional change and calcium ion signaling. It has been reported that PAF itself activates NF-$_{\kappa}$B, inducing cytokine production and PAFR expression [30,31]. Our results show that, there was a significant elevation of systemic cytokine levels and that PAFR blockade significantly reduced the levels of these cytokines. The mechanism of the PAFRA action on LPS- induced cytokine inhibiton may be due to these effects.

In the present experiment, endotoxin injection caused statistically significant increases in serum CRP, AST, ALT, creatinine, BUN, LDH, cholesterol, triglyceride concentration *(Table 1)*, however, it caused statistically significant decrease in platelet count, total protein, albumin and glucose levels compared to control group. Serum CRP markedly increased after LPS infusion. PAFRA administration was not effective on serum CRP levels at 6 hour when compared to endo-toxemic animals receiving LPS alone ($P>0.05$) *(Table 1)*. Jeschke et al.[8] showed that serum CRP levels significantl increased in endotoxemic rats. Diaz Padilla et al.[32] concluded that rat CRP, similarly to human CRP, could activate autologous comlement, supporting that opsonization of ligands with complement is an important biological function of CRP. As has been previously demonstrated in endotoxaemic animal models by several authors [1,3,33,34], liver damage and loss of organ integrity, with subsequently the increases in plasma AST and ALT levels occur during endotoxemia as a consequence of LPS damage. We determined that LPS significantly increased hepatic enzymes AST and ALT which are markers of hepatic injury. PAFRA administration didn't exhibit protective effects on the liver, kidney and lipid metabolism of rats as judged from biochemical profile in this endoto-xaemia model. A number of studies have reported that PAF is involved in inflammatory tissue alterations associated with acute liver injury [21,35]. Earlier studies demonstrated PAF is one of the key mediators of a variety of liver injuries and that inhibition of PAF through the use of its receptor anta-gonists attenuates the extended injury [36,37]. Grypioti et al.[38] have previously reported that PAF was increased almost at the same time with all biochemical parameters (AST, ALT, ALP) indicative of liver injury in acetaminophen-induced liver toxicity in rats. Also, Grypioti et al.[10] has demonstrated that PAF-R antagonist (ginkgolide B, BN52021) attenuates liver damage and can provide important means of improving liver function following APAP intoxication. Our observation contradicts that of Grypioti et al.[10] who showed a significant improvementin the plasma levels of AST and ALT. In harmony with earlier findings [4,22,39], In this study the endo toxin increased serum cholesterol and triglycerid levels. Previous studies reported that LPS and TNF-α infusion stimulated hepatic lipogenesis with subsequent incerase cholesterol and triglycerides. This increase may be related to increased hepatic production of VLDL [40,41]. Al-Dughaym [42] reported that in endotoxaemia the decreases in TP, albumin level at 6 h may be attributed to hypovoloemia due to increased capillary permeability and reduced liver synthesis or decrease intestinal absorption which is in agreement with our observations. In harmony with earlier

findings [2,8,42], In the present study, a significant decrease in glucose concentration was observed in the endotoxaemic animals as compared to the controls. This hypoglycaemia was not suppressed by the administration of PAFRA.

Platelet count determined at 6 h after LPS injection displayed significant decreases, In endotoxaemia, the decreases in platelet count is thought to be a consequence of platelet aggregation in the lungs and other capillary beds, and of shortened platelet survival. The LPS-induced thrombocytopenia in rats is not directly mediated by PAF, because rat platelets are devoid of specific PAF receptors [43]. Thus, PAF seems to produce thrombocytopenia in rats through TNF-α production [44]. The endotoxin-induced leukopenia related to an increased adherence of activated neutrophils (expressing adhesion molecules) to endothelial cells is mainly mediated by TNF-α [45]. In our study, PAFRA significantly suppressed disturbances in leukocyte count, neutrophil and lymphocyte percentage associated with endotoxaemia. The neutropenia is followed by significant neutrophilia over the next several hours due to increased levels of activated complement products due to granulocyte colony-stimulating factor (G-CSF) and proinflammatory cytokines. Platelet-activating factor (PAF) stimulates leukocyte-endothelial cell (EC) adhesion through its effects either on leukocytes or on ECs [46]. The platelet activating factor (PAF) has been shown to play a significant role in endotoxin-induced leukocyte adherence. In harmony with our findings, The PAF receptor antagonist BN52021 attenuated the leukocyte adherence [47]. Beyer et al.[48] examined the effect of intra-abdominal contamination induced by cecal ligation and puncture (CLP) on neutrophil infiltration into the gastrointestinal tract. They found that CLP significantly increased the infiltration and a PAF receptor antagonist, WEB-2086, significantly attenuated it. In a recent study In endotoxin-induced uveitis models of rats PAF inhibitors, antagonize LPS induced leukocyte accumulation [49]. The mechanisms involved in the impairment of neutrophil migration may be due to the reduction in the levels of proinflammatory cytokines by PAFRA [50]. Leukocyte adhesion to vascular endothelium during endotoxemia was suppressed by a PAFRA in rats [51]. PAFRA blocked development of LPS-induced rat neutropenia [51,52]. Consistently vascular hyper permeability was inhibited by PAFRA [53]. This effects on hematological variables may be ascribed to the inhibiting effect of PAFRA on leukocyte migration.

In conclusion, in the current study, at the administered dose and route, PAFRA has a partial effect on inflammatory and haematological parameters; however, it has no useful effect as required by treatment with PAFRA on biochemical disturbances. Further experimental studies including administration route and the combination of PAFRA with other antiinflammatory agents are necessary to clarify its effects in endotoxaemia.

REFERENCES

1. Møller S, Laigaard F, Olgaard K, Hemmingsen C: Effect of 1,25-dihydroxy-vitamin D3 in experimental sepsis. *Int J Med Sci*, 4, 190-195, 2007.

2. Çöl R, Durgun Z: Effect of recombinant interleukin-10 on some haematological and biochemical parameters in a rat endotoxaemic model. *Acta Vet Hung,* 59, 237-245, 2011.

3. Hryszko T, Inaba K, Ihara H, Suzuki Y, Mogami H, Urano T: Nafamostat attenuated the impairment of fibrinolysis in animal sepsis model by suppressing the increase of plasminogen activator inhibitor type 1. *J Trauma*, 60, 859-864, 2006.

4. Çöl R, Durgun Z: Effect of vasoactive intestinal peptide (VIP) on cytokine levels and haemostatic and biochemical parameters in a rat endotoxaemic model. *Bull Vet Inst Pulawy*, 56, 63-67, 2012.

5. van Westerloo DJ, Giebelen IAJ, Meijers JCM, Daalhuisen J, de Vos AF, Levi M, van der Poll T: Vagus nerve stimulation inhibits activation of coagulation and fibrinolysis during endotoxemia in rats. *J Thromb Haemost*, 4, 1997-2002, 2006.

6. Gonzalez-Rey E, Chorny A, Robledo G, Delgado M: Cortistatin, a new antiinflammatory peptide with therapeutic effect on lethal endotoxemia. *J Exp Med*, 203, 563-571, 2006.

7. Cheng T, Mathews K, Abrams-Ogg A, Wood D: The link between inflammation and coagulation: influence on the interpretation of diagnostic laboratory tests. *Compend Contin Educ Vet,* 33, E1-E12, 2011.

8. Jeschke MG, Rensing H, Klein D, Schubert T, Mautes AE, Bolder U, Croner RS: Insulin prevents liver damage and preserves liver function in lipopolysaccharide-induced endotoxemic rats. *J Hepatol*, 42, 870-879, 2005.

9. Fernández-Martínez E, Morales-Ríos MS, Pérez-Alvarez V, Muriel P: Immunomodulatory effects of thalidomide analogs on LPS-induced plasma and hepatic cytokines in the rat. *Biochem Pharmacol*, 68, 1321-1329, 2004.

10. Grypioti AD, Kostopanagiotou G, Demopoulos CA, Roussos A, Mykoniatis M: Platelet activating factor (PAF) antagonism with ginkgolide B protects the liver against acute injury. importance of controlling the receptor of PAF. *Dig Dis Sci*, 53 (4): 1054-1062, 2008.

11. Koltai M, Hosford D, Guınot P, Esanu A, Braquet P: PAF, a review of its effects, antagonists and possible future implications (Part 1). *Drugs*, 42, 9-29, 1991.

12. Suputtamongkol Y, Intaranongpai S, Smith MD, Angus B, Chaowagul W, Permpikul C, Simpson JA, Leelarasamee A, Curtis L, White NJ: A double-blind placebo-controlled study of an infusion of lexipafant (Platelet-activating factor receptor antagonist) in patients with severe sepsis. *Antimicrob Agents Chemother*, 44, 693-696, 2000.

13. Horkheimer I, Schuster DP: The role of platelet-activating factor in sepsis: A bench-to-bedside review. *Advances In Sepsis*, 2 (1): 2-7, 2002.

14. Yost CC, Weyrich AS, Zimmerman GA: The platelet activating factor (PAF) signaling cascade in systemic inflammatory responses. *Biochimie*, 92 (6): 692-697, 2010.

15. Xia SH, Hu CX, Zhao ZL, Xia GD, Di Y: Significance of platelet activating factor receptor expression in pancreatic tissues of rats with severe acute pancreatitis and effects of BN52021. *World J Gastroenterol*, 13 (21): 2992-2998, 2007.

16. Travers J, Pei Y, Morin SM, Hood AF: Antiinflammatory activity of the platelet-activating factor receptor antagonist A-85783. *Arch Dermatol Res*, 290 (10): 569-573, 1998.

17. SPSS: SPSS Release 15.0 Statistical packet program, SPSS for Windows. SPSS Inc., Chicago, IL. 2006.

18. Hopper, K, Bateman, S: An updated view of hemostasis: mechanisms of hemostatic dysfunction associated with sepsis. *J Vet Emerg Crit Care*, 15, 83-91, 2005.

19. Han SJ, Ko HM, Choi JH, Seo KH, Lee HS, Choi EK, Choi IW, Lee HK, Im SY: Molecular mechanisms for lipopolysaccharide-induced biphasic activation of nuclear factor-kappa B (NF-kappa B). *J Biol Chem*, 277 (47): 44715-44721, 2002.

20. Moreno SE, Alves-Filho JC, Rios-Santos F, Silva JS, Ferreira SH, Cunha FQ, Teixeira MM: Signaling via platelet-activating factor receptors accounts for the impairment of neutrophil migration in polymicrobial sepsis. *J Immunol*, 177 (2): 1264-1271, 2006.

21. McManus LM, Pinckard RN: PAF, a putative mediator of oral inflammation. *Crit Rev Oral Biol Med*, 11, 240-258, 2000.

22. Asakura H, Takahashi Y, Kubo A, Ontachi Y, Hayashi T Omote M, Arahata M, Kadohira Y, Maekawa M, Yamazaki M, Morishita E, Takami A, Yoshida T, Miyamoto K, Nakao S: Immunoglobulin preparations attenuate organ dysfunction and hemostatic abnormality by suppressing the production of cytokines in lipopolysaccharide-induced disseminated intravascular coagulation in rats. *Critical Care Medicine*, 34, 2421-2425, 2006.

23. Iba T, Miyasho T: Danaparoid sodium attenuates the increase in inflammatory cytokines and preserves organ function in endotoxemic rats. *Critical Care*, 12, R86, 2008.

24. Mathiak G, Grass G, Herzmann T, Luebke T, Zetina CC, Boehm SA, Bohlen H, Neville LF and Hoelscher AH: Caspase-1-inhibitor ac-YVAD-cmk reduces LPS lethality in rats without affecting haematology or cytokine responses. *Br J Pharmacol*, 131, 383-386, 2000.

25. Ishii S, T Nagase, F Tashiro, K Ikuta, S Sato, I Waga, K Kume, J Miyazaki, T Shimizu: Bronchial hyperreactivity, increased endotoxin lethality and melanocytic tumorigenesis in transgenic mice overexpressing platelet-activating factor receptor. *EMBO J,* 16, 133-142, 1997.

26. Tang, HM, Teshima DY, Lum BKB: Effects of the PAF antagonist Befafant and L-659,989 in endotoxic and septic shock. *Drug Dev Res*, 29, 216-221, 1993.

27. Ogata M, Nandate K, Kawasaki T, Kawasaki C, Ozaki M, Shigematsu A: A platelet activating factor receptor antagonist inhibits cytokine production in human whole blood by bacterial toxins and live bacteria. *Anesth Analg*, 98 (6): 1767-1772, 2004.

28. Lo CJ, Cryer HG, Fu M, Kim B: Endotoxin-induced macrophage gene expression depends on platelet-activating factor. *Arch Surg,* 132, 1342-1347, 1997.

29. Ishii S, Nakamura M, Waga I, Kume K, Izumi T, Shimizu T: Cloning and characterization of a murine platelet-activating factor receptor gene. *Adv Exp Med Biol*, 407, 347-355, 1997.

30. Im SY, Han SJ, Ko HM, Choi JH, Chun SB, Lee DG, Ha TY, Lee HK: Involvement of nuclear factor kappa B in platelet-activating factor-mediated tumor necrosis factor-alpha expression. *Eur J Immunol,* 27, 2800–2804, 1997.

31. Wang H, Tan X, Chang H, Gonzalez-Crussi F, Remick DG, Hsueh W: Regulation of platelet-activating factor receptor gene expression *in vivo* by endotoxin, plateletactivating factor and endogenous tumour necrosis factor. *Biochem J*, 322, 603-608, 1997.

32. Diaz Padilla N, Bleeker WK, Lubbers Y, Rigter GM, Van Mierlo GJ, Daha MR, Hack CE: Rat C-reactive protein activates the autologous complement system, *Immunology*, 109, 564-571, 2003.

33. Çöl R, Keskin E, Atalay B: Effect of pentoxifylline on endotoxin-induced haemostatic disturbances in rabbits. *Acta Vet Hung*, 53, 325-335, 2005.

34. Yang H, Li QW, Han Z S, Hu JH, Li WY, Liu ZB: Recombinant human antithrombin expressed in the milk of non-transgenic goats exhibits high efficiency on rat DIC model. *J Thromb Thrombolysis*, 28, 449-457, 2009.

35. Zhou W, Chao W, Levine BA, Olson MS: Role of plateletactivating factor in hepatic responses after bile duct ligation in rats. *Am J Physiol*, 263, G587-G592, 1992.

36. Coker A, Coker I, Huseyinov A, Sokmen S, Karademir S: Is mannitol effective against platelet-activating factor (PAF)-induced liver damage in obstructive jaundice? *Hepatogastroenterology*, 48, 1134-1137, 2001.

37. Yang Y, Nemoto EM, Harvey SA, Subbotin VM, Gandhi CR: Increased hepatic platelet activating factor (PAF) and PAF receptors in carbon tetrachloride induced liver cirrhosis. *Gut*, 53, 877-883, 2004.

38. Grypioti AD, Theocharis SE, Papadimas GJ, Demopoulos CA, Papadopoulou-Daifoti Z, Basayiannis AC, Lykoniatis LG: Platelet-Activating Factor (PAF) involvement in acetaminophen- induced liver toxicity and regeneration. *Arc Toxicol*, 79, 466-474, 2005.

39. Yazar E, Col R, Uney K, Atalay B, Elmas M, Tras B: Effect of pentoxifylline on biochemical parameters in endotoxaemic New Zealand White Rabbits. *Bull Vet Inst Pulawy*, 48, 297-299, 2004.

40. Memon RA, Grunfeld C, Moser AH, Feingold KR: Tumor necrosis factor mediates the effects of endotoxin on cholesterol and triglyceride metabolism in mice. *Endocrinology*, 132, 2246-2253, 1993.

41. Fraunberg P, Schaefer S, Werdan K, Walli AK, Seidel D: Reduction of circulating cholesterol and apolipoprotein levels during sepsis. *Clin Chem Lab Med*, 37, 357-362, 1999.

42. Al-Dughaym AM: Some endotoxin-induced clinical and biochemical changes in plasma of camels *(Camelus dromedarius). Vet Res Commun*, 28, 711-718, 2004.

43. Inarrea P, Gomez-Cambronero J, Nieto M, Sanchez Crespo M: Characteristics of the binding of plateletactivating factor to platelets of different animal species. *Eu J Pharmacol*, 105, 309-315, 1984.

44. Rabinovici R, Esser KM, Lysko PG, Yue T, Griswold DE, Hillegass LM, Bugelski PJ, Hallenbeck JM, Feuerstein G: Priming by platelet-activating factor of endotoxin-induced lung injury and cardiovascular shock. *Circ Res*, 69, 12-25, 1991.

45. van Devender SJH, Buller HR, ten Cate JW, Aareden LA, Hack CE, Sturk A: Experimental endotoxemia in humans: Analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. *Blood*, 76, 2520-2526, 1990.

46. Ding Z, Li S, Wu Z: Mechanisms of endothelial cell-dependent leukocyte adhesion stimulated by platelet-activating factor. *Inflammation*, 16 (2): 179-186, 1992.

47. Schmidt H, Ebeling D, Bauer H, Bŏhrer H, Gebhard MM, Martin E: Influence of the platelet-activating factor receptor antagonist BN52021 on endotoxin-induced leukocyte adherence in rat mesenteric venules. *J Surg Res*, 60 (1): 29-35, 1996.

48. Beyer AJ, Smalley DM, Shyr YM, Wood JG, Cheung LY: PAF and CD18 mediate neutrophil infiltration in upper gastrointestinal tract during intra-abdominal sepsis. *Am J Physiol,* 275, G467, 1998.

49. Garland RC, Sun D, Zandi S, Xie F, Faez S, Tayyari F, Frimmel SAF, Schering A, Nakao S, Hafezi-Moghadam A: Noninvasive molecular imaging reveals role of PAF in leukocyte-endothelial interaction in LPS-induced ocular vascular injury. *FASEB J,* 25, 1284-1294, 2011.

50. Moreno SE, Alves-Filho JC, Rios-Santos F, Silva JS, Ferreira SH, Cunha FQ, Teixeira MM: Signaling via platelet-activating factor receptors accounts for the impairment of neutrophil migration in polymicrobial sepsis. *Immunol*, 177 (2): 1264-1271, 2006.

51. Schmidt H, Ebeling D, Bauer H, Böhrer H, Gebhard MM, Martin E: Influence of the platelet-activating factor receptor antagonist BN52021 on endotoxin-induced leukocyte adherence in rat mesenteric venules. *J Surg Res*, 60, 29-35, 1996.

52. Coughlan AF, Hau H, Dunlop LC, Berndt MC, Hancock WW: P-selectin and platelet-activating factor mediate initial endotoxin-induced neutropenia. *J Exp Med*, 179, 329-334, 1994.

53. Defaux JP, Thonier F, Baroggi N, Etienne A, Braquet P: Involvement of platelet-activating factor (PAF) in endotoxin- or ischaemia-induced intestinal hyperpermeability in the rat. *J Lipid Mediat*: 7, 11-21, 1993.

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RESEARCH ARTICLE

Pathological and Microbiological Investigations of Pneumonic Pasteurellosis in Sheep [1]

Zafer OZYILDIZ * Osman Yasar TEL ** Rahsan YILMAZ *
Sule Yurdagul OZSOY *** Oktay KESKIN *

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* Harran University, Faculty of Veterinary Medicine, Department of Pathology, TR-63200 Eyyubiye Campus, Sanliurfa - TURKEY
** Harran University, Faculty of Veterinary Medicine, Department of Microbiology, TR-63200 Eyyubiye Campus, Sanliurfa - TURKEY
*** Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Pathology, TR-31040 Antakya, Hatay - TURKEY

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Summary

In this study, between March 2010 and March 2011, 110 pneumonia suspected lung tissues were examined histopathologically, immunohistochemically and microbiologically, in Sanliurfa province. After definition of the macroscopic localization of the consolidated areas in the lungs, tissue samples were taken and preserved in cold chain and 10% formalin for microbiological and pathological examinations, respectively. For bacteriological examination of *Pasteurella* spp. lung specimens were inoculated in 7% sheep blood agar and Mc Conkey agar. After routine pathological tissue follow up procedure, tissues were embedded in paraffin and obtained sections stained with Hematoxylin&Eosin (H&E). The cases, having histopathological findings consistent with pneumonia, were forwarded to immunohistochemical (IHC) examinations to know whether lesions related to *Mannheimia haemolytica* and *Pasteurella multocida* using hyperimmune polyclonal rabbit sera in Avidin Biotin Complex Peroxidase (ABC-P). Microbiological, histopathological and immunohistochemical findings were comparatively evaluated in examined animals. *Pasteurella multocida* as a cause of pneumonia were detected in 38 cases of microbiological inoculations. Immunohistochemical staining resulted *Mannheimia haemolytica* (n=35) and *Pasteurella multocida* (n=30) positive. Immunohistochemically both *Mannheimia haemolytica* and *Pasteurella multocida* were positive in 23 cases and 45 animals were negative for both bacteria. The aim of this study is to show importance and role of *Pasteurella* spp, in sheep pneumonia in Sanliurfa region.

Keywords: *Pneumonic Pasteurellosis, Pathology, Microbiology, Immunohistochemistry, Sheep*

Koyun Pnömonik Pastörelloz'unda Patolojik ve Mikrobiyolojik İncelemeler

Özet

Bu çalışmada, Şanlıurfa ilinde Mart 2010-Mart 2011 tarihleri arasında, 110 adet pnömoni şüpheli akciğer dokuları histopatolojik immunohistokimyasal ve mikrobiyolojik olarak incelendi. İncelenen akciğerlerde makroskobik olarak konsolide alanların lokalizasyonu belirlendikten sonra bu bölgelerden alınan doku örneklerinin bir kısmı soğuk zincirde muhafaza edilerek mikrobiyolojik inceleme için kullanıldı. Doku örneklerinin bir kısmı %7 lik koyun kanlı agar ve Mc Conkey agara ekildi. Diğer bir kısım ise %10'luk tamponlu formaldehitte tespit edildikten sonra, rutin doku takibinden geçirilip, histopatolojik incelemeler için Hematoksilen&Eozin (H&E) ile boyandı. Histopatolojik olarak pnömoni teşhisi konulan kesitler, poliklonal tavşan *Mannheimia haemolytica* ve *Pasteurella multocida* hiperimmun serumları kullanılarak Avidin Biyotin Kompleks Peroksidaz (ABC-P) Yöntemi ile immunohistokimyasal olarak boyandı. İncelenen hayvanlarda mikrobiyolojik, histopatolojik ve immunohistokimyasal bulgular karşılaştırmalı olarak değerlendirildi. Mikrobiyolojik ekimlerde 38 vakada *Pasteurella multocida* pnömoni etkeni olarak belirlendi. Yapılan immunohistokimyasal boyamalar sonrası toplam 35 hayvanda *Mannheimia haemolytica*, 30 hayvanda *Pasteurella multocida*, 23 hayvanda her ikisinin birden ve 45 hayvanda ise nedeni belirlenmeyen farklı etkenlerin pnömoni sebebi olduğu ortaya konuldu. Bu çalışmanın amacı Şanlıurfa İlindeki koyun pnömonilerinde *Pasteurella* spp.'lerin yeri ve öneminin belirlenmesidir.

Anahtar sözcükler: *Pnömonik Pastörellozis, Patoloji, Mikrobiyoloji, İmmunohistokimya, Koyun*

İletişim (Correspondence)
+90 414 3183909
zaferozyildiz@hotmail.com

INTRODUCTION

Pasteurella spp. bacteria colonize in normal bacterial flora of the nasopharynx, and oral mucosa [1]. Transportation, crowd, climate changes, stress situations such as bad housing conditions are predisposing causes to pasteurellosis. When the respiratory defences of animals are weaken by viral infections *Pasteurella* spp. can colonize the lower respiratory tract in large numbers and induce severe fibropurulent bronchopneumonia. Thus, secondary bacterial infection can be as a major complication of acute respiratory viral diseases, such as those due to: Peste Des Petits Ruminants, Parainfluenza 3, Adenovirus and Respiratory syncytial virus [2-5].

Pneumonic pasteurellosis is one of the most lethal forms of pneumonia with bronchopneumonic and lobar pattern. It is common in many species of pets or wild animals [1,2,10]. Previously *M. haemolytica* and *P. multocida* reported in sheep in different states of disease [6,7]. Pneumonic pasteurellosis is sporadic or enzootic disease, mainly caused by *M. haemolytica* and *P. multocida* and can be seen in all age groups and in every season of the year [3,7-9]. While the acute events tend to form hemorrhagic and fibrino-necrotic bronchopneumonia, subacute to chronic events tend to form fibrinopurulent bronchopneumonia that causes fibrinous pleural adhesion and abscess [6,10]. Macroscopical aspects of lung lesions of pasteurellosis are characterized by red-black to gray-brown in color. There are consolidation areas with significantly gelatinous interlobular septal thickening, fibrinous pleuritis and coagulation necrosis areas in cranioventral lobes [10].

In pneumonic pasteurellosis, responsible agents can be placed within hemorrhagic and fibrinous exudate in bronchus, bronchiole epithelia, alveolar capillary and alveolar lumen sometimes can be placed in the periphery of syncytial cell formations and degenerated spindle-shaped leukocytes (oat cells) and at necrotic areas [6,10].

Pasteurella spp. well reproduce at blood agar, is routinely used at isolation [6]. A selective medium is required for the isolation of *M. haemolytica* and *P. multocida* when contaminated with other microorganisms [2]. When samples were taken from slaughterhouse conditions, other saprophytes contamination were often formed [11-13]. Therefore, as well as pneumonic form of the disease, for the detection of acute hemorrhagic-septicemic forms, rapid and sensitive immunoperoxidase technique are important to implement as routine [14]. There are several studies made on sheep and cattle to diagnose the disease with immunoperoxidase technique by using polyclonal *anti-Mannheimia haemolytica* and anti-*Pasteurella multocida* hyperimmune serum [1,3,14,15]. The purpose of this study was to determine the role and incidence of *Pasteurella* spp., in clinical or subclinical pneumonia cases of sheep in Sanliurfa region.

MATERIAL and METHODS

Between the dates of March 2010 and March 2011, age between 0-2, totally 110 pieces (100 from private DEM-ET Slaughter House, 10 was handed to Harran University, Faculty of Veterinary Medicine, Department of Pathology) pneumonia suspected lung tissues were examined histopathologically, immunohistochemically and microbiologically, in Sanliurfa region.

Bacteriology

For bacteriological examination of *Pasteurella* spp., lung specimens were inoculated in 7% sheep blood agar and in Mc Conkey agar. Petri dishes were incubated at 37°C, in aerobic conditions for 24-48 h. and suspicious colonies were selected and examined for *Pasteurella* spp. Some characteristics of the bacteria such as the colony morphology, hemolytic characteristics, Gram staining, oxidase, catalase, indole and reproduction at Mc Conkey agar, examined according to standard methods [5].

Histopathology

The lung tissues were fixed in 10% neutral buffered formalin and embedded in paraffin by routine methods. Sections were cut 5 µm in thickness and they were stained with H&E [16].

Immunohistochemistry

Hyperimmun sera: Polyclonal anti-*Pasteurella multocida* and anti-*Manheimia haemolytica* hyperimmune sera were obtained from Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, Aydin.

Immunohistochemical staining: The selected tissue sections for immunohistochemical staining were dewaxed and rehydrated by routine methods and stained by avidin-biotin-peroxidase complex (ABC-P) technique (Labvision, USA). Antigen retrieval was facilitated by heating the specimens in a citrate buffer (pH 6.0) for 15 min in a microwave owen at a power of 360 W and 600 W respectively. Then the slides were dipped in freshly prepared 0.3% hydrogen peroxide in methanol for 15 min, to block endogenous peroxide activity and followed by incubation with normal goat serum for 20 min at 40°C. The sections were incubated for 60 min at room temperature with one of the following primary antibodies: Polyclonal anti-*Pasteurella multocida* and anti-*Manheimia haemolytica* hyperimmune sera (in 1:1000 dilutions). Colour labeling was developed by a final incubation step using 3.3' Diaminobenzidin (DAB, Labvision, USA). Finally, sections were counterstained with Harris' haematoxylin and mounted. For positive control, previously *Pasteurella* spp. positive lung tissues (confirmed by Uludag University, Faculty of Veterinary Medicine, Department of Microbiology and Pathology) were restained. For the negative control, same sections were incubated with normal goat sera instead of primary antibody.

RESULTS

Determination of localization of consolidated areas in the sheep lungs, the each lobe of the lungs labeled by given characters A, B, C, D, E, and F *(Fig. 1)*. The localization of consolidated sites in the lungs was shown in *Table 1*. Lung samples were evaluated due to form of pneumonia such as lobar and/or lobular. According to this, the percentage of pneumonia localization was found as 66% (73/110) lobular, 16% (18/110) lobar and 17% (19/110) both of two.

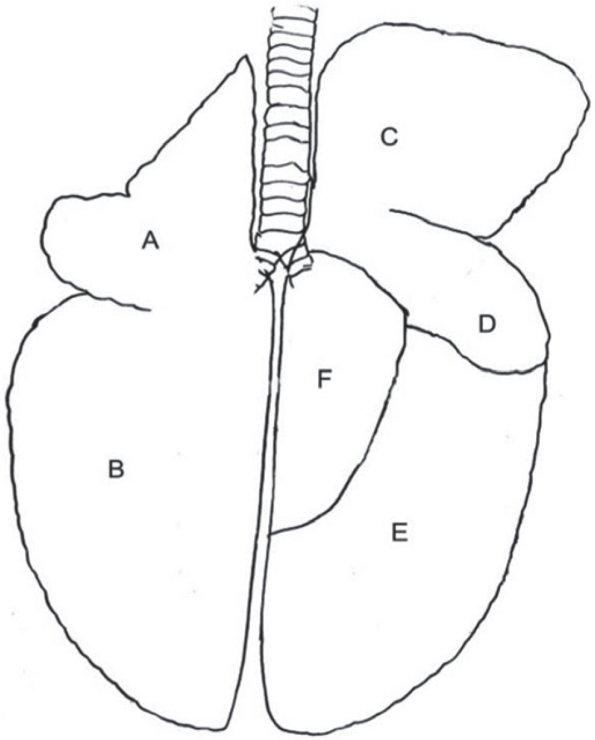

Fig 1. Denotation of lobes

(A- Left cranial lobe, B- Left caudal lobe, C- Right cranial lobe, D- Medial lobe, E- Right caudal lobe, F- Accessor lobe)

Şekil 1. Lobların isimlendirilmesi

(A- Sol cranial lob, B- Sol caudal lob, C- Sağ cranial lob, D- Medial lob, E- Sağ caudal lob, F- Accessor lob)

Table 1. *Distribution of pneumonia according to their locations*
Tablo 1. *Lezyonların makroskobik dağılımı*

Localization	Number	Localization	Number
A	10	C+D	8
B	1	C+E	2
C	56	A+B+C	1
D	1	B+C+D	1
E	1	A+C+D	6
A+C	10	C+D+E	5
D+E	1	C+D+F	2
B+E	1	A+C+D+E	1
A+D	1	A+B+C+D+E	2

Macroscopical examinations revealed that the most frequently affected lobe was right cranial lobe (C). Other affected lobes were the left cranial lobe (A), together with left and right cranial lobe (A+C), together with the right cranial and medial lobe (C+D), both two cranial lobe with right medial lobe (A+C+D) respectively. Because of the low-level combinations of the other lobes were excluded from consideration.

Consolidated areas of the lungs were swollen and dark red in color. Affected lung tissues were mostly palped as liver and crusty in consistence *(Fig. 2-A, B, C)*. At cross-section of the lungs; fine foamy fluid or creamy suppuration yellowish, gray in color were detected in some bronchus and bronchiole lumen *(Fig. 2-D)*.

Microscopically, catarrhal-purulent (n=22) and fibrinous (n =24) broncho-pneumonia which has characterized by neutrophil leukocytes and mono-nuclear cell infiltration with fibrin were seen in the bronchi, bronchiole, alveolar lumen and pleura *(Fig. 3-B, C, D)*. Multinucleated syncytial cell formations with presence of spindle-shaped oat cells were observed in the alveolar lumen. In some sections (n =23), widespread neutrophilic infiltration, coagulation necrosis with purulent-necrotic bronchopneumonia were observed in and around of the bronchus and bronchioles *(Fig. 3-A)*. In 21 lung tissue sections, bronchointerstitiel pneumonia was characterized as diffuse mononuclear cell infiltration in and around of bronchus, bronchiole and in 16 lung tissue sections, interstitial pneumonia was characterized as mononuclear cell infiltration in interstitial areas (n =16). In 4 lung tissue sections, pulmonary adenomatosis was seen. The immunohistochemical and microbiological results are shown in *Table 2*. At the end of the immunohistochemical stainings pneumonic pasteurellosis was found in 65 of 110 animals. The immunopositive areas were localized in bronchi, bronchiole *(Fig. 4-B, C)* and alveolar *(Fig. 4-A, D)* lumen and epithelia, interstitium, vein lumen and peribronchial glands. The microscopical distributions of agents are shown in *Table 3*.

DISCUSSION

Pneumonic pasteurellosis is one of the important infectious diseases of the respiratory system observed in Turkey. Recent studies have shown that the disease is an important health problem at ruminants [10,14,17-19]. In many geographic regions of Turkey, *Pasteurella* spp. responsible for the most pneumonia cases in small ruminants [9,14,15,19]. According to our study *Pasteurella* spp. is holding an important place in sheep pneumonia in Sanliurfa region.

Pasteurella pneumonia is usually indicated lobar distribution and often fibrinous, purulent, necrotic lung infections [10]. In this study pneumonia was lobular rather than lobar (68 animals). This situation may be associated with animals being butchered, thus the lesions are not formed

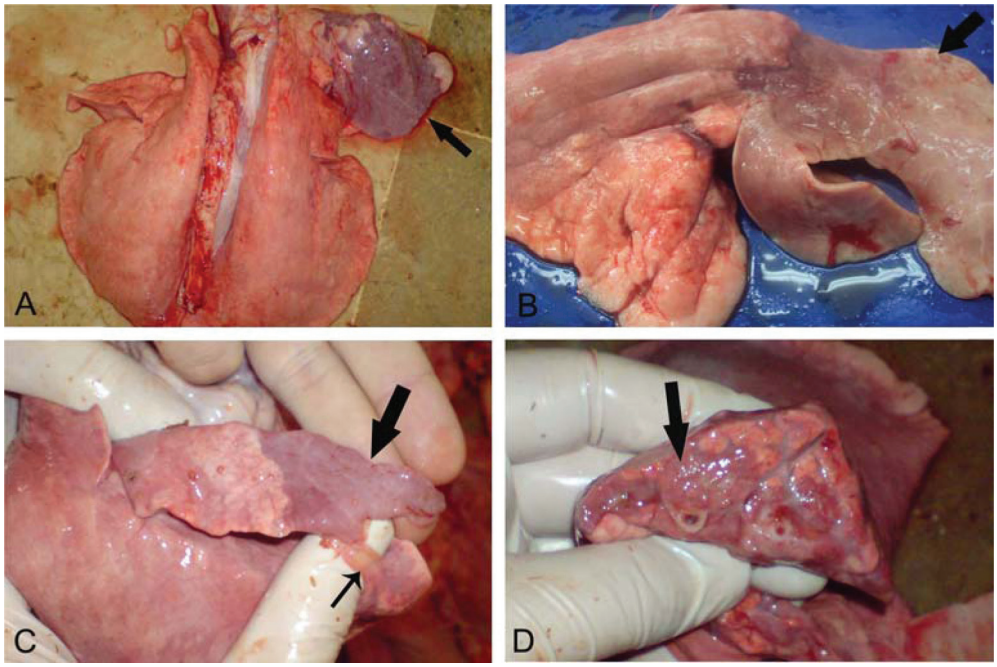

Fig 2. A- Right cranial lobe, lobar pneumonia *(arrow)*, dorsal appearance, B- Right cranial lobe with medial lobe, lobar pneumonia *(arrow)*, dorsal appearance, C- Medial lobe lobular pneumonia *(arrow)*, adhesion on visceral pleura *(thin arrow)*, dorsal appearance, D- The cut section of pneumonic areas

Şekil 2. A- Sağ kranial lob, lober pnömoni *(ok)*, dorsalden görünüm, B- Sağ kranial lob ile medial lobda lober pnömoni *(ok)*, dorsalden görünüm, C- Medial lobda lobuler pnömoni *(ok)*, viseral plörada adezyon *(ince ok)*, dorsalden görünüm, D- Pnömonik alanların kesit yüzü

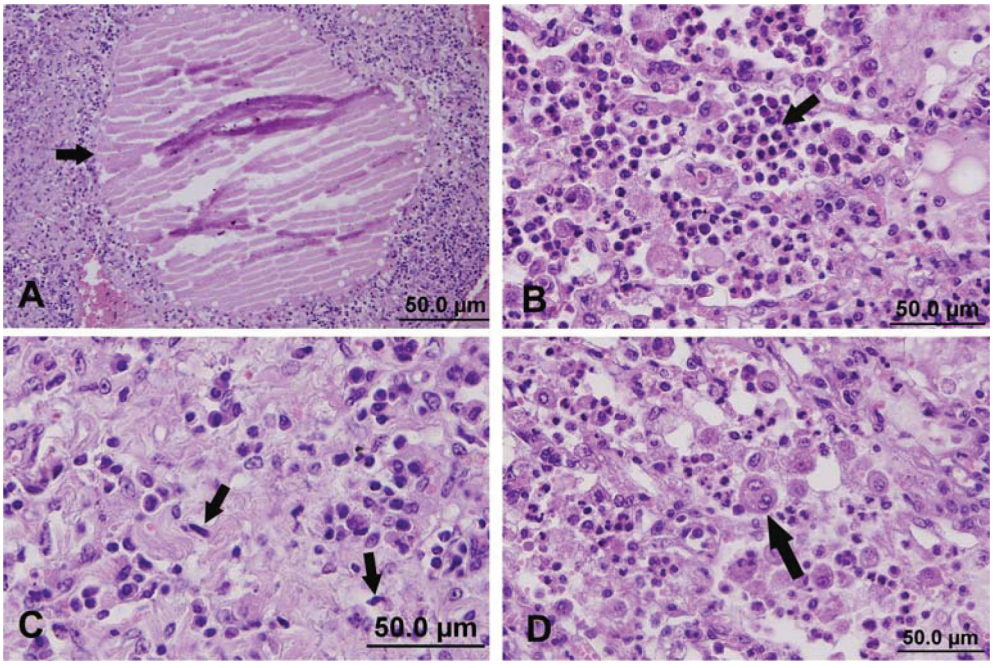

Fig 3. A- Coagulation necrosis *(arrow)* surrounded with inflammatory cells, H&E x200, B- Neutrophil leukocytes and mononuclear cell infiltration *(arrow)* in alveoli lumen, H&E x400, C- Oat cells *(arrows)* H&E x400, D- Syncytial cell formation *(arrow)*, H&E x400

Şekil 3. A- Etrafı yangı hücreleri ile çevrili koagulasyon nekrozu (ok), H&E x200, B- Alveol lümeninde nötrofil lökosit ve mononükleer hücreler *(ok)*, H&E x400, C- Yulaf hücreleri *(oklar)* H&E x400, D- Sinsityal hücre oluşumu *(ok)*, H&E x400

whole. In addition, animals with the complaint of cough, nasal flow and respiratory distress cannot be completely treated, should be considered.

In the study, consolidated areas were present at the contact surfaces of lobes close to each other, and often detected at the right cranial lobe together with medial lobe.

Table 2. *Pasteurella positive animal number according to results of immunohistochemical staining and microbiological isolation*
Tablo 2. *IHC boyama ve mikrobiyolojik muayene sonuçlarına göre pastörella pozitif hayvan sayısı*

Microorganism	IHC (n=110)	Microbiological isolation (n=110)
M. Haemolytica (+)	35	0
P. Multocida (+)	30	38
Both of them (+)	23	-
Both of them (-)	45	72

pneumonia (21 cases) was also significant. These findings indicated that important part of pneumonia was also possibly a complication of viral infections. Therefore, this study revealed that in this region viral infections should also be considered.

In this study the microbiological results obtained from a total of 110 animals, were not exactly the same with positive reactions at immunohistochemical stainings. However, immunohistochemically *M. haemolytica*-positive samples were found (n=33), *M. haemolytica* was not isolated

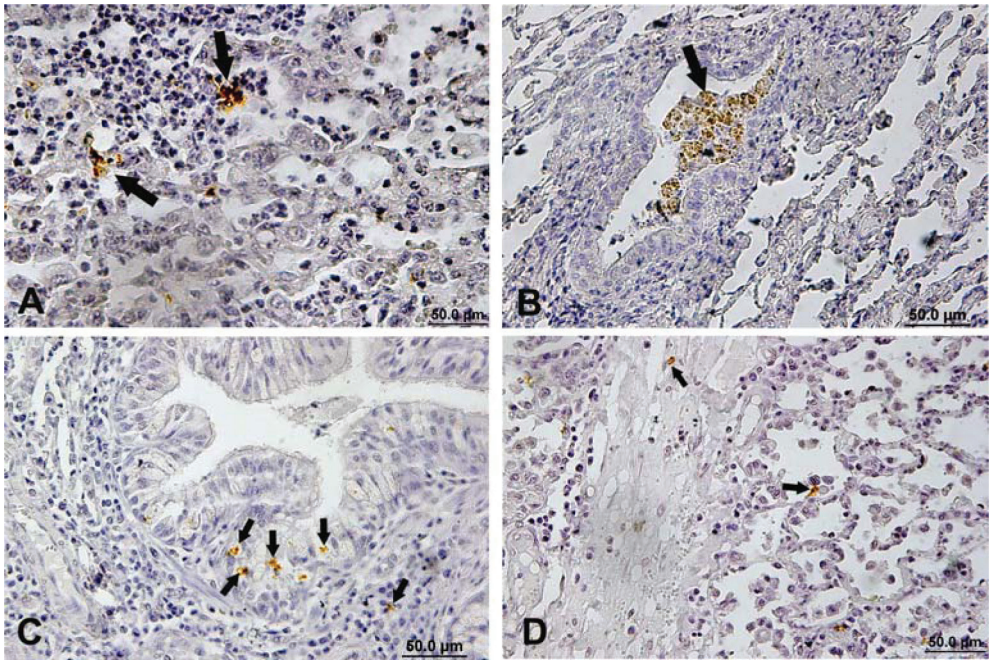

Fig 4. A- Alveolar lumen, immunpositive staining in neutrophil leukocytes *(arrows)*, ABC method counterstained with Harris haematoxylin x400, B- Bronchiole lumen, immunpositive staining in exudate *(arrow)*, ABC method counterstained with Harris haematoxylin x400, C- Bronchiole epithelium and peribronchial area, immunpositive staining in epithelium and mononuclear cell *(arrows)*, ABC method counterstained with Harris haematoxylin x400, D- Alveolar lumen and interstitial area, immunopositive agents (arrows), ABC method counterstained with Harris haematoxylin x400

Şekil 4. A- Alveol lümeni, nötrofil lökositler içinde pozitif boyanmış etkenler *(oklar)*, ABC, Harris haematoxylin X400, B- Bronşiol lümeni, eksudat içerisinde pozitif boyanmış etkenler *(ok)*, ABC, Harris haematoxylin x400, C- Bronşiol epiteli ve peribronşiol bölge, bronşiol epiteli ve mononükleer hücre içerisinde pozitif boyanmış etkenler *(oklar)*, ABC, Harris haematoxylin x400, D- Alveol lümeni ve intersitisyel alan, serbest haldeki etkenler *(oklar)*, ABC, Harris haematoxylin x400

Table 3. *The distribution of agents at lung tissues with IHC staining*
Tablo 3. *IHC boyamalarda etkenlerin akciğer dokusundaki dağılımı*

IHC (+) Agent	Alveolar epithelium	Alveolar lumen	Bronchi, bronchiole epithelium	Bronchi, bronchiole lumen	Interstitium	Vein Lumen	Peribronchial glands
P. Multocida	23	17	25	23	17	2	5
M. Haemolytica	27	30	29	24	10	3	4

This situation supports that the disease either spread by the endobronchial way or spread by contact to the adjacent lobe pleura like other bacterial agents such as *E. coli*, *Corynebacterium* spp, *Mycoplasma* spp. [11,13]. In histopathological examination, the predominant lesion was bronchopneumonia (catarrhal-purulent: 22, fibrinous: 24, purulent-necrotic: 23). However broncho-interstitiel

in terms of any microbiological cultivation results. Classic inoculation methods give reliable results in the diagnosis of pasteurellosis [20,21]. The histopathologic diagnosis shows the type and the presence of pneumonia, the presence of the agent in the tissue can be detected by immunohistochemical methods [1,14,15]. Barely hygienic conditions in slaughter houses is not satisfactory, the lungs are put in

bulk containers gregariously and hence the inevitability of contamination with other bacteria, because of unconscious use of antibiotics, the reproductive ability of the agent is weak or dead, possible causes of incoordination between the results of microbiological and immunohistochemical examinations. Similar problems were encountered in previous studies [13,14,19]. Immunohistochemical methods are revealing to bacterial agent either free or inside of phagosome. Because of this, immunohistochemical staining method is more sensitive than classic microbiological methods. Present results showed that IHC can be used routinely for the diagnosis of pasteurellosis. Nevertheless for the definitive diagnosis of pneumonic pasteurellosis, combined results of microbiological isolation and immunohistochemical staining will give more reliable results.

Pneumonic pasteurellosis causes serious field losses and animal death [17]. Biggest problems in this subject are; breeders in the region does not take precautions or treat their animals unless there are large number of deaths and incompleted treatments or random medicine use makes disease asymptomatic or chronic thus tricking breeders into thinking that their animals are get well. At the same time because of the animals are housed in closed barns, in inadequate ventilation especially during the winter months and as a result of ammonia irritation *Pasteurella* spp. opportunism is increases. By changing the care and feeding conditions and the vaccination studies in the region, is thought to be important steps in solving the problem.

As a result, by this study we described pneumonic pasteurellosis in sheep by pathologic, immunohistochemical and microbiologic methods in Sanliiurfa region.

Acknowledgement

We are thankful to Adnan Menderes University Veterinary Faculty Department of Microbiology for polyclonal *Pasteurella multocida* and *Manheimia haemolytica* hyper-immun serum supply.

REFERENCES

1. Ackermann MR, Brogden KA: Response of the ruminant respiratory tract to *Mannheimia (Pasteurella) haemolytica. Microbes and Infection*, 2, 1079-1088, 2000.

2. Aydın N: Pasteurellaceae familyası. **In,** Arda M, Minbay A, Leloğlu N, Kahraman M, Akay Ö, Ilgaz A, İzgür M, Diker KS (Eds): Özel Mikrobiyoloji, Epidemiyoloji, Bakteriyel ve Mikotik İnfeksiyonlar. 4. Baskı, s. 64-74. Medisan, Ankara, 1997.

3. Blanco-Viera FJ, Trigo FJ, Jaramillo-Meza L, Aquilar-Romero F: Serotypes of *Pasteurella multocida* and *Pasteurella haemolytica* isolated from pneumonic lesions in cattle and sheep from Mexico. *Rev Latinoaaemericana de Microb,* 37, 121-126, 1995.

4. Brogden KA, Lehmkuhl HD, Cutlip RC: *Pasteurella haemolytica* complicated respiratory infections in sheep and goats. *Vet Res*, 29, 233-254, 1998.

5. Quinn PJ, Carter ME, Markey BK, Carter GR: Clinical Veterinary Microbiology. Mosby-Year Book. pp. 254-258. Dublin, 1994.

6. Gilmour NJL: P*asteurella haemolytica* infections in sheep. *Vet Quart*, 2, 191-198, 1980.

7. Gilmour NJL, Gilmour JS: Pasteurellosis of sheep. **In,** Adlam CF, Rutter JM (Eds): Pasteurella and Pasteurellosis. pp. 223-262. Academic Press Inc, New York, 1989.

8. Holt JG, Kreig NR, Sneath PHA, Staley JT, Williams ST: Bergey's manual of determinative bacteriology. **In,** Hensley WR (Ed): Pasteurella and Pasteurellosis. pp. 281-284. Williams and Wilkins, Baltimore, USA, 1994.

9. Kaya O, Erganis O: Koyun ve kuzu pnömonilerinde etiyolojik survey. *Veterinarium*, 32, 27-29, 1991.

10. Hazıroglu R, Milli UH: Veteriner Patoloji. Cilt 2, s. 80-85. Özkan Matbaacılık, Ankara, 1998.

11. Hazıroglu R, Diker KS, Gulbahar MY, Akan M, Guvenc T: Studies of the pathology and microbiology of pneumonic lungs of lambs. *Dtsch Tierarztl Wschr*, 101, 441-443, 1994.

12. Ilhan Z, Keles I: Biotyping and serotyping of *Mannheimia (Pasteurella) haemolytica* isolated from lung samples of slaughtered sheep in the Van region. *Turk J Vet Anim Sci*, 31, 137-141, 2007.

13. Oruc E: The pathologic and bacteriologic comparison of pneumonia in lambs. *Turk J Vet Anim Sci,* 30, 593-599, 2006.

14. Hazıroglu R, Diker KS, Turkaslan J, Gulbahar MY: Detection of *Mycoplasma ovipneumoniae* and *Pasteurella haemolytica* antigens by an immunoperoxidase technique in pneumonic ovine lungs. *Vet Pathol*, 33, 74-76, 1996.

15. Yener Z, Ilhan F, Ilhan Z, Saglam YS: Immunohistochemical detection of *Mannheimia (Pasteurella) haemolytica* antigens in goats with natural pneumonia. *Vet Res Commun*, 33, 305-313, 2009.

16. Luna LG: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed., pp. 232-241, The Blakiston Division, Mc Graw-Hill Book Company, New York, 1968.

17. Batmaz H: Pasteurella pneumonileri ve ekonomik önemi. **In,** *Sığırların Solunum Sistemi Hastalıkları Sempozyumu, Uludağ Universitesi, Veteriner Fakultesi,* TUBITAK: Bursa, s. 58-64, 2006.

18. Erbas G, Kaya O: Aydın ve İzmir Bölgesindeki sığırlardan *Pasteurella multocida*'nın izolasyonu, tiplendirilmesi ve antibiyotiklere olan duyarlılıkları. *Bornova Vet Kont Araşt Enst Derg,* 30, 7-14, 2008.

19. Kiran MM, Berkin S, Kaya O, Dincer Z: Konya bölgesi koyun pnömonilerinde patolojik ve etiyolojik araştırmalar. *SÜ Vet Fak Derg*, 9, 3-9, 1993.

20. Kırkan S, Kaya O: Serotyping of *Mannheimia haemolytica* strains isolated from pneumonic lungs of sheep in the Aydın region of Turkey. *Turk J Vet Anim Sci*, 29, 491-494, 2005.

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RESEARCH ARTICLE

Kars İli Sığır İşletmelerinde Barınakların Mevcut Durumu ve Yetiştirici Talepleri: I. Mevcut Durum [1]

Muammer TİLKİ * Mehmet SARI * Erol AYDIN ** Serpil IŞIK * Ali Rıza AKSOY *

[1] *Kars İl Gıda, Tarım ve Hayvancılık Müdürlüğü tarafından hazırlanan TRA2-11-DFD-009 nolu proje Serhat Kalkınma Ajansı (SERKA) tarafından desteklenmiştir*

* Kafkas Üniversitesi Veteriner Fakültesi, Zootekni Anabilim Dalı, TR-36100 Kars - TÜRKİYE
** Kafkas Üniversitesi Veteriner Fakültesi, Hayvancılık Ekonomisi ve İşletmeciliği Anabilim Dalı, TR-36100 Kars - TÜRKİYE

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Özet

Bu araştırmada, Kars ili merkez ve ilçelerine ait sığır işletmelerinin genel yapısı incelenmiş ve değerlendirilmiştir. Araştırmada anket metoduyla Kars ili Merkez ilçeden 87, Akyaka'dan 50, Arpaçay ve Digor'dan 44'er, Kağızman'dan 40, Sarıkamış'tan 49, Selim'den 47 ve Susuz'dan 51 anket olmak üzere toplamda 412 işletme değerlendirilmiştir. Bölgede en yaygın kullanılan barınak tipinin kapalı bağlı barınaklar olduğu tespit edilmiştir. Kapalı bağlı sistem yetiştiricilik yapmayı seçen 326 işletme sahibinin (%79.13), bu sistemi tercih etmesinin nedenleri arasında ilk sırayı "*hayvanların bakım-besleme ve idaresinin kolay olduğu*" seçeneği almıştır. Bunu "*geleneksel olduğu*" ve "*diğer sistemlere göre daha sağlıklı olduğu*" için yapılan tercihler takip etmiştir. Anket uygulaması yapılan 26 işletmeye ait barınakta hiç havalandırma bacası olmadığı, 15 işletmede ise yalnızca 1 adet havalandırma bacası olduğu tespit edilmiştir. Araştırmada 43 işletmeye ait barınakta pencere bulunmadığı, 23 işletmede ise yalnızca 1 adet pencere olduğu saptanmıştır. İşletmelerin genelinde kış şartlarının ağır olması gerekçesi ile pencere ebatlarının standartların altında olduğu tespit edilmiştir. Araştırmada işletmelerde hayvanların büyük bir oranda elle sağıldığı, bunu seyyar süt sağım makineleri ile sağımın takip ettiği belirlenmiştir. Sağım üniteleri bölgede yeni yaygınlaşmaya başlamıştır. Özellikle sağım öncesi memelerin temizliği ve sağım sonrası sütlerin muhafazasında hijyen kurallarına yeterince dikkat edilmediği görülmüş, geleneksel işletmelerin hemen tamamında sütlerin plastik bidonlarda depolandığı belirlenmiştir. Araştırma sonucunda, Kars ve çevresinde mevcut barınaklarda birçok yetersizlik ve eksiklikler tespit edilmiştir. Ayrıca bölgedeki yetiştiricilerin bazı konularda bilgilerinin yetersiz olduğu da gözlemlenmiştir. Bunun için yetiştiricilere bakım, besleme, sağlık, hijyen gibi konularda eğitim verilmesinin yararlı olacağı sonucuna varılmıştır.

Anahtar sözcükler: *Kars, Sığır barınağı, Çiftlik demografisi, İşletme yapısı*

Current Status of Cattle Shelters in Livestock Enterprises and Breeder Demands in Kars: I. Current Status

Summary

In this study, the overall structure of cattle livestock from all the districts of Kars was evaluated. The research data consisted of a total of 412 questionnaires of which 87 gathered from central district of Kars, 50 from Akyaka, 44 from Arpacay, 44 from Digor, 40 from Kağızman, 49 from Sarıkamış, 47 from Selim, and 51 from Susuz. The study revealed that closed system shelters were the most widely used system in livestock. Among the 326 animal producers who used closed binded system barns "*convenience in the management*" ranked first reason (79.13%) to use this system. The other reasons for the system were listed as "*traditional*" and "*healthier than other systems*". The study also revealed that the 26 livestock barns did not have any air ventilation and 15 barns had only one concluding insufficient air flow. A total of 43 barns surveyed did not have any chimneys and 23 had only one small size window. The reduction in the window number and size is associated with the severe winter conditions in the region. The research results indicated that hand milking is the most common method followed by portable milking machines and the usage of milking units found to be new in the region. Cleaning udder pre-milking and preservation of milk after milking are the two main deficiencies in the region compare to the standard hygiene rules and the milk is almost exclusively stored in plastic drums. As a result, Kars and its environs have been identified deficiencies and many lack in existing enterprises. Therefore, we concluded that animal husbandry in the region is hampered with underutilization mainly due to the insufficient farmer training. Hence, it is advisable to train the farmers on the ground of the animal welfare, feeding, health, hygiene.

Keywords: *Kars, Cattle shelter, Farm demography, Livestock structure*

İletişim (Correspondence)
+90 474 2126807/5112
mtilki@hotmail.com

GİRİŞ

Nüfus bakımından Türkiye'nin orta büyüklükteki illeri arasında yer alan Kars, 2011 yılı Adrese Dayalı Nüfus Sistemi (ADNS) sonuçlarına göre toplam 305.755 kişi nüfusa sahiptir. Türkiye İstatistik Kurumu (TÜİK)'nun 2010 yılı verilerine göre Kars ilinin ülke toplam nüfusu içindeki payı yaklaşık %0.4 olup, il nüfusunun %57.8'i kırsalda, %42.2'si ise şehirde yaşamaktadır. Kars coğrafi bölge sınıflandırmasına göre Doğu Anadolu Bölgesi'nde (DAB), İstatistikî Bölge Birimleri Sınıflandırması (İBBS) düzey-1'e göre Kuzeydoğu Anadolu Bölgesi'nde (KDAB-TRA), düzey-2'ye göre TRA2'de bulunmaktadır. Türkiye geneli sosyo-ekonomik gelişmişlik sıralamasında 2011 yılı itibariyle Kars ili 68. sırada yer almaktadır [1-3].

Kars ilinin merkez ilçesi dâhil 8 ilçesi, 10 belediyesi ve 384 köyü vardır. Yüzölçümü 9.442 km^2'dir. Rakımı ortalama 1768 metreyi bulan Kars ili arazisinin büyük bölümü yaylalardan oluşmuştur [4,5].

Toplam istihdamın içinde tarım ve hayvancılık sektörünün payı, Türkiye genelinde %23.7, TRA2'de ise %70.2 oranındadır. Gayri Safi Milli Hasıla (GSMH)'dan sektörlerin aldıkları paylar incelendiğinde; tarım ve hayvancılık sektörü, Türkiye genelinde %8.5, TRA2'de %24.6 oranında paya sahiptir [6]. İstihdam ve GSMH'nın sektörel dağılımına bakıldığında Kars ili kırsal ekonomi karakteri göstermektedir [7].

Kars ilinin merkezi, ilçeleri ve köylerindeki en temel ekonomik sektör hayvancılıktır. Yöredeki coğrafi şartlar nedeniyle diğer yörelere göre; tarımsal üretime uygun zaman diliminin kısa ve birim alan başına verimin düşük olması, beraberinde hayvancılık sektörünün güçlenmesini getirmektedir. Yöre insanı mera ve çayırların fazlalığı sayesinde hayvancılıkla uğraşarak geçimlerini sağlamaktadırlar. Çayır ve mera arazileri %39.1 ile tarımsal araziden daha geniştir. Bu oranın büyük oluşu ilde hayvancılığın gelişimine büyük katkı sağlamaktadır. Ancak ilde otlak alanları çok olmasına rağmen, modern usullerle hayvancılık yapılmamaktadır. Hayvancılık il genelinde genellikle aile ihtiyaçlarını karşılamak amacıyla yapılan geçimlik bir faaliyettir. Kars ili ekonomisinde önemli yeri olan hayvansal ürünlerin pazarlanmasında sorunlar bulunmaktadır. Hayvan yetiştiricisi sütünü eder fiyatından düşük değerde avans sistemi ile pazarlarken, canlı hayvanını ise besi olgunluğuna ulaşmadan batı bölgelerine satmaktadır [5,7,8].

Kars ilinde kültür ırkı hayvan sayısı istenilen düzeyde değildir. Türkiye geneli sığır varlığı içinde kültür ırkı sığırların payı %36.92 oranında iken, Kars ilinde %3.98 düzeyindedir. Melez sığırların sayısında ise geçmiş yıllara oranla bir artış söz konusudur. Ancak ildeki yerli sığırların sayısı oldukça fazladır. Bu durum sığırlardan alınan süt ve et gibi bazı verimlerin istenilen miktarda olmasına engel olmaktadır. İlde sayısal üstünlüğe sahip yerli ırkların et ve süt veriminin, kültür ve melez ırklara göre düşük olması, ilde hayvancılıkta verimliliğin artırılması önünde en büyük engellerden birisidir [8,9].

Sığır yetiştiriciliğinde en önemli çevre koşullarından birisi barınakların durumu ve uygun barınak tipinin seçimidir. Yapılan çalışmalar, çevresel faktörlerin sığırların sağlık ve performansı üzerinde önemli etkileri olduğunu ortaya koymuştur. Hayvan barınakları konusunda bölgede ciddi sorunlar olduğu bilinmektedir. Çoğu barınakta yem deposu, gübre deposu ya da gübre yeri gibi fiziki imkânlar bulunmamaktadır. Diğer taraftan barınaklarda hayvanların temiz hava, sıcaklık ve nem gibi çevre isteklerinin en iyi biçimde karşılanmasına dikkat edilmemektedir. Ayrıca barınaklarda sağım, gübre temizliği ve yemleme gibi bakım işlerine yönelik uygun şartlar sağlanamamaktadır. Bölgedeki barınakların birçoğunda hayvan başına olması gereken alan yetersizdir. Öte yandan barınaklarda hayvanların yaşlarına, cinsiyetlerine, gebe veya hasta olmalarına göre ayrı yerlerde tutulmaları gerekirken bu ayrım yapılmamakta ve genelde hayvanların tamamı bir arada bulundurulmaktadır. Bazen ahırların bir kısmında buzağı bölmesi bulunduğuna ve erkek damızlıklarında bağlı olarak ahırın bir köşesinde olduğuna rastlanılmaktadır. Bölgede hayvan barınaklarının önemli bir kısmı, hem et ve süt verimini düşürecek hem de hayvan ve insan sağlığını tehdit edecek nitelikte gelişmemiş yapılar olup, bazı barınaklar ise evler ile bitişik şekildedir [10-13].

Bu araştırma, Kars ili merkez ve ilçelerinde mevcut sığır işletmelerinin genel özelliklerini ortaya koymak ve bu özellikleri değerlendirmek amacıyla yapılmıştır.

MATERYAL ve METOT

Bu araştırmada, Kars ili Merkez, Akyaka, Arpaçay, Digor, Kağızman, Sarıkamış, Selim ve Susuz ilçelerinde yer alan sığır işletmelerinin genel yapısı incelenmiş ve değerlendirilmiştir. Araştırmanın materyalini işletme sahipleri veya sorumlu kişilerle yüzyüze anket metodu ile elde edilen veriler oluşturmuştur. İşletmelerin seçiminde tesadüfü örnekleme yönteminden yararlanılmıştır. Anket soruları iki kısımdan oluşmuştur. Bunlardan birincisi demografik yapı, ikincisi ise barınak ve çevre düzenlemesi ile ilgili bilgilerdir.

Çalışma kapsamında; Kars ili Merkez ilçeden 87, Akyaka'dan 50, Arpaçay ve Digor'dan 44'er, Kağızman'dan 40, Sarıkamış'tan 49, Selim'den 47 ve Susuz'dan 51 anket olmak üzere toplamda 412 anket yapılmıştır. Anket çalışmaları yapılırken İl Gıda, Tarım ve Hayvancılık Müdürlüğü personelinden de faydalanmıştır. Bunun için anket yapacak personelle önceden toplantı yapılmış, anket soruları ile ilgili kapsamlı bilgiler verilmiştir.

Anket uygulanan 8 ilçe ve 189 köyde toplam sığır sayısı 16.066 baş olup ilçelere göre dağılım ise şu şekildedir. Kars ili Merkez ilçesinde 3.560, Akyaka'da 2.493, Arpaçay'da 1.568, Digor'da 1.790, Kağızman'da 1.601, Sarıkamış'ta 1.589, Selim'de 1.789 ve Susuz'da 1.676 baş sığır işletmelerde belirlenmiştir.

Araştırma sonucu elde edilen verilerin değerlendirilmesinde karşılaştırılmalı istatistikî analizlerin kullanılmasına

gerek görülmemiştir. Elde edilen verilerin tanımlayıcı istatistikleri, frekansları ve yüzde dağılımları hesaplanmıştır. Verilerin elektronik ortama girilmesi ve hesaplamalarda Microsoft Office 2010 Excel ve SPSS for Windows (16.0) programları kullanılmıştır.

BULGULAR

Araştırma kapsamında Kars merkez ve ilçelerinde toplam 412 anket uygulanmıştır. Anket sayısı ilçelere göre 40 ile 87 arasında değişmekte olup, en az anket Kağızman'da en fazla ise Merkez ilçede yapılmıştır *(Tablo 1)*. Anket yapılan işletme sahiplerinin hane büyüklüğü ortalama 7.17±0.17 kişi olarak belirlenmiştir. Kaç yıldır sığır yetiştiriciliği yapıldığı sorusuna ortalama 30.20±0.69 yıl olarak cevap verilmiştir. İldeki barınakların ortalama yaşı 18.19±0.71 yıl olarak belirlenmiş olup, Susuz ve Arpaçay ilçelerindeki barınakların yaşı diğer ilçelere göre daha yüksek bulunmuştur.

Sığır yetiştiriciliğinin ekonomik etkinliği ve üretimde verimlilik açısından eğitimin öneminin büyük olduğu bilinmektedir. Araştırmada işletme sahiplerinin %75 oranında ilk ve ortaokul mezunu olduğu belirlenmiştir *(Tablo 2)*.

Anket yapılan işletmelerde kullanılan ahır tipleri *Tablo 3*'te verilmiştir. Buna göre 412 işletmede en yaygın ahır tipi kapalı bağlı ahırlardır. Bu barınakları ilde bulunan çok az miktarda kapalı serbest dolaşımlı ahırlar takip etmektedir.

Kapalı bağlı sistem yetiştiricilik yapmayı seçen 326 işletme sahibinin (%79.13), bu sistemi tercih sebepleri *Tablo 4*'te gösterilmiştir. Buna göre yetiştiricilerin kapalı bağlı sistemi tercih etmesinin nedenleri arasında ilk sırayı "hayvanların bakım-besleme ve idaresinin kolay olduğu" seçeneği almıştır.

İlde mevcut barınakların yapı malzemesi ile ilgili sonuçlar *Tablo 5*'te verilmiştir. Buna göre en yaygın yapı malzemesinin %39.81 ile taş olduğu belirlenmiştir. Bunu betonarme yapılar takip etmektedir (%35.44).

Yetiştiricinin elinde bulunan barınakların kapasiteleri *Tablo 6*'da verilmiştir. Buna göre en yüksek barınak kapasitesi %44.42 oranı ile 21-50 baş arası işletmelerde belirlenmiştir. Bunu %29.37 oranı ile 11-20 baş arası işletmeler takip etmektedir.

Mevcut işletme sahiplerinin üye olduğu üretici birlikleri *Tablo 7*'de verilmiştir. İşletme sahiplerinden 222 (%53.88) kişi herhangi bir üretici örgütüne üye olmadığını ifade ederken, 190 (%46.12) katılımcı en az bir üretici örgütüne kayıtlı olduğunu beyan etmiştir.

Mevcut işletmelerde kullanılan sağım yöntemleri

Tablo 1. *Büyükbaş hayvancılık yapılan işletmelere ait demografik bilgiler*
Table 1. *The demographic data of livestock*

İlçeler	Anket Sayısı (n)	Hane Halkı Büyüklüğü (Kişi) (X+Sx)	Sığır Yetiştiriciliği Tecrübesi (Yıl) (X+Sx)	Barınak Yaşı (Yıl) (X+Sx)
Merkez	87	6.64±0.28	33.06±1.64	15.01±1.29
Akyaka	50	5.98±0.41	31.74±2.09	19.78±2.28
Arpaçay	44	6.27±0.41	30.59±1.80	23.89±2.72
Digor	44	9.70±0.77	21.63±1.58	14.02±1.70
Kağızman	40	8.88±0.64	25.60±1.86	14.68±1.86
Sarıkamış	49	7.73±0.44	30.80±1.99	16.88±1.86
Selim	47	6.62±0.35	32.43±1.88	18.09±2.18
Susuz	51	6.45±0.46	31.71±2.02	24.82±2.02
Kars İl Toplamı	412	7.17±0.17	30.20±0.69	18.19±0.71

Tablo 2. *Sığır yetiştiriciliği yapan işletme sahiplerinin eğitim durumları*
Table 2. *Educational status of the cattle livestock owners*

İlçeler	Okur-Yazar Olmayan		İlkokul Mezunu		Ortaokul Mezunu		Lise Mezunu		Üniversite Mezunu	
	Frekans	%	Frekans	%	Frekans	%	Frekans	%	Frekans	%
Merkez	2	2.30	47	54.02	16	18.39	20	22.99	2	2.30
Akyaka	6	12.00	30	60.00	6	12.00	7	14.00	1	2.00
Arpaçay	-	0.00	21	47.73	8	18.18	11	25.00	4	9.09
Digor	1	2.27	33	75.00	8	18.18	1	2.27	1	2.27
Kağızman	1	2.50	19	47.50	12	30.00	5	12.50	3	7.50
Sarıkamış	1	2.04	29	59.18	13	26.53	5	10.20	1	2.04
Selim	1	2.13	22	46.81	10	21.28	14	29.79	-	0.00
Susuz	4	7.84	28	54.90	7	13.73	10	19.61	2	3.92
Kars İl Toplamı	16	3.88	229	55.58	80	19.42	73	17.72	14	3.40

Tablo 3. *İşletmelerdeki mevcut ahır tipleri*
Table 3. *Type of barns*

İlçeler	Kapalı Bağlı Sistem		Kapalı Serbest Dolaşımlı Sistem		Diğer	
	Frekans	%	Frekans	%	Frekans	%
Merkez	83	95.40	3	3.45	1	1.15
Akyaka	47	94.00	2	4.00	1	2.00
Arpaçay	42	95.45	0	0.00	2	4.55
Digor	44	100.00	0	0.00	0	0.00
Kağızman	38	95.00	1	2.50	1	2.50
Sarıkamış	48	97.96	0	0.00	1	2.04
Selim	47	100.00	0	0.00	0	0.00
Susuz	47	92.16	2	3.92	2	3.92
Kars İl Toplamı	396	96.12	8	1.94	8	1.94

Tablo 4. *Kapalı bağlı sistem yetiştiricilik yapmayı seçen işletme sahiplerinin, tercih sebepleri*
Table 4. *The reasons of preferring a closed system of breeding binded livestock by owners*

Kapalı Bağlı Sistem Yetiştiriciliği Tercih Sebepleri	Değerlendirme		İlk Sırada Söylenme	
	Puan	Sıralama	Frekans	Oran (%)
Hayvanların bakım, besleme, idaresi kolay olduğu	1751	1	118	36.65
Geleneksel olduğu	1327	2	111	34.47
Diğer sistemlere göre daha sağlıklı olduğu	1141	3	47	14.60
Hayvan daha iyi verim gösterdiği	970	4	24	7.45
Daha kârlı olduğu	641	5	10	3.11
Kredi ve teşviklerden yararlanabilme	263	6	5	1.55
Diğer	88	7	7	2.17

Tablo 5. *Mevcut barınakların yapı malzemesi*
Table 5. *The building materials of existing shelters*

İlçeler	Betonarme		Toprak		Taş		Ahşap		Kerpiç-Briket	
	Frekans	%	Frekans	%	Frekans	%	Frekans	%	Frekans	%
Merkez	37	42.53	13	14.94	33	37.93	4	4.60	0	0.00
Akyaka	17	34.00	6	12.00	26	52.00	0	0.00	1	2.00
Arpaçay	17	38.64	8	18.18	17	38.64	1	2.27	1	2.27
Digor	21	47.73	11	25.00	12	27.27	0	0.00	0	0.00
Kağızman	13	32.50	9	22.50	15	37.50	2	5.00	1	2.50
Sarıkamış	12	24.49	9	18.37	24	48.98	4	8.16	0	0.00
Selim	11	23.40	10	21.28	24	51.06	2	4.26	0	0.00
Susuz	18	35.29	19	37.25	13	25.49	1	1.96	0	0.00
Kars İl Toplamı	146	35.44	85	20.63	164	39.81	14	3.40	3	0.73

ilçelere göre *Tablo 8*'de verilmiştir. İlde hayvanların büyük bir oranda (%81.07) elle sağıldığı görülmektedir. Bunu seyyar süt sağım makineleri takip etmektedir. Sağım ünitelerinin kullanımı ise bölgede yeni kullanılmaya başlanmıştır.

Hayvan barınakları ile ilgili bazı tespitler *Tablo 9*'da gösterilmiştir. İşletme sahiplerinin mevcut işletmelerinde su kaynağının bulunup bulunmadığı ile ilgili soruya %73.30 oranında evet su kaynağı bulunuyor cevabı verilmiştir. Yine işletme sahiplerinin yaklaşık %42.48'lik oranı mevcut barınaklarının yeterli olmadığına inanmaktadır.

Hem silaj hem de gübre çukurunun işletmelerin büyük bir kısmında olmadığı belirlenmiştir. Ayrıca işletme sahiplerinin %60.92'si barınaklarının yapısı nedeniyle hayvanların süt verimlerinin düşük olduğunu belirtmişlerdir. Yine barınakların yapısı nedeniyle hayvanların gelişimine olumsuz etkisi olduğuna inanan yetiştiricilerin oranı da %57.04 olarak tespit edilmiştir. Mevcut barınakların yapısı nedeniyle, çalışanların sağlıklarının da olumsuz etkilendiğine inananların oranı da yüksek bulunmuştur (%48.79).

Tablo 6. *Mevcut ahırların kapasiteleri*
Table 6. *Capacities of the existing barns*

İlçeler	≤10 (Baş)		11-20 (Baş)		21-50 (Baş)		51≥ (Baş)	
	Frekans	%	Frekans	%	Frekans	%	Frekans	%
Merkez	5	5.75	23	26.44	35	40.23	24	27.59
Akyaka	2	4.00	9	18.00	27	54.00	12	24.00
Arpaçay	2	4.55	15	34.09	23	52.27	4	9.09
Digor	1	2.27	13	29.55	22	50.00	8	18.18
Kağızman	4	10.00	9	22.50	17	42.50	10	25.00
Sarıkamış	3	6.12	15	30.61	24	48.98	7	14.29
Selim	2	4.26	17	36.17	18	38.30	10	21.28
Susuz	3	5.88	20	39.22	17	33.33	11	21.57
Kars İl Toplamı	22	5.34	121	29.37	183	44.42	86	20.87

Tablo 7. *Üye olunan üretici birlikleri*
Table 7. *Membership to associations of producers*

İlçeler	Damızlık Sığır Yetiştiricileri Birliği		Köy-Koop.		Diğer	
	Frekans	%	Frekans	%	Frekans	%
Merkez	19	67.86	5	17.86	4	14.29
Akyaka	15	75.00	3	15.00	2	10.00
Arpaçay	20	66.67	5	16.67	5	16.67
Digor	13	68.42	1	5.26	5	26.32
Kağızman	20	64.52	5	16.13	6	19.35
Sarıkamış	20	71.43	7	25.00	1	3.57
Selim	31	91.18	2	5.88	1	2.94
Susuz	15	88.24	0	0.00	2	11.76
Kars İl Toplamı	153	73.91	28	13.53	26	12.56

Tablo 8. *İşletmelerde kullanılan sağım yöntemleri*
Table 8. *Milking methods used in livestocks*

İlçeler	Elle		Seyyar Sağım Makinesi		Sağım Ünitesi	
	Frekans	%	Frekans	%	Frekans	%
Merkez	66	75.86	18	20.69	3	3.45
Akyaka	43	86.00	5	10.00	2	4.00
Arpaçay	32	72.73	9	20.45	3	6.82
Digor	40	90.91	3	6.82	1	2.27
Kağızman	26	65.00	9	22.50	5	12.50
Sarıkamış	43	87.76	6	12.24	0	0.00
Selim	38	80.85	8	17.02	1	2.13
Susuz	46	90.20	5	9.80	0	0.00
Kars İl Toplamı	334	81.07	63	15.29	15	3.64

TARTIŞMA ve SONUÇ

Kars ili sığır varlığı ırk kompozisyonu ile Türkiye geneli sığır varlığı ırk kompozisyonu karşılaştırıldığında; ilde halen kültür ırkı ve melezi sığır varlığının yeterli düzeyde olmadığı ve Türkiye ortalamasının gerisinde kaldığı görülmektedir [9]. Bu ilde hayvansal üretimin geliştirilmesi ve kırsal sosyo-ekonomik kalkınmanın başarılabilmesinde yeterli seviyeye gelinemediğinin bir göstergesi olarak ifade edilebilir.

Anket yapılan işletme sahiplerinin hane büyüklüğü ortalama 7.17±0.17 kişi olarak belirlenmiştir. Hane halkı büyüklüğü en fazla Digor ve Kağızman ilçelerinde, en az ise Akyaka ve Arpaçay ilçelerinde belirlenmiştir. Kaç yıldır sığır yetiştiriciliği yapıldığı sorusuna ortalama 30.20±0.69 yıl olarak cevap verilmiştir. Bu soruya bazı kişiler "doğduğumdan beri", "ata baba mesleği", "kendimi bildim bileli" gibi cevaplar vermiştir.

Tablo 9. *İşletmelere yönelik bazı tespitler*
Table 9. *Some information about livestocks*

Değerlendirme	**Aydınlatma Yeterlidir**	**Elektrik Vardır**	**Su Kaynağı Vardır**	**Hayvan Atıkları Düzenli Olarak Atılmaktadır**	**Hayvan Atık Suları İçin Drenaj Vardır**
Evet (%)	88.83	98.06	73.30	91.26	30.83
Hayır (%)	11.17	1.94	26.70	8.74	69.17
Değerlendirme	**Zemin Meyillidir**	**Durak Demiri Vardır**	**Kaşıntı Fırçası Vardır**	**Barınağım Yeterlidir**	**Termometre Vardır**
Evet (%)	84.95	26.70	54.37	57.52	8.74
Hayır (%)	15.05	73.30	45.63	42.48	91.26
Değerlendirme	**Süt Soğutma Tankı Vardır**	**Süt Sağım Ünitesi Vardır**	**Fabrika Yemi İçin Yer Vardır**	**Her Hayvan İçin Yemlik Vardır**	**Her Hayvan İçin Suluk Vardır**
Evet (%)	3.16	15.29	58.98	63.35	25.49
Hayır (%)	96.84	84.71	41.02	36.65	74.51
Değerlendirme	**Samanlık Vardır**	**Silaj Çukuru Vardır**	**Gübre Çukuru Vardır**	**Düzenli Veteriner Hekim Kontrolü Yapılır**	**Hayvan Sigortası Vardır**
Evet (%)	63.59	10.19	15.78	70.15	13.59
Hayır (%)	36.41	89.81	84.22	29.85	86.41
Değerlendirme	**Ahırların Yapısı Nedeniyle Hayvanların Süt Verimi Düşüktür**		**Ahırlarımız Hayvanların Gelişimine Olumsuz Etkide Bulunuyor**		**Ahırlarımızın Yapısı Bizlerin de Sağlığını Olumsuz Etkiliyor**
Evet (%)	60.92		57.04		48.79
Hayır (%)	39.08		42.96		51.21

Özkan ve Erkuş [14] tarafından yapılan bir araştırmada sığırcılık işletmesi sahiplerinin iş tecrübesi 20 yıl olarak saptanmıştır. Öte yandan Şahin ve Yılmaz [15] Van ilinde tarım ve hayvancılıkla uğraşan işletme sahiplerinin iş tecrübesi 23 yıl, Aydın [7] Kars ilinde sığır besi işletme sahiplerinin iş tecrübesini ise 18 yıl olarak bildirilmiştir. Görüldüğü üzere çalışmada işletme sahipleri diğer araştırma bulgularına göre daha fazla yetiştiricilik tecrübesine sahiptir.

İldeki barınakların ortalama yaşı 18.19±0.71 yıl olarak saptanmış olup, Susuz ve Arpaçay'daki barınakların yaşı diğer ilçelere göre daha yüksek bulunmuştur. İldeki barınakların bazılarının oldukça eski olduğu, bunların yaşının tespitinde ana yaşı, baba yaşı, çocuk yaşı, evlenme zamanı vb. çeşitli belirleyici tarihler dikkate alınarak tespitler yapıldığı gözlemlenmiştir.

Çalışmada Kars ilinde okur-yazar olmama oranı %4, ilkokul ve ortaokulu bitirme oranı %75 olarak elde edilmiştir. Okur-yazar olma ve ilköğretimi bitirme oranını Aksoy ve Yavuz [16] Erzurum ilinde hayvancılık sektöründeki faaliyet gösteren işletme sahiplerinde %86, Aydın [7] Kars ilinde entansif sığır besi işletmele sahiplerinde %79, Kara [17] tarım sektöründeki işletme sahiplerinde ilkokul mezun olma oranını Erzurum ilinde %77, Kars ilinde %72, Özkan ve Erkuş [14] Bayburt ilinde %88, Cevher ve Karakurt [18] Ankara ilinde %86 olarak bildirmişlerdir.

Sığır yetiştiriciliğinin ekonomik etkinliği ve üretimde verimlilik açısından eğitimin öneminin büyük olduğu bilinmektedir. Kars ilinde faaliyet gösteren yetiştiricilerin büyük çoğunluğunun eğitim düzeyinin düşük olmasının yanı sıra yetiştiricilikle ilgili herhangi bir resmi eğitim almadıkları gözlenmiştir. Türkiye'de üreticilerin eğitim düzeyi yükseldikçe, işletmelerin karlılığının arttığını ortaya koyan araştırmalar bulunmaktadır [7,19].

Kars ilinde en yaygın ahır tipi kapalı bağlı ahırlardır. Bu ahır tipini ilde bulunan çok az miktarda kapalı serbest dolaşımlı ahırlar takip etmektedir. Kapalı serbest dolaşımlı ahırların hemen hemen tamamı devletin vermiş olduğu desteklerle yapılmıştır. Yetiştiricilerin kapalı bağlı sistemi tercih etmesinin nedenleri arasında ilk sırayı hayvanların "bakım-besleme ve idaresinin kolay olduğu" belirlenmiştir. Bunu "geleneksel olduğu" ve "diğer sistemlere göre daha sağlıklı olduğu" için yapılan tercihler takip etmektedir.

İlde kapalı bağlı ahır tiplerinin yetiştirici gözünde yaygın olmasının çeşitli nedenleri vardır. Bunlardan birisi ilde yetiştirilen hayvanların bir kısmının yerli ve melez olması, bu hayvanların serbest dolaşımlı pozisyonda bulunması durumunda birbirlerine zarar vereceklerine inanılmasıdır. Bir diğeri bölgede yapılan yetiştiricilikte son yıllarda kısmen artmakla birlikte hemen hemen işletmelerin hiçbirinde boynuzsuzlaştırma işlemi uygulanmamakta, bu durumda da hayvanlar birbirlerine zarar verebilmektedir. Başka bir bağlı sistem nedeni de bölgede kış mevsiminin yaklaşık 7-8 ay sürmesidir. Ahır içerisinde bulunan hayvanların serbest dolaşımlı pozisyonda olması durumunda, fazla enerji harcayacakları ve daha fazla yem tüketeceklerine inanılmaktadır.

İlde mevcut barınakların en yaygın yapı malzemesinin %39.81 ile taş ve %35.44 ile betonarme yapılar takip etmektedir. Ancak bu yapı malzemesi bazı barınaklarda sadece taş değil, bazen toprakla birlikte yer aldığı gözlemlenmiştir.

Benzer durum betonarme ve ahşap yapı malzemesi içinde geçerlidir. Birçok barınakta bu iki yapı malzemesinin birlikte bulunduğu tespit edilmiştir.

Yetiştiricinin elinde bulunan en yüksek ahır kapasitesi %44.42 oranı ile 21-50 baş arası sığır kapasiteli ahırlardır. Bunu %29.37 oranı ile 11-20 baş arası sığır kapasiteli ahırlara sahip işletmeler takip etmektedir. Hayvancılık bölgesi olması nedeniyle 10 baş sığır kapasitesinden düşük olan ahıra sahip işletme oranının son derece düşük olduğu tespit edilmiştir. Yine işletme sahiplerinin yaklaşık %42.48'lik oranı mevcut ahırının yeterli olmadığına inanmaktadır. Ayrıca yetiştiricilerin, bu faaliyeti hayvancılık ekonomisinin temel prensiplerine dikkat etmeksizin, geçimlik olarak yaptığı da gözlemlenmiştir. Aksoy ve Yavuz [16], Erzurum ilinde üreticilerin %44'ünün hayvancılığı ev ihtiyacını karşılamak amacıyla, %55'inin ise ev ihtiyacı, istihdam ve ticari (hepsi) amaçla yaptığını bildirmiştir. Diğer taraftan Kara ve ark.[20], Doğu Anadolu Bölgesi'nde kendi ihtiyaçlarını karşılamak üzere hayvancılık yapan işletmelerin oranını %89 olarak bildirmişlerdir. Aydın [7], Kars ilinde süt sığırcılığına göre sığır besiciliğinin daha fazla ticari amaçla yapılıyor olduğunu belirtmiştir. Buna göre araştırma kapsamında incelenen süt sığırcılık işletmelerinin küçük ölçekli olması ve geçimlik olması diğer araştırma bulgularıyla örtüşmektedir.

Hayvancılık işletmelerinde genel olarak işletme ölçeği büyüdükçe kapasite kullanım oranı artmaktadır. Böylece büyük ölçekli işletmelerde birim kapalı alana düşen sığır sayısı küçük ölçekli işletmelere oranla daha yüksek düzeyde olmaktadır. Bu sayede büyük ölçekli işletmeler sabit varlıklarını (ahır, süt sağım ünitesi, yem silosu vs) daha verimli kullanmakta ve birim hayvan başına düşen sabit maliyet daha düşük seviyede olmaktadır. Başka bir ifadeyle hayvancılık işletmeleri işletme ölçeği büyüdükçe daha kârlı çalışmaktadır [21].

İşletme sahiplerinden %53.88'i herhangi bir üretici örgütüne üye olmadığını ifade ederken, %46.12'i en az bir üretici örgütüne kayıtlı olduğunu beyan etmiştir. Üye olunan üretici birlikleri arasında ilk sırada Damızlık Sığır Yetiştiricileri Birliği yer almaktadır. Ancak 412 işletme sahibinden 222 işletme sahibinin herhangi bir birliğe üye olmaması oldukça düşündürücü olup, birliklerin yapılarının veya faaliyetlerinin gözden geçirilmesinin yararlı olacağı düşünülmektedir. Acar [22] kooperatif bünyesinde çalışan insanların bu konu hakkında eğitimsiz olmasının, kooperatiflerin verimli bir şekilde işletilmesini önlediğini ve bu nedenle Gıda, Tarım ve Hayvancılık Bakanlığı'nın kooperatifçilik eğitimine yönelik düzenli seminerler vermesinin daha faydalı olacağı belirtmiştir.

Kars ve ilçelerinde hayvanların büyük bir kısmının (%81.07) elle sağıldığı tespit edilmiştir. Bunu seyyar süt sağım makineleri takip etmektedir. Sağım ünitelerinin kullanımı ise bölgede yeni kullanılmaya başlanmıştır. Elle sağımın en az olduğu ilçe Kağızman olarak belirlenmiştir. Özellikle sağım öncesi memelerin temizliği ve sağım sonrası sütlerin muhafazasında sorunlar yaşandığı, hijyen kurallarına yeterince dikkat edilmediği görülmüştür.

Anket çalışması yapılan işletmelerin hemen hemen hiçbirinde soğutma tankı bulunmamaktadır. İlde yapılan modern işletmelerin birçoğunda bile süt soğutma tankının olmadığı tespit edilmiştir. Bu işletmelerde de sütün depolanması için büyük plastik kaplar kullanıldığı belirlenmiştir. Geleneksel işletmelerin hemen hemen tamamında sütlerin plastik bidonlarda depolandığı belirlenmiştir (%88.35). Bu sütler kısa sürede mandıra sahipleri tarafından alınıp süt işletmelerine getirilmektedir.

İşletme sahiplerinin %60.92'si ahırlarının yapısı nedeniyle hayvanların süt verimlerinin düşük olduğunu belirtmişlerdir. Yine ahırların yapısının hayvanların gelişimine olumsuz etkisi olduğuna inanan yetiştiricilerin oranı da %57.04 olarak tespit edilmiştir. Mevcut ahırların yapısı nedeniyle ahırda çalışanların sağlıklarının da olumsuz etkilendiğine inananların oranı da yüksek bulunmuştur (%48.79). Kısacası, mevcut ahır şartlarından dolayı hem hayvanların gelişimine, hem hayvanların verimlerine, hem de insan sağlığı üzerine olumsuz etkilerinin olduğuna inanan yetiştirici sayısı oldukça fazladır. Ancak; bu yetiştiricilerin işletmelerini modernize etme ya da yeniden yapma imkânlarının kısıtlı olduğu da gözlemlenmiştir.

Araştırmada elde edilen bazı bulgular tablo olarak sunulmamış olup, metin olarak verilmiştir. Bunlardan birisi ahırların eve göre konumudur. Kars ve çevresinde bulunan ahırların büyük kısmının evin yanında (%51.46), bir kısmının ise ev ile bitişik (%38.83) olduğu tespit edilmiştir. Bunun nedenleri güvenlik ve işçiliğin daha az olduğunun düşünülmesinden dolayıdır. Özellikle evlerle bitişik olan hatta evin yanında olan ahırların, hane halkının sağlığı için problemler oluşturduğu ve çevre kirliliğine neden olduğu da gözlemlenmiştir.

Anket uygulaması yapılan 26 (%6.31) işletmeye ait ahırda hiç havalandırma bacası olmadığı, 15 (%3.64) işletmede ise yalnızca 1 adet havalandırma bacası olduğu tespit edilmiştir. Mevcut havalandırma bacalarının da genellikle yetersiz ölçülerde olduğu belirlenmiştir. Ankete katılan 43 (%10.44) işletmeye ait ahırlarda pencere bulunmadığı, 23 (%5.58) işletmede ise yalnızca 1 adet pencere olduğu saptanmıştır. İşletmelerin genelinde ise kış şartlarının ağır olması gerekçesi ile pencere ebatlarının standartların altında olduğu tespit edilmiştir. Hem havalandırma bacası hem de pencerelerin bazı işletmelerde ahır içerisinin soğuk olduğu gerekçesiyle kış aylarında kapatıldığı da gözlemlenmiştir. Bu tespit, Türkiye'de yapılan birçok çalışma sonuçları ile benzerlik göstermektedir [23-25].

İşletmelerin büyük çoğunluğunda ahır içerisinde doğan tüm buzağıların konulduğu, buzağı bölmesi olduğu belirlenmiştir (%76.67). Buzağı bölmesi olmayan işletmelerin ise, buzağılarını mevcut sürüleri ile birlikte barındırdıkları tespit edilmiştir. Ahırda doğum yapacak düve ve inekler için bölme var mı sorusuna %82.77 oranında hayır cevabı verilmiştir. Doğum yapacak hayvanlar ahır içerisinde bağlı bulundukları yerde doğum yapmaktadırlar. Doğan buzağılarında analarının başının yanına ayaklarından bağlandığı gözlemlenmiştir.

İşletmelerde hayvanlardan elde edilen gübrenin en çok

ahır yakınında bir yerde biriktirerek depolandığı belirlenmiştir (%81.07). Bunu gübre deposunda depolama ve diğer uygulamalar takip etmektedir. Elde edilen gübrenin bir kısmı "basmalık" diye ifade edilen tezek yapımında kullanılmaktadır. Bir kısmı da tarlalarda doğal gübre olarak değerlendirilmektedir.

Hayvanların ahır içerisinde ve merada su ihtiyaçları tüm ilçelerde ortalama %54.85 oranında şebeke suyundan karşılandığı belirtilmiştir. Bunu akarsular takip etmektedir. Bölgede oldukça fazla su kaynağı olmasına rağmen hem insanların hem de hayvanların su ihtiyaçlarının karşılayabilecekleri yeterli alt yapı imkânlarının olmadığı gözlemlenmiştir. İşletmelerin %26.70'inde ahır içinde su kaynağının olmaması, iş gücü açısından önemli bir problemdir. Bu tür işletmeler genelde hayvanlarını günün belirli saatlerinde ahır dışında bulunan bir su kaynağına götürerek su içirmektedirler.

Bölgedeki yetiştiricilerin birçoğunun sığır yetiştiriciliği hakkında herhangi bir eğitimi bulunmamakta, yetiştiricilik faaliyetlerini tamamen geleneksel yöntemlerle yapmaktadır. İşletme sahiplerinin çoğu hayvancılığı geçimlik olarak görmekte ve hayvancılık sektörü dışında başka gelir kaynağı bulunmamaktadır. İldeki bulunan yetiştiricilere hayvancılıkla ilgili teknik bilgilerin uygulamalı olarak verilmesi, işletmecilik faaliyetlerini daha rasyonel yürütmelerini sağlayacaktır.

Hayvancılık ile uğraşan yetiştiricilerin çoğu, geçmişten kalan alışkanlıklarını devam ettirmekte ve yüksek verimli hayvanlara dahi geleneksel yöntemlerle bakım-besleme uygulamaktadırlar. Kars iline yurt içinden ve özellikle yurt dışından getirilen kombine verimli sığır ırkları, getirildikleri işletmede henüz çevre koşullarına uyum sağlamadan meraya çıkarılmaktadır.

Bölgede yapılan yetiştiricilikte, ineklerin doğumdan sonra oldukça kısa süre sağıldıkları, hatta bazı aileler ineklerini günde bir kez sağdıkları gözlemlenmiştir. Bunun için elde edilen sütün düzenli satışı gerçekleştirilememektedir. Bu haliyle büyükbaş hayvancılık verimli ve rasyonel olmaktan uzaktır. Ancak besleme sorunlarının olmadığı, yüksek verimli kültür ırklarıyla, modern barınaklarda, teknolojik makine-ekipmanla yapılan hayvancılık faaliyeti sonucunda işletmenin ölçeğini büyütmek ve yıl boyunca süt üretimi yapmak mümkün görünmektedir.

Araştırma sonucunda, Kars ve çevresinde mevcut ahırlarda birçok yetersizlik ve eksiklikler tespit edilmiştir. Ayrıca bölgedeki yetiştiricilerin bazı konularda bilgilerinin yetersiz olduğu gözlemlenmiştir. Bunun için yetiştiricilere barınak, bakım, besleme, sağlık, hijyen gibi konularda eğitim verilmesinin yararlı olacağı sonucuna varılmıştır.

KAYNAKLAR

1. Anonim: 22 Eylül 2002 tarih ve 2002/4720 sayılı Bakanlar Kurulu Kararı, 2002.

2. DPT: İllerin ve bölgelerin sosyo-ekonomik gelişmişlik sıralaması araştırması. http://www.dpt.gov.tr/DocObjects/view/14197/BASIN_AÇIKLAMASI-sege_2011-v6.pdf, *Erişim tarihi:* 01.06.2012.

3. TÜİK: Türkiye İstatistik Kurumu. Nüfus istatistikleri. http://www.tuik.gov.tr, *Erişim tarihi:* 10.03.2012.

4. Anonim: http://tr.wikipedia.org/wiki/Kars, *Erişim tarihi:* 01.03.2012.

5. Anonim: http://www.karstarim.gov.tr, *Erişim tarihi:* 20.02.2012.

6. TÜİK: Türkiye İstatistik Kurumu. İşgücü istatistikleri. http://www.tuik.gov.tr, *Erişim tarihi:* 10.03.2012

7. Aydın E: Kars ve Erzurum İlleri Sığır Besi İşletmelerinin Ekonomik Analizi. *Doktora Tezi,* Ankara Üniv. Sağlık Bil. Enst., 2011.

8. Anonim: Kars İl Gıda Tarım ve Hayvancılık Müdürlüğü Brifing, 2011.

9. TÜİK: Türkiye İstatistik Kurumu. Hayvancılık istatistikleri. http://www.tuik.gov.tr, *Erişim tarihi:* 10.03.2012

10. Alpan O, Aksoy AR: Sığır Yetiştiriciliği ve Besiciliği. 5. Baskı. Zafer Ofset ve Matbaacılık, Erzurum, 2009.

11. Akman N: Barınaklar. http://www.amasyadsyb.org/docs/Amasya_DSYB_Yayin_ 001.pdf, *Erişim tarihi:* 05.03.2012.

12. Elmaz Ö, Sipahi C, Saatcı M, Özçelik Metin M: Determining the current status of the dairy cattle enterprises in the Mediterranean region, Turkey. *Outlook on AGRICULTURE,* 41 (2): 133-138, 2012.

13. SERKA: Serhat Kalkınma Ajansı, Doğu Anadolu Bölgesi Büyükbaş Hayvancılık Çalıştay Raporu. Temmuz, 2011.

14. Özkan U, Erkuş A: Bayburt ilinde sığır besiciliğine yer veren tarım işletmelerinin ekonomik analizi. *Tarım Bil Derg,* 9 (4): 467-472, 2003.

15. Şahin K, Yılmaz İH: Van ilinde yem bitkileri tarımı, mera kullanımı ve sosyo ekonomik yapı üzerine bir araştırma. *Tarım Bil Derg,* 14 (4): 414-419, 2008.

16. Aksoy A, Yavuz F: Hayvancılık işletmelerinin Avrupa Birliğine uyumu ve rekabet edebilirliği. Doğu Anadolu örneği. *Tarım Ekonomisi Derg,* 14 (1): 37-45, 2008.

17. Kara A: Erzurum ve Kars illerinde tarım işletmelerinin sermaye yapısı ve tarımsal faaliyet sonuçları bakımından karşılaştırılması. *Yüksek Lisans Tezi,* Atatürk Üniv. Fen Bil. Enst., 1999.

18. Cevher C, Karakurt E: Mera ıslah çalışması yürütülen köylerde yem bitkisi üretimini artırmaya yönelik yayım çalışmasının değerlendirilmesi. *Tarla Bitkileri Merkez Araştırma Enstitüsü Dergisi,* 19 (1-2): 17-23, 2010.

19. Köknaroğlu H, Demircan V, Yılmaz H, Dernek Z: Besi sığırcılığı üretim faaliyetinde üreticilerin eğitim düzeylerinin besi performansı ve kârlılığa etkisi. *V. Ulusal Zootekni Kongresi,* 5-8 Eylül, Van, 2007.

20. Kara A, Kadıoğlu S, Küçüközdemir A, Günay G: Kuzeydoğu Anadolu tarım işletmelerinde hayvan yetiştiriciliği uygulamaları ve ilgili problemler. *V. Zootekni Bilim Kongresi,* 5-8 Eylül, Van, 2007.

21. Aydın E, Sakarya E: Kars ve Erzurum illeri entansif sığır besi işletmelerinin ekonomik analizi. *Kafkas Univ Vet Fak Derg,* 18 (6): 997-1005, 2012.

22. Acar İ: Mandıra İşleten Dönerdere Tarımsal Kalkınma Kooperatifi ile Üyelerin Ekonomik Yapısı ve Kooperatif-Ortak İlişkileri. *Yüksek Lisans Tezi,* Yüzüncü Yıl Üniv. Fen Bil. Enst., 2001.

23. Uğurlu N, Şahin S: Kayseri ili süt sığırı barınaklarının yapısal özellikleri. *Selçuk Tarım ve Gıda Bilimleri Dergisi,* 24 (2): 23-26, 2010.

24. Kaygısız A, Tümer R: Kahramanmaraş ili süt sığırı işletmelerinin yapısal özellikleri; 2. Barınak özellikleri. *KSÜ Doğa Bil Derg,* 12 (1): 40-47, 2009.

25. Bayraktar H, Uğurlu N, Yılmaz AM: Bitlis ili Ahlat ve Adilcevaz ilçeleri süt sığırı işletmelerinde barınakların değerlendirmesi. *Selçuk Tarım ve Gıda Bilimleri Dergisi,* 24 (2): 17-22, 2010.

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RESEARCH ARTICLE

The Effects of Udder Dermatitis Due to Sarcoptic Mange on Milk Yield in Dairy Cows

Deniz ALIC URAL * Kerem URAL ** Ozgur KOSKAN ***

* Bozdogan Vocational School, Adnan Menderes University, Bozdogan, Aydin, TURKEY
** Department of Internal Medicine, Faculty of Veterinary, Adnan Menderes University, Isikli, Aydin, TURKEY
*** Department of Animal Science Faculty of Agriculture, Suleyman Demirel University, Isparta, TURKEY

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Summary

In the present study the aim was to elucidate the relationship between udder dermatitis due to naturally occuring sarcoptic mange infestation and milk yield in dairy cattle. Field observation was carried out in a private dairy farm in Bozdogan, Aydin comprising 18 out of 80 cattle were diagnosed with udder dermatitis in association with sarcoptic mange on the basis of clinical and parasitological examinations. For assessment of milk yield production, the data set included 5490 test day yields. The actual milk yield evaluated in second lactation changed between 2594-7742 kg (with a mean milk yield: 5394±327.5 kg). The lactation periods of cows were detected between 181-306 day (the mean lactation period: 275±9.5 days). The first occurence of each lesions were included in the analysis and 6 cows had udder dermatitis in first 5 month and other cows at later lactation period. The mean daily milk loss was calculated 8.17 kg and daily milk yields loss for a cow was 0.44 kg. This pruritic disease invoving udder led severe infection and dramatic drop of milk yield among dairy cattle enrolled in the present study. Results of the present study reported herein suggested that udder dermatitis in relation to scabies could be identified in cows in all stages of the lactating period, especially the prevalence was higher in later lactation period. The milk losses consequent to udder dermatitis may cause significant economic problems.

Keywords: *Udder, Dermatitis, Sarcoptic mange, Milk yield, Cow*

Sütçü İneklerde Sarkoptik Uyuza Bağlı Meme Dermatitisinin Süt Verimi Üzerine Etkileri

Özet

Bu çalışmada sütçü ineklerde doğal olarak oluşan sarkoptik uyuz enfestasyonuna bağlı meme dermatitisi ile süt verimi arasındaki ilişkinin incelenmesi amaçlanmıştır. Aydın ili Bozdoğan ilçesine bağlı özel bir süt sığırcılığı işletmesinde klinik ve parazitolojik muayeneler ile sarkoptik uyuza ilişkin meme dermatitisi tanısı konulan 18/80 inekte saha gözlemi yapıldı. Süt veriminin değerlendirilmesi amacıyla 5490 kontrol günü verimi kullanıldı. İkinci laktasyondaki gerçek süt veriminin 2594-7742 kg arasında (ortalama süt verimi: 5394±327.5 kg) değiştiği saptandı. Çalışmaya dahil edilen sütçü ineklerde laktasyon periyodunun 181-306 gün arasında (ortalama laktasyon periyodu: 275±9.5 gün) olduğu belirlendi. Analizlerde her bir lezyonun ilk ortaya çıkışı dikkate alınarak 6 inekte meme dermatitisinin ilk 5 ayda diğerlerinde ise daha sonraki laktasyon periyodunda meydana geldiği belirlendi. Günlük ortalama süt kayıbı 8.17 kg olarak hesaplanırken, bir inek başına günlük süt verim kayıbı 0.44 kg'dı. Bu çalışma kapsamına alınan sütçü ineklerde meme derisinde kaşıntıyla seyreden bu hastalığın şiddetli infeksiyona ve dramatik biçimde süt veriminin azalmasına neden olduğu belirlendi. Çalışmanın sonuçlarına bakıldığında, sarkoptik uyuzla ilişkili meme dermatitisinin laktasyon periyodunun tüm dönemlerindeki ineklerde tespit edilebileceği, özellikle de laktasyonun geç dönemlerinde prevalansın daha yüksek olabileceği tespit edildi. Meme dermatitisine ilişkin süt kayıplarının önemli ekonomik kayıplara neden olabileceği ortaya konuldu.

Anahtar sözcükler: *Meme, Dermatitis, Sarkoptik uyuz, Süt verimi, İnek*

INTRODUCTION

Profitability of disease control interactions at a herd level could be assessed by evaluation of relations among lossess consequent to disease condition and expenditures for prevention or control measures [1-3]. In a dairy cattle herd level

İletişim (Correspondence)
+90 256 4143738
alicdeniz@gmail.com

losses may be defined as reduction of the output/input ratio relevant production process [4]. The probable effect of any disease condition on milk yield, may be expressed as the difference in milk yield of any ill cow, in comparison to that expected yield of that cow with no disease condition [1].

Up to date the relationship between udder diseases and milk yield has been elucidated in the veterinary literature, however little is known about the exact causes of udder dermatitis, also known as udder scald and intetrigo, or udder rot [5,6].

Udder dermatitis has been recognized infrequently in the literature however may be an important problem for milkers, veterinary surgeons on large animal practice and herd managers [5-7]. Dermatitis detected between the udder and upper thigh may be observed in early lactation, as a sequele to skin damage by udder edema pressure into the upper thigh [5]. Lesions are frequently characterized within necrosis of the udder skin and a bad odour [6]. In the present study the aim was to elucidate the relationship between udder dermatitis due to naturally occuring sarcoptic mange infestation and milk yield in dairy cattle in Aydin, Turkey.

MATERIAL and METHODS

Study Area

The present study (field observation) was conducted retrospectively among 18 dairy cattle (out of 80) in Bozdogan province and at the Department of Internal Medicine, Faculty of Veterinary, Aydin city. The material was obtained from second lactation records of 18 Holstein Friesian cows raised between the years of November 2010 and October 2011 in a privately owned dairy farm in Bozdogan province in Aydın, Turkey. The data set included 5490 test day yields (TDYs). *The milk* samples were collected during the *morning* and evening *milking*. Data included the cow's identification number, herd code, the type of herd or production sector, the lactation number, the test-day milk yield, and the number of times the cow was milked per day.

In the final analysis only complete lactations within study period were used because of the potential that a cow might have become udder dermatitis before and after the data collection period within the same lactation (second lactation). Lesions causing clinical udder dermatitis were recorded and descriptions of the lesions were performed by the farmer to assist with lesions recognation for researchers.

Data Analysis

TDYs were the outcome variable; it followed a normal distribution. The data were hierarchically structed with TDY within cow wihin farm. The TDYs were obtained from the morning and evening milkings of each cow. Data from the first 306 days of lactation. The data were analysed using SPSS Statistics Release 17.0[a]. The lactation curve modelled using days in milk (DIM) and TDYs. The Gamma model was used for the estimation of the parameters of lactation curve [8]. The incomplete gamma function was used, as suggested previously [8]:

$$\mathbf{Y_t = a * t^b * e^{(-c*t)}},$$

where Y_t is the milk yield in DIM t,

a is a constant representing the level of initial yield of the cow,

b is a parameter representing the rate of increase to peak,

c is the rate of decline after peak,

t is the time period (daily),

e is the Neper number.

For fitting of the model the non-linear module of SPSS[a] program was used. The lactation curves were drawn for the herd's mean benefit from the estimation of the parameters.

Parasitological Examination

In a convenience sample of Holstein cows with suspected lesions consistent with sarcoptic mange, skin scrapings were withdrawn from the tail, the area among the hind limbs dorsal to the udder and ventral to the vulva and especially whole udder. Skin scrapings were microscopically examined following KOH digestion. A complete history of each animal and date of examination were recorded and all the samples were processed within 12 h after collection. Briefly, 10% KOH solution was added to each sample container and boiled for 5-10 min [9]. Than samples were centrifuged at 1500 *g* for 5 min, supernatant and sediment were examined microscopically. Identification of mites was performed by morphological characteristics [10].

RESULTS

Assessment of Milk Yield

In this study, the actual milk yield was evaluated in second lactation. The actual milk yields of cows at second lactation changes between 2594 kg and 7742 kg (the mean of milk yield: 5394±327.5 kg). The lactation periods of cows changes between 181-306 days (the means of lactation periods: 275±9.5 days).

The first occurence of each lesions were included in the analysis and 6 cows could have had udder dermatitis in first 5 month and other cows have at the later lactation period. The mean daily milk loss was calculated 8.17 kg and daily milk yields loss for a cow was 0.44 kg.

The lactation curve parameters estimated by Gamma model were given *Table 1*.

The lactation curve of estimated by Gamma model was shown in *Fig. 1*. The fitted values of milk yield were plotted for udder dermatitis

Table 1. *The lactation curve parameters estimated by Gamma model (R^2: 0.55)*
Tablo 1. *Gama modeli kullanılarak tahmin edilen laktasyon eğrisi parametreleri*

Variable	N	Min	Max	Mean	S.E.
a	18	14.93	40.79	27.5	1.78
b	18	-0.20	0.16	0.007	0.0225
c	18	-0.001	0.007	0.002	0.0047

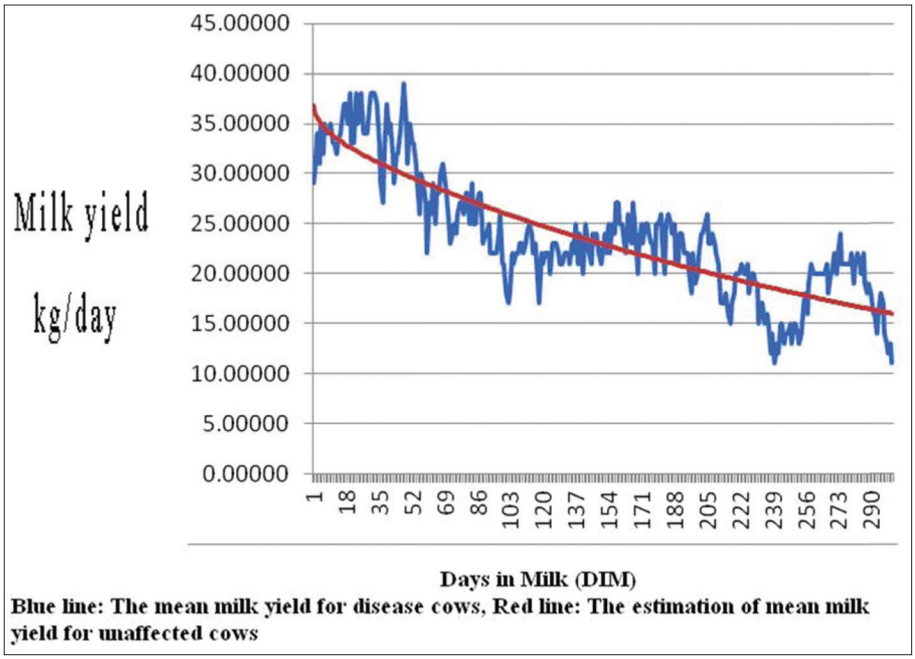

Fig 1. The lactation curve of estimated using by Gamma model of 18 Holstein Friesian cows

Şekil 1. Gama modeli kullanılarak tahmin edilen 18 baş Siyah Alaca ineğin laktasyon eğrisi

Parasitological Findings

Of the 80 cows examined 18 (22.5%) were found to have udder dermatitis consistent with sarcoptic mange. Anorexia was evident in most of the animals involved. Lesions were located on whole area of the udder in all 4 quarters. Lesions had hyperpigmentation, crusting, mild erythema and intense pruritus was evident during physical examination. All 18 cows had skin scrapings from lesional sites, were positive for live *Sarcoptes scabiei* mites.

DISCUSSION

Mange in cattle may be caused by different species of mite infestation with *Sarcoptes scabiei var. bovis* (syn. *Sarcoptes bovis*) [11-13], *Chorioptes bovis* or *Psoroptes ovis* [12]. Although mites may infest cattle of all classes and ages, chorioptic mange is frequently prevalent in dairy cows whereas sarcoptic mange is often associated with growing cattle. Under appropriate conditions, the latter types of mange may spread all over the body of cattle and consequently cause considerable economic losses [12,14,15], involving decreased milk and meat production [9,10]. Especially udder dermatitis may be associated with sarcoptic mange [5,6]. *Sarcoptes spp.* may lead to itch, dermatitis and intense pruritus due to which animals loose much of the rumination time and hence loose general body condition [5,6,9,16].

The 22.5% prevalence rate, noticed in this multdiciplinar study (Agricultural Department of Zootechnics and Veterinary Internal Medicine), may be attributed to a combination of factors such as poor nutrition, hygiene level, herdsmen poor knowledge of udder health and overcrowding conditions where the cattle were kept. Prolonged anorexia due to intense pruritus prone the cattle to debility and emaciation, or predisposed cattle to other secondary diseases, all finally leading to significant economic losses of the affected animals. Bacterial complications may also be involved as the cause of death in scabietic cattle [16].

Apart from the diseased animal, the farmer was worried about the dramatic drop of milk yield also reported in similarly affected sheep [9] and cattle [5,6] and may be attributed to the significant reduction of food intake secondary to intense pruritus [16].

In a university practice study with unpublished results, the veterinarians detected 1600 cow herd case that udder dermatitis were mostly prevalent in later lactation aged cows, probably could have been associated to suspected sarcoptic mange, however this was not proved [5]. A recent University study has shown that udder dermatitis in early lactation caused high milk losses. Milk production losses averaged 681 pounds for each cow having this disease, which was approximately equal to digestive disorders [5].

In the present study the actual milk yield was assessed in second lactation and changed between 2594-7742 kg. with (the mean of milk yield: 5394±327.5 kg). Mean lactation periods of cows was 275±9.5 days. Regarding the stage of lactation and its interactions with milk yield and udder dermatitis were evaluated, the present findings indicate that

udder dermatitis was more common in later lactation period (12/18, 66.6%) in this herd, however could be identified in all stages of the lactating period, as detected in the first 5 month of lactation in 6/18 cows. The mean daily milk loss was calculated 8.17 kg and daily milk yields loss for a cow was 0.44 kg. This value was similar to that reported previously [5]. This pruritic disease invoving udder led severe infection and dramatic drop of milk yield among dairy cattle enrolled in the present study.

Despite its economic and zoonotic importance, sarcoptic mange has not been received fully attention and its real impact on milk yield is still unknown in many areas of Turkey. Keeping in view the importance of sarcoptic mange, the present study was planned to determine the existence of sarcoptic mange on udder and to investigate the probable milk yield disturbance associated with mange in dairy cattle.

Although little has been documented about udder dermatitis in association with sarcoptic mange and its correlation with milk yield, particular attention should be paid to potential confounding when the risk of sarcoptic mange varies according to production level. Results of the present study reported herein suggested that udder dermatitis in relation to Scabies could be identified in cows in all stages of the lactating period, especially the prevalence was higher in later lactation period. The milk losses consequent to udder dermatitis may cause significant economic problems. Furthermore it was considered that sarcoptic mange adversly affects the production of the infested cattle.

REFERENCES

1. Fourichon C, Seegers H, Bareille N, Beaudeau F: Effects of disease on milk production in the dairy cow: A review. *Prev Vet Med,* 41 (1): 1-35, 1999.

2. McInerney JP, Howe KS, Schefers JA: A framework for the economic analysis of disease in farm livestock. *Prev Vet Med*, 13 (2): 137-154, 1992.

3. Seegers H, Fourichon C, Malher X, L'Hastis M: A framework for animal health. *Vet Res*, 25 (2-3): 165-173, 1994.

4. Dijkhuizen AA: Economic aspects of diseases and disease control. *PhD Thesis.* University of Utrecht, 155 p, 1983.

5. Reneau JK: Udder scald may be more costly than you think. *University of Minnesota Quality Counts Programs*, pp. 1-2, 2008.

6. Warnick LD, Nydam D, Maciel A, Guard CL, Wade SE: Udder cleft dermatitis and sarcoptic mange in a dairy herd. *JAVMA,* 221(2): 273-276, 2002.

7. Roy C, Roque JL, François PM, Ferrieres A, Raboisson D: Investigation of the aetiology of udder-thigh dermatitis in French dairy cattle. *Vet J,* [In press], 2011.

8. Wood PDP: Algebratic model of the lactation curve in cattle. *Nature,* 216 (511): 164-165, 1967.

9. Fthenakis GC, Papadopoulos E, Himonas C, Leontides L, Kritas S, Papatsas J: Efficacy of moxidectin against sarcoptic mange and effects on milk yield of ewes and growth of lambs. *Vet Parasitol,* 87 (2-3): 207-216, 2000.

10. Soulsby EJL: Helminths, Artropods and Protozoa of Domesticated Animals. 7th ed., pp. 765-777, Baillier Tindall, London, 1982.

11. Barth D, Preston JM: Efficacy of topically administered ivermectin against chorioptic and sarcoptic mange of cattle. *Vet Rec,* 123 (4): 101-104, 1988.

12. Rehbein S, Visser M, Winter R, Maciel AE: Efficacy of a new long-acting formulation of ivermectin and other injectable avermectins against induced *Psoroptes ovis* infestations in cattle. *Parasitol Res,* 88 (12): 1061-1065, 2002.

13. Soll MD, d'Assonville JA, Smith CJZ: Efficacy of topically applied ivermectin against sarcoptic mange (*Sarcoptes scabiei* var. *bovis*) of cattle. *Parasitol Res,* 78 (2): 120-122, 1992.

14. Elbers AR, Rambags PG, van der Heijden HM, Hunneman WA: Production performance and pruritic behaviour of pigs naturally infected by *Sarcoptes scabiei* var *suis* in a contact transmission experiment. *Vet Q,* 22 (3): 145-149, 2000.

15. Rehbein S, Visser M, Winter R, Trommer B, Matthes HF, Maciel A, Marley SE: Productivity effects of bovine mange and control with ivermectin. *Vet Parasitol,* 114 (4): 267-284, 2003.

16. Rebhun WC: Skin Diseases. **In,** Rebhun WC (Ed): Diseases of Dairy Cattle. p. 256, Lippincott Williams & Wilkins Philadelphia, 1995.

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RESEARCH ARTICLE

Prevalence of Subclinical Paratuberculosis in Dairy Cattle in Uşak Region

Deniz YILDIRIM * Turan CİVELEK *

* Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Internal Medicine, TR-03200 ANS Campus, Afyonkarahisar - TURKEY

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Summary

Paratuberculosis caused by *Mycobacterium avium* subs. *paratuberculosis* a chronic, inflammatory and fatal disease of ruminants. The infection is characterized by chronic "subclinic" phase. Cattle in this phase is capable of infecting other animals in the herd. Although the presence of paratuberculosis has been known, the scientific studies on the disease and the prevalence appears to be scarced in Turkey. Thus, there is no scientific report on prevalence of the disease in dairy cattle in Uşak region. In this study, it was aimed to determine the prevalence of subclinic paratuberculosis in dairy cattle farming in Uşak region in Turkey. In the study a total of 200 Holstein dairy cattle aged between 3-7 years clinically healthy with optimum milk yield and body condition score were used. The MAP were identified in feces and milk samples using direct bacterioscopy technique, and in positive samples by culturing and Polymerase Chain Reaction (outer and nested) techniques. In Uşak region in dairy cattle the prevalence of paratuberculosis was determined to be 17% by Ziehl-Neelsen staining, 9.5% by Outer Polymerase Chain Reaction and 20% Nested Polymerase Chain Reaction in feces; 4% according to bacteriologic culture results; 15.5% by Ziehl-Neelsen staining, 5.5% by Outer Polymerase Chain Reaction and 17.5% by Nested Polymerase Chain Reaction in milk samples and 2.5% according to bacteriologic culture results.

Keywords: *Johne's disease, Mycobacterium paratuberculosis, PCR, Ziehl-Neelsen staining*

Uşak Yöresi Sütçü Sığırlarda Subklinik Paratuberkülozun Prevalansı

Özet

Paratuberküloz, *Mycobacterium avium* subs. *paratuberculosis*'in etken olduğu, ruminantların yangısal ve ölümcül bir hastalığıdır. Enfeksiyon, kronik "subklinik" evre ile karakterizedir. Bu dönemdeki sığırlar sürüdeki diğer hayvanları enfekte edebilir. Türkiye'de paratuberküloz'un varlığı uzun yıllardır bilinmesine rağmen, hastalık ve hastalığın prevalansı üzerine yürütülmüş bilimsel çalışma sayısı oldukça azdır. Uşak yöresi süt sığırlarında hastalığın prevalansı üzerine gerçekleştirilmiş bilinen bir bilimsel rapor ise mevcut değildir. Bu çalışmada, yoğun olarak süt sığırı yetiştiriciliği yapılan Uşak yöresinde subklinik paratuberküloz'un prevalansının ortaya konulması amaçlandı. Araştırma, optimal süt verimine ve optimal vücut kondüsyon skoruna sahip, klinik olarak sağlıklı, 3-7 yaşlı 200 Holştayn süt sığırı üzerinde yürütüldü. Etken, dışkı ve süt örneklerinde direk bakterioskopi tekniği ve pozitif bulunan örneklerde ise kültür ve Polimeraz Zincir Reaksiyonu (Outer ve Nested) teknikleri kullanılarak tespit edildi. Uşak yöresi süt sığırlarında subklinik paratuberküloz'un prevalansı, dışkı örneklerinde; Ziehl-Neelsen boyama ile %17, Outer Polimeraz Zincir Reaksiyonu tekniği ile %9.5, Nested Polimeraz Zincir Reaksiyonu tekniği ile %20, Bakteriyolojik Kültür sonuçlarına göre %4, süt örneklerinde ise; Ziehl-Neelsen boyama ile %15.5, Outer Polimeraz Zincir Reaksiyonu tekniği ile %5.5, Nested Polimeraz Zincir Reaksiyonu tekniği ile %17.5 ve Bakteriyolojik Kültür sonuçlarına göre %2.5 olarak tespit edildi.

Anahtar sözcükler: *Johne's hastalığı, Mycobacterium paratuberculosis, PCR, Ziehl-Neelsen boyama*

INTRODUCTION

Paratuberculosis (pTB) is a slowly progressing infection affecting normally ruminants. Its cause is shown to be the *Mycobacterium avium subs. paratuberculosis (MAP)*, an acid-fast bacillus. *MAP* is demonstrated to stay alive more than a year in the feces of cattle and around 160 days within the surface water [1]. The bacterium has also a wide array of host

İletişim (Correspondence)
+90 272 2281312/202
tcivelek@aku.edu.tr

distribution, infecting mainly ruminants but less frequently muflon [2] and buffalo [3]. It also affects non-ruminant animals such as birds, wild pigs, mice, rats, foxes, bears, and rabbits [4] as well as domestic pigs and primates [5]. Moreover, *MAP* is critical for possessing zoonotic potential since it is reported to be possible cause of Crohn's disease in human [6,7]. Although pTB cases are encountered across the continents, its regional and territorial distributions show differences. While the prevalence of pTB infection in Germany is reported to be 84.7%, some parts of Australia is stated to be free of the infection. Moreover, pTB prevalence is very low in Sweden. The studies performed on dairy cattle in different countries indicate that the prevalence of pTB infection is similar and reported to be 47% in Denmark, 43% in Canada, and 50% in the United States [8-10].

MATERIAL and METHODS

Animals and Samples

The animals used in the present study were randomly collected from 31 different dairy cattle farms located in Uşak -a province in western Turkey and surrounding central villages (Çamyazı, n=44; Beylerhan, n=14; Kaşbelen, n=36; Aşağı-karacahisar, n=4; Köprübaşı, n=12; Güneli, n=21; Sarıdere, n=2; İkisaray, n=12; Bozkuş, n=8; Karahasan, n=32; Karaağaç, n=3; Çevre, n=9; and Selvioğlu, n=3). We used 200 dairy Holstein cattle that were at age between 3 to 7 years old, healthy, optimum milking, not inoculated for pTB, showing cyclic activities, and possessing optimum body condition score.

We collected feces and milk samples from the animals as study materials and kept them in sterile transfer containers under maintained cold environment till the samples were transferred to the laboratory for analyses.

Bacteriological Analyses

Ziehl-Neelsen's stain: Direct examinations of the fecal and milk samples were performed after reacting them with Ziehl-Neelsen's (ZN) stain.

Bacteriologic culture: Before performing cultures from the fecal and milk samples found to be positive with ZN stain and Nested Polymerase Chain Reaction (PCR), 10-20 g of the samples were crushed in glass homogenizers to inactivate other contaminating microorganisms. The homogenized samples were treated with equal amounts of decontaminants (20 g NaOH and 4.5 mL Bromcreasol purple), stirred for 5-10 sec, and kept at room temperature for 10 min. Consequently, after adding equal amounts of neutralizers (82.5 mL %37.5 HCl) on the homogenized samples, they were centrifuged at 1.500 rpm for 10 min and at the end of the centrifugation the sediments were saved. While some of the sediments of the samples were used for microscopic analyses, the reaming sediments were cultured in BBL™ Herrold's Egg Yolk Agar Slant with M J and ANV (USA, Cat No: 8015750) for 6-8 weeks at 37°C. The caps of the culture tubes were opened twice or trice in a week for proper oxygenation [11,12]. Entire of the current studies were performed in a Class II biosafety cabinet.

PCR procedure: We used Qiagen QIAamp® DNA stool mini kit (Cat. No. 51504: Qiagen, Hilden, Germany) for DNA extraction from the cultures and fecal samples. We selected *MAP* specific primers targeted to the IS900 gene region (*M. paratuberculosis* IS900A sequence on the EMBL GenBank DNAsequence database, accession number X16293). The samples were amplified using nested PCR approach. The oligonucleotide primers selected for the present study and specific to gene region were as follows:

MAPOF1: 5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3', Outer

MAPOR1: 5'-GGCGTTGAGGTCGATCGCCCACGTGAC-3', Outer

MAPNF1: 5'-CAGGGACGTCGGGTATGGCTTTCA-3', Nested

MAPNR1: 5'-CGTCACCGCCGCAATCAACTCCAG-3', Nested

DNA isolation from the fecal samples: After putting MAP 316 F reference culture and fecal samples (200 mg) in 2 mL-sterile eppendorf tubes, 1.4 mL ASL buffer solution was added to the tubes. After stirring, the tubes were vortexed for 1 min and incubated on a thermal block (VWR 460-3208, USA) for 5min at 95°C. At the end of the incubation the tubes were stirred for 15 sec and spun at 14.000 g for 1 min. Subsequently, 1.2 mL supernatants were collected from the centrifuged samples and placed in 2 mL-sterile eppendorf tubes. An InhibitEX tablet coming with commercially available DNA isolation Kit was added into each of these tubes and the tubes were kept at room temperature for 1-2 min and vortexed until the tablets were melted. The tubes were again centrifuged at 14.000 g for 3 min. Then 200 µL supernatant was pulled from the generated supernatant and placed in 1.5 mL eppendorf tubes containing 15 µg proteinase K and 200 µL AL buffer was added to these tubes which were then incubated at 70°C for 10 min. After the incubation, the tubes were reacted with 200 µL of 96% ethyl alcohol and the samples were transferred into the QiAamp spin colon and spun at 14.000 g for 1 min and the filtrate was discarded. Subsequently, after putting the QiAamp spin colons back in 2 mL centrifuge tubes, 500 µL AW2 was added into the tubes which were then again spun at 14.000 g for 3 min and the filtrate was removed. After putting QiAamp spin colons in 1.5 mL centrifuge tubes, 200 µL AE buffer was added to the tubes, incubated for 1 min at room temperature. Finally, QiAamp spin colons were centrifuged at 14.000 g for 1 min and the QiAamp spin colons were disposed. By this way, DNA was extracted in 1.5 mL eppendorf tubes that were kept at -20°C until use.

DNA isolation from the milk samples: For DNA isolation from the milk samples, 10 mL milk samples collected from the animals were centrifuged at 20.000 rpm for 1 h and the yielding

supernatants were discarded. The pellets at the bottom of the tubes were resuspended in 1 mL distilled water and vortexed, the 500 µL of the suspensions were transferred into the eppendorf tubes and the tubes were centrifuged at 13.000 g for 10 min and the supernatants were disposed. Subsequently, the pellets at the tip of the tubes were resuspended in 300 µL Tris-EDTA and vortexed. The tubes were inactivated on a thermal block at 95°C for 15 min and then cooled down at room temperature. Following the inactivation, phenol saturated with 300 µL Tris-HCl was added to the tubes and hand mixed for 4-5 min, spun at 13.000 g for 10 min, and the supernatants were relocated to new tubes where DNA precipitation process was initiated. For DNA precipitation, 0.1 volume 3M Na-acetate and 2.5 volume pure ethyl alcohol were added to the tubes, thoroughly mixed, kept at -20°C for 1 h. Aftermath, 300 µL 90% and 70% ethyl alcohol was added to the suspension. The tubes were centrifuged at 13.000 g for 5 min between these stages and the supernatants were discarded at each time. The pellets were resuspended in 50 µL distilled water, denatured at 60°C for 1 h, and then used for PCR analyses.

DNA amplification with PCR: DNA amplification was performed at two stages using Nested PCR. The primers MAPOF1 and MAPOR1, and MAP 316 F were used for outer PCR in order to extract DNA from the fecal and milk samples. The MAPOF1 was designed to code 413bp fragment on the IS900 gen region on MAP strains and the MAPOR1 was used as reference primer. The sequences of MAPOF1 and MAPOR1 were 5′-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3′ and 5′-GGCGTTGAGGTCGATCGCCCACGTGAC-3′, respectively. After visualizing PCR products on agarose gel, 1 µL product was taken from positive and negative samples for performing Nested PCR where we used the primers MAPNF1 and MAPNR1. MAPNF1 was designed to code 326bp. The sequences of MAPNF1 and MAPNR1 were 5′-CAGGGACGTCGGGTATG GCTTTCA-3′ and 5′-CGTCACCGCCGCAATCAACTCCAG-3′, respectively.

Outer PCR amplification was carried out in a 50 µl mixture containing 5 µl PCR buffer (containing KCL), 3 µL $MgCl_2$ (25 mM), 1 µL dNTP set (10mM), 1 µL (1 µM) primer (MAPOF1, MAPOF1), 0.25 µL (1.25 U) Taq DNA polymerase, 33.75 µL ultra pure water, and 5µL targeted DNA. Targeted DNA was amplified after an initial 15 min denaturation at 95°C, 30 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min, and followed by a 2 min extension at 72°C [13] using a thermal cycler (Eppendorf, Matercycler gradient 5331 000.010, Germany). Afterward, 5µL DNA was stained in 1 µL of loading dye and loaded on a 1.5% agarose gel containing 5 µg/mL ethidium bromide and run on electrophoresis.

For Nested PCR amplification, 1 µL was taken from positive and negative samples and PCR amplification was carried out in a 25 µl mixture containing 2.5 µL PCR buffer (containing KCL), 1.5 µL $MgCl_2$ (25 mM), 0.5 µL dNTP set (10 mM), 0.5 µL (1 µM) primer (MAPNF1, MAPNR1), 0.25 µL (1.25 U) Taq DNA polymerase, 18.55 µL ultra pure water. Targeted DNA was amplified after an initial 15 min denaturation at 95°C, 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min, and followed by a 2 min extension at 72°C [13] using a thermal cycler (Eppendorf, Matercycler gradient 5331 000.010, Germany). Moreover, 1000 bp DNA ladder was used as marker and DNA extraction of *MAP* 316 F reference strain was used as positive control.

RESULTS

The results of the present study are summarized in *Table 1*. The present results showed that the prevalence of subclinical pTB in dairy cows was 17% with ZN stain, 9.5% with outer PCR technique, 20% with Nested PCR technique, and 4% with bacteriologic culture method in fecal samples whereas it was 15.5% with ZN stain, 5.5% with outer PCR technique, 17.5% with Nested PCR technique, and 2.5% with bacteriologic culture method in milk samples.

DISCUSSION

Mycobacterium avium subs. paratuberculosis, often abbreviated *MAP* is the ethological agent for paratuberculosis

Table 1. *Prevalence of subclinical paratuberculosis in feces and milk samples*
Tablo 1. *Dışkı ve süt örneklerinde subklinik paratüberkülozun prevalansı*

Results	Feces Samples				Milk Samples			
	ZN	PCR		BC	ZN	PCR		BC
		Outer	Nested			Outer	Nested	
(+) n=200	34/200	19/200	40/200	8/200	31/200	11/200	35/200	5/200
(-) n=200	166/200	181/200	160/200	192/20	169/200	189/20	165/200	195/20
(?) n=200	2/200	-	-	-	5/200	-	-	-
(+)%	17%	9.5%	20%	4%	15.5%	5.5%	17.5%	2.5%
(-)%	82%	90.5%	80%	96%	82%	94.5%	82.5%	97.5%
(?)%	1%	-	-	-	2.5%		-	-

BC: *Bacteriologic culture;* ***ZN:*** *Ziehl-Neelsen staining;* ***(+):*** *Positive results;* ***(?):*** *Suspicious results;* ***(-):*** *Negative results*

or Johne's disease. Mainly, pTB causes considerable economic losses in dairy cow farms [14,15]. The animals infected with *MAP* disseminate the bacterium via their feces and milk [14]. Moreover, since the animals carrying subclinical pTB infection can transfer bacilli to healthy animals, proper identification of the subclinical pTB infection is critical to prevent spread of the bacteria. Although the presence of pTB infection has been known throughout Turkey, the numbers of the studies inspecting the infection and its prevalence are limited [16,18,19]. Likewise, no studies are available concerning pTB infection in Uşak province in Turkey (Web of Science). Therefore, at the present study we aimed to establish the prevalence of subclinical pTB infection dairy cows in Uşak area.

In the present study, we detected the presence of *MAP* in fecal and milk samples using direct bacterioscopy technique (ZN staining) and the samples turned out to be positive with ZN stain were further analyzed using culture and PCR (Outer and Nested) approaches.

The diagnosis of *MAP* can be done using conventional IS900 PCR primers in real-time PCR. For this purpose, different strategies have been tried in time to determine more effective, dependable and useful techniques for the determination of *MAP* [20-23]. Nested PCR was used for the first time in 1993 to compare effectiveness of Nested PCR and fecal culture approach in the determination of *MAP* [24]. Later on, Nested PCR has been used particularly in diagnoses of *MAP* associated Crohn's disease and in numerous other studies on pTB cases [13,16,25-31]. The specificity and sensitivity of the PCR tests used in the diagnoses of *MAP* in fecal samples have been greatly improved in recent years and new PCR tests can allow us to work with small amounts of fecal samples in determination of *MAP*. The main reason for the increased sensitivity of the PCR tests is the availability of improved DNA extraction and purification processes [14].

Nested PCR is shown to be more sensitive than conventional PCR technique in determination of *MAP* infected animals [14,28]. Accurate diagnose rates of *MAP* with both techniques are shown to be higher in fecal samples than milk samples (in fecal samples, Nested PCR: 49.3%; simple PCR: 45.3%; in milk samples: Nested PCR: 32%; simple PCR: 27.3%) [14]. These observations are consistent with our findings here (in fecal samples, Nested PCR: 20%; outer PCR: 9.5%; in milk samples: Nested PCR: 17.5%; simple PCR: 5.5%) and further support present results. *MAP* is mainly disseminated to environment via infected animal feces. However, contami-nation of *MAP* via milk is also very significant and one third of the clinically infected animals are reported to spread *MAP* via their milks [32]. Relatively lower prevalence of the pTB in milk samples with regard to fecal samples in the present study can be accounted for the fact that the main excretion of *MAP* is via feces with respect to milk [17]. Since *MAP* possesses zoonotic potential and also contaminates milk, precise identification of its presence in milk is critical for public health [17].

Significant improvements have been obtained in PCR based methods for diagnosing *MAP*. Modified PCR methods are almost as sensitive as bacterial culture methods accepted as gold standard today. Nested PCR has been reported to be simple, fast, reliable, and highly sensitive technique that can be used alternatively in determination of *MAP* in fecal and milk samples [14]. Nonetheless, in the present study the prevalence of subclinical pTB in fecal and milk cultures was 4% and 2.5%. These acquired results showed differences according to the prevalence rates obtained using Nested and Outer PCR *(Table 1)*. This result can be explained either with the fact that PCR is more sensitive than culture method [17,28] or with the ability of PCR to be able to replicate residual DNA of dead microorganisms, thereby enabling multiplication of contents of dead microorganisms in our study samples and leading negative growth in cultures [17]. An earlier study indicate that while 50% of PCR positive samples show factor growth in cultures, this percentage decreases to 36% in PCR negative samples [19]. Moreover, at their study Gao et al.[28] reported that the amount of the *MAP* disseminated by infected cows would be less than the sum determined with culture in the Nested PCR positive but culture negative cases. When compared to culture method, Nested PCR is suggested to be convenient and an alternative approach for the diagnosis of *MAP* in fecal and milk samples cases [28]. The use of decontaminants to inactivate other micro-organism, high incubation temperature (37°C), and longer incubation times causes death of more *MAP*, a major reason for getting reduced prevalence in cultures [28]. At the same time, the prevalence values obtained in the present study using ZN stain are consistent with those acquired by PCR (in fecal samples; ZN stain: 17%, Nested PCR: 20%; in milk samples, ZN stain: 15.5%, Nested PCR: 17.5%). Obtainment of possible false positivity owing to cross reaction with even very low probability may explain the one of the reasons for the difference between prevalence values gained using PCR and culture.

The prevalence of pTB infection in cows is demonstrated to be between 3-30% in European countries [15]. In Turkey, a study using complement fixation technique indicate that the prevalence of the infection in the Central Anatolia Region is shown to be between 2.7% and 4.3% [19]. Nonetheless, pTB infection generally shows a subclinical course. While the use of complement fixation technique in the determination of the animals showing clinical symptoms is indicated to be more useful [33], it is reported to be insufficient in the diagnoses of the subclinical pTB cases [17]. Therefore, observation of lower prevalence in other studies with regard to the present study is expected. The studies performed in England report the prevalence of clinical pTB as 1%. Moreover, the prevalence of pTB in the animals obtained from the abattoirs and showing no clinical signs for the infection is shown to be 3.5% [34,35].

Majority of the animals carrying subclinical pTB infection do not present infection associated symptoms all through their live. Studies indicate that 5-10% of the animals inflicted with subclinical pTB infection in herd show clinical symptoms [36].

A recent study on the pTB infection in dairy cows in Burdur area reported that seroprevalence for the infection was 6.2% [33]. ELISA is not only widely used in diagnosis of pTB but also it has easy application and high specificity. Nevertheless, the specificity of ELISA in the animal not showing clinical symptoms and presenting clinical signs are reported to be around 15% and 87%, respectively [37,38]. Furthermore, the specificity of ELISA is shown to be lower in the cows younger than two years old [39]. Therefore, the studies suggest that sole use of ELISA could be inadequate in the diagnosis of the subclinical pTB infections.

In the present study, our results showed that subclinical pTB prevalence in the fecal samples was ranged from the lowest 4% (culture) to the highest 20% (Nested PCR), in the milk samples it was ranged from the lowest 2.5% (culture) to the highest 17.5% (Nested PCR).

Central significant factors determining prevalence of pTB are shown to be climate, nourishment, and barn conditions [35]. Therefore, the prevalence of pTB can show marked differences throughout Turkey.

This study was done on Holstein dairy cows and we encountered no reported strain based predisposition to pTB infection in these animals (Web of Science).

The results of the present study demonstrate the national necessity for the studies examining the presence of subclinical pTB infections in dairy cows in Turkey to determine their prevalence and estimate the infection-associated economical losses. Present results also indicate that Nested PCR technique is possibly more sensitive than bacterial culture method that is accepted to be the gold-standard although whose significant disadvantages for the detection of the pTB infection is not fully clarified. PCR technique can be used for the detection of subclinical pTB infection as an alternative to bacterial culture method. Nevertheless, the use of combination of discrete techniques for the detection of the pTB infection would increase accuracy of the diagnosis. Proper diagnoses and treatments of pTB infections are critical when we consider the high transmission rate of *MAP* with milk, its zoonotic potential, and its resistance to pasteurization [40-42]. In addition, the animals carrying subclinical pTB infection can transmit the infection other healthy animals causing additional health problems for other animals and people. In summary, PCR techniques indicate higher prevalence rates (20% in fecal and 17.5% in milk samples) in Uşak province when compared to the other regions in Turkey.

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REFERENCES

1. Baumgartner W, Khol JL: Paratuberculosis (Johne disease) in Ruminants ongoing story. *Slow Vet Res,* 43, 5-10, 2006.

2. Machackova M, Svastova P, Lamka J: Paratuberculosis in farmed and free-living wild ruminants in the Czech Republic. *Vet Microbiol,* 101, 225-234, 2004.

3. Sivakumar P, Tripathi BN, Singh N: Detection of *Mycobacterium avium subsp. paratuberculosis* in intestinal and lymph node tissues of water buffaloes *(Bubalis bubalois)* by PCR and bacterial culture. *Vet Microbiol,* 108, 263-270, 2005.

4. Judge J, Kyriazakis I, Greis A: Clustering of Mycobacterium avium subsp. paratuberculosis in rabbits and the environment: how hot is a hot spot? *Appl Environ Microbiol,* 71, 6033-6038, 2005.

5. Klee W: Paratuberkulose (Johnesche Krankheit). **In,** Dirksen D, Gründer HD, Stöber M (Eds): Innere Medizin und Chirurgie des Rindes. 4 Aufl Berlin Blackwell, pp. 586-591, Parey, Stuttgart, 2006.

6. Nakase H, Nishio A, Tamaki H: Specific antibodies against recominant protein of insertion element 900 of *Mycobacterium avium subspecies paratuberculosis* in Japanese patients with Crohn´s disease. *Inflamm Bowel Dis,* 12, 62-69, 2006.

7. Pickup RW, Rhodes G, Arnott S: *Mycobacterium avium subspecies paratuberculosis* in the catchment aera and water of the river Taff in 11. South Wales, United Kingdom, and its potential relationship to clustering of Crohn´s disease in the city of Cardiff. *Appl Environ Microbiol,* 71, 2130-2139, 2004.

8. Collins MT, Sockett DC, Goodger WJ: Herd prevalence and geographic distribution of, and risk factors for bovine paratuberculosis in Wisconsin. *J Am Vet Med Assoc,* 204, 636-641, 1994.

9. Jakobsen MB, Alban L, Nielsen SS: A cross-sectional study of paratuberculosis in 1155 Danisch dairy cows. *Prev Vet Med,* 46, 15-27, 2000.

10. Van Leeuwen JA, Keefe GP, Tremblay R: Seroprevalence of infection with *Mycobacterium avium subspecies paratuberculosis*, bovine leukemia virus and bovine viral diarrhea virus in Maritime Canada dairy cattle. *Can Vet J,* 42, 193-198, 2001.

11. Ambrosio SR, Márcia D.E, Deus OE, Rodriguez, CAR, Amaku, M: Comparison of three decontamination methods for *Mycobacterium bovis* isolation. *Braz J Microbiol*, 39, 241-244, 2008.

12. Petroff SA: A new and rapid method for the isolation and cultivation of tubercle bacilli directly from the sputum and feces. *J Exp Med,* 21, 38-42, 1915.

13. Ryan P, Bennett MW, Aaron S, Lee G, Collins JK: PCR detection of *Mycobacterium paratuberculosis* in Crohns disease granulomas isolated by laser capture microdissection. *Gut,* 51, 665-670, 2002.

14. Moghadam MT, Sarv S, Moosakhani F, Badiie A: Detection of *Mycobacterium avium subspecies paratuberculosis* in milk and facel samples in dairy cattle by PCR and Nested-PCR. *J Anim Vet Adv,* 9 (24): 3055-3061, 2010.

15. Nielsen SS, Toft N: Review of prevalences of paratuberculosis in farmed animals in Europe. *Prev Vet Med,* 88, 1-14, 2009.

16. Civelek T, Celik HA, Ozenc E, Avci G, Kav K, Cingi CC, Yilmaz O: Effects of PCR-confirmed subclinical paratuberculosis on retinol and β-carotene levels in dairy cattle. *Arch Med Vet,* 41, 281-284, 2009.

17. Cetinkaya B, Ergan K, Harbour DA, Morgan KL: An abattoir-based study of the prevalence of subclinical Johnes disease in adult cattle in south west England. *Epidemiol Infect,* 116, 373-379, 1999.

18. Ikiz S, Bagcigil AF, Ak S, Ozgur NY, Loaz A: Paratuberculosis in cattle in Turkey detected by PCR. *Medicina Wet,* 61, 881-883, 2005.

19. Vural B, Atala N: Serological study on bovine paratuberculosis in central Anatolia using the microcomplement fixation and tube complement fixation tests. *Etlik Vet Mikrobiol Derg,* 6, 87-97, 1998.

20. Ayele WY, Svastova P, Roubal P, Bartos M, Pavlik I: *Mycobacterium*

avium subspecies paratuberculosis cultured from locally and commercially pasteurized cows milk in the Czech Republic. *Applied Environ Microbiol,* 71, 1210-1214, 2005.

21. Herthnek D, Bolske G: New PCR systems to confirm real-time PCR detection of *Mycobacterium avium subsp. paratuberculosis*. *BMC Microbiol,* 6, 87, 2006.

22. Khare S, Ficht TA, Santos RL, Romano J, Ficht AR: Rapid and sensitivedetection of *Mycobacterium avium subsp. paratuberculosis* in bovine milk and feces by a combination of immunomagnetic bead separation-conventional PCR and real-time PCR. *J Clin Microbiol,* 42, 1075-1081, 2005.

23. Tasara T, Stephan R: Development of an F57 sequence-basedreal-time PCR assay for detection *of Mycobacterium avium subsp. paratuberculosis* in milk. *Applied Environ Microbiol,* 71, 5957-5968, 2005.

24. Collins DM, Stephens DM, De Lısle, GW: Comparison of polymerase chain reaction tests and fecal culture for detecting *Mycobacterium paratuberculosis* in bovine feces. *Vet Microbiol,* 36, 289-299, 1993.

25. Autschbach F, Eisold S, Hinz U, Zinser S, Linnebacher M: High prevalence of *Mycobacterium avium subsp. paratuberculosis* IS900 DNA in gut tissues from individuals with Crohns disease. *Gut,* 54, 944-949, 2005.

26. Bull TJ, Mcminn EJ, Sidi-boumedine K, Skull A, Durkin D: Detection and verification of *Mycobacterium avium subsp. paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without crohn's disease. *J Clin Microbiol,* 41, 2915-2923, 2003.

27. Fang Y, Wu WH, Pepper JL, Larsen JL, Marras SA: Comparison of realtime, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *Mycobacterium avium subsp. paratuberculosis* in bovine fecal samples. *J Clin Microbiol,* 40, 287-291, 2002.

28. Gao A, Odumeru J, Raymond M, Handrick S, Duffied T, Mutharia L: Comparison of milk cultures, direct and nested polymerase chain reaction (PCR) with fecal culture based on samples from dairy herds infected with *Mycobacterium avium subs.* P*aratububerculosis. Can J Vet Res,* 73, 58-64, 2009.

29. Lisby G, Andersen J, Engbaek K, Binder V: *Mycobacterium paratuberculosis* in intestinal tissue from patients with Crohns disease demonstrated by a nested primer polymerase chain reaction. *Scand J Gastroenterol,* 29, 923-929, 1994.

30. Meadus WJ, Gill CO, Duff P, Badoni M, Saucier L: Prevalence on beef carcasses of *Mycobacterium avium ssp. paratuberculosis* DNA. *Int J Food Microbiol,* 124, 291-294, 2008.

31. Pinedo PJ, Rae DO, Williams JE, Donovan GA, Melendez P, Buergelt CD: Association among results of serum ELISA, faecal culture and nested PCR on milk, blood and faeces for the detection of Paratuberculosis in dairy cows. *Transbound Emer Dis,* 55, 125-133, 2008.

32. Taylor TK, Wilks CR, Mcqueen DS: Isolation of *Mycobacterium paratuberculosis* from the milk of a cow with Johne's disease. *Vet Rec,* 109, 532-533, 1981.

33. Burnham WR, Stanford JL, Lennard-Jones JE: Evidence for Mycobacterial aetiology of Crohns disease. *Gut,* 18, 965, 1978.

34. Cetinkaya B, Ergan K, Morgan KL: A practice-based survey of the frequency of Johne's disease in southwest England. *Vet Rec,* 134, 494-497, 1994.

35. Cetinkaya B: An epidemiological study of clinical and subclinical Johne's Disease (Paratuberculosis) in cattle. *Dep Vet Clin Sci, Univ Bristol, England,* 1996.

36. Wilson DJ, Rossiter C, Han HR, Sears PM: Association of Mycobacterium paratuberculosis infection with reduced mastitis, but with decreased milk production and increased cull rate in clinically normal cows. *Am J Vet Res,* 54, 1851-1857, 1993.

37. Bassey EOE, Collins MT: Study of T-lymphocyte subset of helaty and *M. paratuberculosis* infected cattle. *Infect Immun,* 65, 4869- 4872, 1997.

38. Hietala SK: The option in diagnosing ruminat paratuberculosis. *Vet Med,* 87, 1122- 1132, 1992.

39. Ozturk D, Pehlivanoglu F, Tok AA, Gunlu S, Guldali Y, Turutoglu H: Seroprevalence of paratuberculosis in the Burdur province (Turkey) in dairy cattle using the Enzime Linked Immunosorbent. *Assay Isr J Vet Med,* 65, 53-57, 2010.

40. Chiodini RJ, Hermon-Taylor J: The thermal resistance of *Mycobacterium paratuberculosis* in raw milk under conditions simulating pasteurization. *J Vet Diagn Invest,* 5, 629-631, 1993.

41. Gao A, Mutharia L, Chen S, Rahn K, Odumeru J: Effect of pasteurization on survival of *Mycobacterium paratuberculosis* in milk. *J Dairy Sci,* 85, 3198-3205, 2002.

42. Grant IR, Ball HJ, Neill SD, Rowe MT: Inactivation of *Mycobacterium paratuberculosis* in cows milk at pasteurization temperatures. *Appl Environ Microbiol,* 62, 631-636, 1996.

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RESEARCH ARTICLE

Türkiye'deki İki Farklı İşletmede Yetiştirilen Holştayn Boğalarda Faktör XI Yetmezliği (FXID) Allel Frekansının Belirlenmesi

Bilal AKYÜZ *

* Erciyes Üniversitesi, Veteriner Fakültesi, Genetik Anabilim Dalı, TR-38039 Kayseri - TÜRKİYE

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Özet

Faktör XI yetmezliği (FXID) otozomal resesif bir hastalıktır. Faktör XI yetmezliğinde, hem homozigot hem de heterozigot bireylerde mastitis, metritis, uzayan buzağılama aralığı ve gebelik başına ikiden fazla tohumlama gibi bazı problemler görülebilir. Bu çalışmada Holştayn boğalarda kalıtsal bir hastalık olan faktör XI yetmezliğine (FXID) neden olan mutant allelin allel frekansının belirlenmesi amaçlanmıştır. Çalışmada 59 baş Holştayn boğa kullanılmıştır. Bu amaçla, incelenen boğalara ait DNA örnekleri fenol-kloroform yöntemi ile elde edilmiştir. Elde edilen DNA'lar PCR ile çoğaltılmıştır. PCR sonunda incelenen örneklerin FXID yönünden genetik durumları %2'lik agaroz jel elektroforezi ile belirlenmiştir. İncelenen Holştayn boğalar içerisinde birinin FXID taşıyıcısı olduğu belirlenmiştir. İncelenen Holştayn boğalarda FXID prevalansının yaklaşık olarak %1.7 olduğu belirlenmiştir.

Anahtar sözcükler: *FXID, Holştayn boğa, Kalıtsal hastalık, PCR*

Determination of Allele Frequency of Factor XI Deficiency (FXID) in Holstein Bulls Raised in Two Different Enterprise in Turkey

Summary

Factor XI deficiency (FXID) is an autosomal recessive disease. Both heterozygotes and homozygote individuals for FXID can be seen problems such as mastitis, metritis, lower calving and repeat breeding. The aim of this study was to determinate the frequency of allele leading to the hereditary factor XI deficiency disease (FXID) in the Holstein bulls. In this study, 59 Holstein bulls were used. For this purpose, the DNA samples of the eveluated bulls were obtained by phenol-chloroform isolation method. These DNAs were amplified by PCR. End of PCR, the genetic conditions of analyzed Holstein bulls about FXID were determined by 2% agarose gel electrophoresis. In the evaluated Holstein bulls, one of the bulls was detected as a FXID carrier. It was determined that FXID prevalence was 1.7% in the investigated Holstein bulls.

Keywords: *FXID, Hereditary disease, Holstein bulls, PCR*

GİRİŞ

Sığır yetiştiriciliğinde kalıtsal hastalıklar, bireyin yaşama gücünü ve döl verimini düşürerek işletmelerin karlılığını düşürebilmektedir. Bu nedenle işletmeler yetiştirme programlarını hazırlarken, damızlık adaylarının, yetiştirilmesi planlanan ırklarda en yaygın görülen kalıtsal hastalıkları taşımadıklarının belirlenmesi gereklidir. Entansif sığır yetiştiriciliğinde, üstün verimli sınırlı sayıda boğanın kullanıldığı suni tohumlama yönteminin yaygınlaşması kalıtsal hastalıkların sebep olduğu kayıpların önemini her geçen gün daha da artırmaktadır. Özellikle süt sığırı yetiştiriciliğinde suni tohumlama ve multiple ovulasyon gibi metotlarının yaygın kullanılması birim hayvan başına verimi artırmış ancak ırk içi varyasyonu azaltmış ve kalıtsal hastalıkların kısa sürede tüm dünyaya yayılmasına neden olmuştur [1]. Kalıtsal hastalıklar yönünden bir ırkın tüm bireyleri taranamaz. Ancak, kalıtsal hastalıkların kontrol altına alınmasında üstün özellikleri nedeniyle suni tohumlama ve embriyo nakli amacıyla kullanılan damızlıklar ve damızlık adaylarının incelenmesi önemlidir.

Karaciğer tarafından sentezlenen ve kanın pıhtılaşmasında görevli bir serin proteazı olan faktör XI proteininin kalıtsal olarak sentezlenememesi sonucu ortaya çıkan faktör XI

İletişim (Correspondence)
+90 207 6666/29721
bakyuz@erciyes.edu.tr

yetmezliğinin (FXID) sığır, insan ve köpeklerde görüldüğü rapor edilmiştir [2,3]. Otozomal resesif bir kalıtım şekli gösteren hastalık, sığırlarda sadece Holştayn ve Japon Siyah Sığırında belirlenmiştir [4]. Hastalık ilk olarak 1969 yılında ABD'de yetiştirilen Holştayn'larda bildirilmiştir [5]. Bu tarihten sonra Kanada ve İngiltere'de de bu kalıtsal hastalığın varlığı hakkında bildirişler vardır [6]. Hastalık Holştayn'larda, faktör XI proteinini kodlayan genin 12. ekzonuna 76 bazlık bir parçanın eklenmesi sonucu ortaya çıkmaktadır [1].

Faktör XI yetmezliği, hasta hayvanlarda klinik olarak tanınmasını sağlayacak özel semptomlar göstermez [2]. Ancak mutant allel yönünden homozigot ve heterozigot buzağıların, homozigot normal buzağılara göre daha düşük doğum ağırlığına sahip oldukları ve bu buzağılarda yaşama gücü düşüklüğü görülebileceği bildirilmiştir [6]. Heterozigot bireylerde faktör XI pıhtılaşma aktivitesinde azalmanın olduğu, homozigotlarda ise bu aktivitenin tamamen ortadan kalktığı görülür. Hasta bireylerde kanama süresinde uzama ve anemi gibi semptomlar da görülebilir [2].

Ayrıca FXID yönünden taşıyıcı ve homozigot hayvanlarda kan östrodiol oranında düşme, foliküler gelişimin tam olmaması ve üreme performansının düşmesi sonucu süt sığırcılığında önemli verim kayıplarına neden olan repeat breeding prevalansında artışa neden olabildiği de bildirilmiştir [2].

Türkiye'de Holştayn ırkı ineklerde FXID'e neden olan mutant allelin varlığı ilk kez 2009 yılında ortaya konmuştur [3]. Ancak Türkiye'de yetiştirilen ve damızlık olarak kullanılan Holştayn boğalarda bu kalıtsal hastalığın varlığının araştırıldığı bir çalışma yoktur. Bu çalışmada, Türkiye'de yetiştirilen ve damızlık olarak kullanılan Holştayn boğalarda faktör XI yetmezliğine neden olan mutant allelinin varlığının araştırılması amaçlanmıştır.

MATERYAL ve METOT

Araştırmada 59 baş Holştayn boğa incelenmiştir. Çalışma materyalinin İzmir-Menemen (n=41) ve Ankara'da (n=18) yetiştirilen damızlık boğalar oluşturmuştur. Bu hayvanlardan alınan kan örneklerinden fenol-kloroform ekstraksiyon yöntemi ile DNA izole edilmiştir. İncelenen bireylerin FXID durumları Maron ve ark.[6] tarafından önerilen (FXIF 5´-CCCACTGGCTAGG AATCGTT-3´; FXIR 5´-CAAGGCAATGTCATATCCA C-3') primer seti kullanılarak yapılan PCR işlemi ile belirlenmiştir. PCR, toplam hacmi 20 µl olacak şekilde hazırlanan; 14.3 µL dH_2O, 2.8 µL 10 x PCR buffer, 2 µL dNTP, forward ve reverse primerlerden 0.4'er µL (20 nmol), 0.1 µL Taq polimeraz enzimi (5 U/µL) karışımla yapılmıştır. Hazırlanan bu karışım üzerine 5 µL genomik DNA'lar ilave edilerek PCR karışımı 25 µL'ye tamamlanmıştır. PCR işlemi hazırlanan karışımın 95°C'de 10 dk. tutulmasından sonra her bir döngüsü; 95°C'de 30 sn, 55°C'de 1 dk ve 72°C'de 30 sn olacak şekilde 34 döngü yapılmıştır. Son döngüden sonra tüpler 72°C'de 10 dk. tutularak PCR işlemi tamamlanmıştır. Yapılan PCR işlemi sonunda incelenen örneklerin FXID yönünden genotiplendirilmesi, elde edilen PCR ürünlerinin %2'lik agaroz jel elektroforezi ile yapılmıştır.

BULGULAR

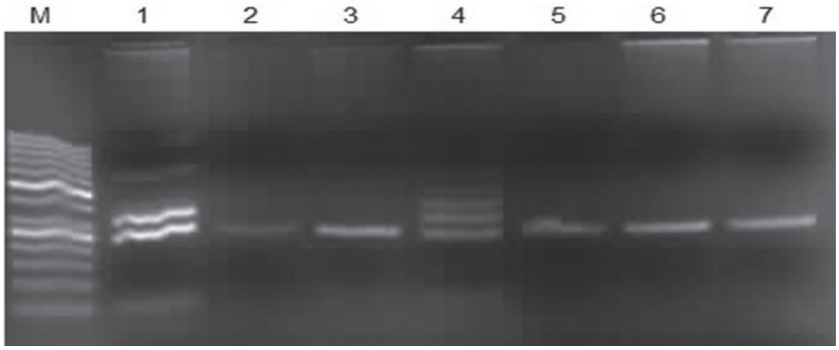
İncelenen boğalardan sadece birinin FXID taşıyıcısı olduğu belirlenmiştir. Çalışma sonunda homozigot hasta bireye rastlanılmamıştır. Yapılan PCR işlemi sonunda heterozigot taşıyıcı bireyde 244 ve 320 bç'lik iki bant, homozigot normal bireylerde ise 244 bç'lik tek bant görülmüştür *(Şekil 1)*.

Yapılan bu çalışma sonunda, Türkiye'de yetiştirilen damızlık boğalar arasında FIXD taşıyıcılarının prevalansı yaklaşık olarak %1.7 ve mutant FXID allelinin frekansının ise yaklaşık %0.85 olduğu belirlenmiştir.

TARTIŞMA ve SONUÇ

Gerek dişilerinin yüksek süt verimi gerekse erkek yavrularının besiye alınabilmeleri nedeniyle süt sığırı yetiştiriciliğinde Holştayn sığır ırkı tüm dünyada en çok tercih edilen sütçü sığır ırkıdır. Ancak damızlık değerlerine göre üstün özelliklere sahip boğaların suni tohumlamada yaygın kullanımı ırk içindeki genetik havuzun daralmasına ve ırk içi varyasyonun azalmasına neden olmaktadır [7,8]. Süt sığır yetiştiriciliğinde suni tohumlamanın yaygın kullanılması sonucu özellikle hastalık yönünden normal görünüşlü taşıyıcı boğalar vasıtasıyla kalıtsal hastalıklar kısa sürede çok sayıda hayvana yayılabilmekte ve populasyon içinde genetik bozukluklara neden olan genlerin frekansını arttırmaktadır [7,9,10].

Resesif kalıtım yolu izleyen kalıtsal hastalığın çiftlik hayvanlarında yayılması hem işletmelerde önemli ekonomik kayıp-



Şekil 1. FXID genotiplerinin %2 agaroz jeldeki görüntüsü. M; 50 bç'lik DNA merdiveni, 1: FXID heterozigot taşıyıcı kontrol, 2: homozigot normal kontrol; 3, 5-7: FXID yönünden homozigot normal boğalar, 4: FXID taşıyıcı birey

Fig 1. The illustration of FXID genotypes on 2% agarose gel. M: 50 bp DNA Ladder, Lane 1: 244 and 320 bp heterozygous carrier control, Lane 2: 244 bp homozygote normal control, Lane 3, 5-7: 244 bp normal bulls, Lane 4: 244 and 320 bp heterozygous bull

lara hem de kullanılan taşıyıcı boğalar yoluyla ıslah programlarının başarısız olmasına neden olabilmektedir [9]. Heterozigot bireyler normal görünüşlü oldukları için populasyon içinde fark edilmeden sürü içinde yaşamlarını sürdürüp sahip oldukları mutant alleli sonraki jenerasyonlara aktarabilirler. Taşıyıcı bir bireyin sahip olduğu mutant alleli sonraki jenerasyona geçirme olasılığı %50'dir. Bu nedenle taşıyıcı bireylerin belirlenerek damızlık sürülerden uzaklaştırılması FXID gibi otozomal resesif kalıtım şekli gösteren kalıtsal hastalıkların sürüden eradikasyonu için gereklidir.

Faktör XI yetmezliğinin 1969 yılında ABD'de bildirilmesinden [5] sonra Kanada [11], İngiltere [12], Çek Cumhuriyeti [13], Hindistan [14], Japonya [15], Polonya [2] ve Avustralya'da [8] yetiştirilen Holştayn'larda bu kalıtsal hastalık yönünden taşıyıcı bireylerin varlığı bildirilmiştir. Türkiye'de yetiştirilen Holştayn'larda, FXID allelinin varlığı ilk olarak Meydan ve ark.[3] tarafından bildirilmiştir. Ayrıca Japonya'da yetiştirilen yerli Japon Siyah Sığır ırkında faktör XI geninin 9. ekzonunda 15 baz çifti uzunluğundaki bir parçanın eklenmesi sonucu bu ırkta da FXID'in geliştiği bildirilmiştir [15,16].

Bu kalıtsal hastalığın moleküler tanısı ilk kez Marron ve ark.[6] tarafından yapılmıştır. FXID'e neden olan mutant allelin varlığı Holştayn ve Japon Siyah Sığırları dışında diğer sığır ırklarında bildirilmemiştir. Örnek olarak, Hindistan'da yetiştirilen 200 baş Karan Fries (Holştayn ve bir Hindistan yerli ırkı olan Tharparkar ırkının melezi) sığırları FXID yönünden incelenmiş ancak taşıyıcı bireylere rastlanılmamıştır [17]. Aynı şekilde Hindistanda yetiştirilen farklı Jersey ırkı, *B. indicus* melezleri, saf *Bos indicus*'lar ve mandaların (*Bubalus bubalis*) erkek örneklerinde FXID allelinin varlığının araştırıldığı bir çalışmada incelenen örneklerde FXID alelline rastlanılmamıştır [14]. Aynı çalışmada incelenen 330 baş Holştayn boğanın ikisinin FXID taşıyıcısı olduğu ve taşıyıcıların prevalansının ise yaklaşık %0.61 olduğu belirlenmiştir [14]. İran'da yetiştirilen yerli bir sığır ırkı olan Khuzestan ırkında FXID'e neden olan mutant allele rastlanılmamıştır [18]. Benzer şekilde Çek Cumhuriyeti'nde yetiştirilen Simmental ırkına ait boğaların hem 9. hem de 12. ekzonda görülen insersiyonlar yönünden incelenmelerinde bu ırkta FXID alleline rastlanılmamıştır [13]. Bu bulgular yerli sığır ırklarında FXID'ye neden olan allelelin bulunması olasılığının düşük olduğu izlenimini vermektedir. Türkiye yerli sığır ırklarında FXID yönünden bir çalışma yapılmamıştır. Ancak yerli ırklarının daha önce mutasyonun bulunduğu bildirilen faktör XI geninindeki hem ekzon 12, hem de ezkon 9 insesiyonları yönünden taranmaları sonucunda Türkiye yerli sığır ırklarının FXID durumları hakkında bilgi elde edilebilir.

Suni tohumlama gibi gelişmiş üreme teknolojilerinin sığır yetiştiriciliğinde yaygın kullanılması, üstün verimli tek bir boğanın tüm dünyada binlerce buzağının babası olmasına imkan vermektedir. Islah çalışmalarında kullanılan bu ileri üreme teknikleri, seleksiyon etkinliğini artırmış ve sığırlarda jenerasyon aralığını kısaltarak genetik iyileştirme ve seleksiyonda başarıyı artırmıştır. Ancak ıslah çalışmalarında az sayıda boğanın yaygın olarak kullanılması mutant allellerin kısa süre de populasyon içinde hızla yayılmasına neden olması bu yöntemin önemli bir dezavantajıdır [8]. Bu nedenle özellikle erkek damızlıkların ırka özgü kalıtsal hastalıklar yönünden taranmaları kalıtsal hastalıkların yayılmasının önlenmesinde önemlidir.

ABD'de 419 baş Holştayn sığırında FXID taşıyıcılarının prevalansının yaklaşık olarak %1.2 olduğu bildirilmiştir [6]. Japonya'nın batısında bulunan 12 farklı çiftlikte 2006-2007 yılları arasında yetiştirilen 500 baş Holştayn ineğin FXID yönünden incelendiği bir çalışmada FXID taşıyıcıların prevalansının %1, mutant allelin frekansının ise %0.5 olduğunu belirlenmiştir [4]. Yine bu çalışmada FXID taşıyıcısı olduğu bilinen sığırların pedigri kayıtlarında bulunan ve FXID taşıyıcısı olduğundan şüphelenilen dört boğa ile incelen 500 baş inek tohumlanmıştır. Deneme sırasında incelenen hayvanların buzağılama aralıkları, gebelik başına tohumlama sayıları da kayıt edilmiştir. Çalışma sonunda bu boğaların birinin FXID taşıyıcısı olduğu ve bu boğanın yavrularından altısının da taşıyıcı olduğu, taşıyıcı buzağılarında sadece birinin annesinin FXID taşıyıcısı olduğu belirlenmiştir. Diğer taraftan FXID mutant alleli yönünden taşıyıcı ineklerde bir gebelik için ortalama 5 tohumlama yapıldığı belirlenmiştir. ABD orijinli FXID taşıyıcısı boğanın aynı zamanda kompleks vertabral malformasyon (CVM) taşıyıcısı olduğu ve bu boğanın doğan üç dişi yavrusunun da CVM taşıyıcısı olduğu belirlenmiştir [4]. Çek Cumhuriyetinde yetiştirilen 229 baş Holştayn boğanın FXID'e neden olan 9. ve 12. ekzon insersiyonu yönünden incelenmelerinde incelenen örneklerde 9. ekzon insersiyonuna rastlanılmamış, ancak 12. ekzon insersiyonu sonucu meydana çıkan FXID prevalansının ise yaklaşık olarak %0.44 olduğu belirlenmiştir [13]. FXID ve döl verimi arasındaki ilişkinin araştırıldığı çalışmalarda repeat breeder olarak adlandırılan gebelik başına üç ve daha fazla tohumlanan dişilerde [19] FXID prevalansının gebelik başına en fazla iki tohumlama yapılan dişilerden 2.5 kat fazla olduğu belirlenmiştir [2]. FXID'e neden olan mutant alleli genotiperinde bulunduran hayvanların östrus sikluslarında luteolizisin yavaş gerçekleştiği bildirilmiştir. Bu durum aynı zamanda ovulasyona yakın zamanda oluşması gereken östradiol pikinin yavaşlığına eşlik eden küçük folikül gelişimiyle ilişkilendirilebilir. Ayrıca hasta hayvanlarda döl tutma probleminin prevalansının diğerlerinden %50 daha fazla olduğu belirlenmiştir [4]. Polonya'da yetiştirilen Holştaynlarda daha önce yapılan bir çalışmada üç taşıyıcı birey belirlenmiş ve bunlardan ikisinin döl tutma problemi olduğu birinin ise normal fertilite gösterdiği bildirilmiştir [2]. Bu da göstermektedir ki FXID yönünden döl tutma problemleri olan hayvanları incelemek taşıyıcı bulma şansını artırabilecektir.

Faktör XI yetmezliği kalıtsal hastalığının görüldüğü diğer bir sığır ırkı olan Japon Siyah sığırından hastalığın frekansının belirlenmesi için yapılan bir çalışmada; Japonya'da rastgele seçilerek incelenen 42 boğa ve 81 dişi incelenmiş ve incelenen boğaların üçünün (%7.1) ve dişilerin ise dördünün (%4.9) FXID yönünden homozigot hasta olduğu ancak hiç birinde aşırı kanama ile ilgili bir durumun görülmediği belirlenmiştir. Ayrıca Japon Siyah sığırına ait incelenen 42 boğanın 22'sinin

(%52.4) ve 81 dişinin 29'unun (%35.8) FXID taşıyıcısı oldukları belirlenmiştir [15]. Bu çalışma göstermektedir ki hastalık her zaman belirgin bir semptom göstermemektedir. Ancak döl tutma problemleri işletmelerin karlılığını azaltmaktadır. Akyüz ve ark.[20] yaptıkları bir çalışma sonunda, Türkiye'de yetiştirilen Holştayn'larda repeat breeder bireylerin normal fertilite gösterenlere göre işletmeye maliyetlerinin iki kat daha fazla olduğunu bildirmişlerdir. Ayrıca Japon Siyah sığırlarında bulunan FXID alleli ile döl verimi arasındaki ilişkinin incelendiği bir çalışmada [16], FXID alleli yönünden homozigot olan bireylerin klinik olarak kanama eğilimlerinin olmadığı, ancak hayatta olan hasta buzağılarda infeksiyöz hastalıklara yüksek hassasiyet ve bu hayvanlar içinde repeat breeder problemlerinin bulunduğu bildirilmiştir. Klinik görünümdeki bu farklılıkların sebebi Japon Siyah sığır ırkında ki FXI geninde meydana gelen mutasyonun Holştaynlarda FXI geninde meydana gelen mutasyondan farklı olmasından kaynaklanmış olabileceği bildirilmiştir [16].

Türkiye'de FXID'e neden olan mutant allelin varlığı ilk kez 2009 yılında 225 baş dişi Holştaynın kullanıldığı bir çalışma sonunda bildirilmiştir. Bu çalışmada incelenen örneklerin dört tanesinin FXID taşıyıcısı olduğu ve mutant allelin frekansının ise yaklaşık olarak %0.9 olduğu bildirilmiştir [3]. Daha sonra, Kayseri ve civarında yetiştirilen 150 baş dişi Holştayn'da bu kalıtsal hastalığın prevalansının araştırıldığı bir başka çalışmada, heterozigotların prevalansının yaklaşık olarak %0.7 olduğunu bildirilmiştir [21]. Antalya ve civarında yetiştirilen 504 baş dişi Holştaynın kullanıldığı bir çalışmada, incelenen hayvanlardan iki tanesinin taşıyıcı olduğu, mutant allelin frekansının yaklaşık olarak %0.2, taşıyıcı bireylerin prevalansının ise yaklaşık olarak %0.4 olduğu bildirilmiştir [22]. Bursa ilinde 2010 yılında yetiştirilen 170 baş Holştayn ineğin incelendiği bir başka çalışmada ise incelenen örneklerden ikisinin FXID taşıyıcısı olduğunu ve FXID'ye neden olan mutant-allelin frekansının %0.6 ve taşıyıcıların prevalansının ise %1.17 olduğunu bildirilmiştir [23]. Bu çalışmada ise incelenen 59 baş Holştayn boğada FXID prevalansının yaklaşık olarak %1.7 mutant allelin frekansının ise yaklaşık olarak %0.85 olduğu belirlenmiştir. Yapılan bu çalışma sonunda, Türkiye'de yetiştirilen Holştayn boğalarda FXID allel frekansı ve taşıyıcıların prevalansının dişilerdekinden yüksek olduğu belirlenmiştir. Bu yüksekliğin sebebinin dişilerde FXID allelinin işetmelerde döl tutma problemli bireylerin ayrılması ile FXID yönünden farkında olmadan bir seleksiyon yapılmış olmasından kaynaklanabileceği düşünülmüştür. Diğer tarafta FXID ile sperma kalitesi ve döl verimi arasında bir ilişkinin olup olmadığı hakkında bir veri yoktur. Bu konuda da çalışmalar yapılabilir.

Bu çalışma göstermiştir ki Türkiye'de yetiştirilen ve damızlık olarak kullanılan Holştayn boğalar arasında da FXID taşıyıcıları vardır. Bu nedenle suni tohumlama istasyonlarında kullanılan damızlık boğalar ve damızlık adaylarının bu kalıtsal bozukluk yönünden taranmaları gereklidir. Çünkü FXID ve diğer kalıtsal hastalıkların yayılmasında bir numaralı sorumlu taşıyıcılığı belirlenmemiş boğalardır. Türkiye'de repeat breederlı dişi Holştaynlarda FXID prevalansının araştırıldığı bir çalışmada, FXID taşıyıcılarının prevalansı repeat breederlı bireylerde %2.33 iken, normal fertilite gösteren bireylerde %0.85 olduğu belirlenmiştir [23]. Bu kalıtsal hastalık, sığır yetiştiriciliğinde bilinen diğer resesif kalıtım şekli gösteren kalıtsal hastalıklardan farklı olarak heterozigot durumunda da döl tutma problemi gibi yetiştiriciler için sorun oluşturabilmektedir. Dolayısıyla FXID'in hem heterozigot durumda hem de homozigot durumda neden olabileceği ekonomik kayıpların önüne geçilmesi için özellikle boğaların taraması gereklidir. Ayrıca bir kalıtsal hastalığı taşıyan boğaların başka kalıtsal hastalıklar yönünden de taşıyıcı olabilecekleri [4] ve bir boğanın bir yıl içerisinde on binlerce buzağının babası olabilecekleri bilgisi unutulmamalıdır.

Türkiye'de sadece, tüm Dünya'da süt sığırcılığında yaygın olarak yetiştirilen Holştayn ırkında en çok görülen FXID, BLAD, CVM ve DUMPS gibi kalıtsal hastalıklara neden olan mutan allellerin varlığı hakkında çalışmalar vardır [21,24-26]. Ancak bu kalıtsal hastalıklardan kaynaklanan ekonomik kayıplar ve Holştayn ırkı dışında Türkiye'de yetiştirilen diğer ırklar ve bu ırklarda görülmesi muhtemel kalıtsal hastalıklar hakkında çalışmalar yoktur.

Yapılan bu çalışmada Türkiye'deki Holştayn boğalarda mutant FXID allelin varlığının araştırılması ve taşıyıcı boğaların prevalansının belirlenmesi amaçlanmıştır. Çalışma sonunda incelenen bireyler arasında bir tane taşıyıcı bireye rastlanılmasının sebebinin örnek sayısının azlığından kaynaklanmış olabileceği düşünülmüştür. Buna rağmen boğalar arasında FXID taşıyıcılarının prevalans ve mutant allel frekansının dişilerden yüksek olduğu görülmüştür.

Daha sonraki çalışmalarda FXID gibi döl verimini düşürerek işletmelerin karlılığını azaltabilecek kalıtsal hastalıklar yönünden özelliklede erkek damızlıklar ve damızlık adaylarının taranması gereklidir. Bu sayede FXID prevalansının Türkiye Holştayn popülasyonunda azaltılması ile bu ve benzeri kalıtsal hastalıkların sebep olabileceği ekonomik kayıplar önlenebilir. FXID ve benzeri kalıtsal hastalıklar yönünden taşıyıcı bireylerin belirlenmesi Türkiye'deki Holştayn yetiştiriciliğinde, üretimin genetik olarak iyileştirilmesi için çok önemli bir adımdır. Ayrıca Türkiye'ye ithal edilen spermalarında bu ve benzeri kalıtsal hastalıklar yönünden incelenmeleri gereklidir.

KAYNAKLAR

1. Mukhopadhyaya PN, Jha M, Muraleedharan P, Gupta RR, Rathod RN, Mehta HH, Khoda VK: Simulation of normal, carrier and affected controls for large-scale genotyping of cattle for factor XI deficiency. *Genet Mol Res,* 5 (2): 323-332, 2006.

2. Gurgul A, Rubis D, Slota E: Identification of carriers of the mutation causing coagulation factor XI deficiency in Polish Holstein-Friesian cattle. *J Appl Genet*, 50 (2): 149-152, 2009.

3. Meydan H, Yildiz MA, Özdil F, Gedik Y, Özbeyaz C: Identification of factor XI deficiency in Holstein cattle in Turkey. *Acta Vet Scand,* 51, 5, 2009.

4. Ghanem ME, Nishibori M: Genetic description of factor XI deficiency

in Holstein semen in Western Japan. *Reprod Dom Anim*, 44, 792-796, 2009.

5. Kociba GD, Ratnoff OD, Loeb WF, Wall RL, Heider LE: Bovine thromboplastin antecedent (factor XI) deficiency. *J Lab Clin Med,* 74, 37-41, 1969.

6. Marron BM, Robinson JL, Gentry PA, Beever JE: Identification of a mutation associated with factor XI deficiency in Holstein cattle. *Anim Genet*, 35, 454-456, 2004.

7. Mirck MH, Von Bannisseht-Wijsmuller T, Timmermans-Besselink WJ, Van Luijk JH, Buntijer JH, Lenstra JA: Optimization of the PCR test for the mutation causing bovine leukocyte adhesion deficiency. *Cell Mol Biol*, 41 (5): 695-698, 1995.

8. Windsor PA, Agerholm JS: Inherited diseases of Australian Holstein-Friesian cattle. *Aust Vet J,* 87 (5): 193-199, 2009.

9. Ghanem ME, Isobe N, Kubota H, Suzuki T, Kasuga A, Nishibori M: Ovarian cyclicity and reproductive performance of Holstein cows carrying the mutation of complex vertebral malformation in Japan. *Reprod Dom Anim,* 43, 346-350, 2008.

10. Janosa A, Baranyai B, Dohy J: Comparison of milk production of the progeny of BLAD-carrier and healthy Holstein bulls in Hungary. *Acta Vet Hung*, 47 (3): 283-289, 1999.

11. Gentry PA, Crane S, Lotz F: Factor XI (plasma thromboplastin antecedent) deficiency in cattle. *Can Vet J,* 16, 160-163, 1975.

12. Brush PJ, Anderson PH, Gunning RF: Identification of factor XI deficiency in Holstein-Friesian cattle in Britain. *Vet Rec,* 121, 14-17, 1987.

13. Čítek J, Řehout V, Hanusová L, Vrabcová P: Sporadic incidence of factor XI deficiency in Holstein cattle. *J Sci Food Agric,* 88, 2069-2072, 2008.

14. Patel RK, Soni KJ, Chauhan JB, Singh KM, Rao KRSS: Factor XI deficiency in Indian *Bos taurus*, *Bos indicus*, *Bos taurus* x *Bos indicus* crossbreds and *Bubalus bubalis*. *Genet Mol Biol,* 30 (3): 580-583, 2007.

15. Ohba Y, Takasu M, Nishii N, Takeda E, Maeda S, Kunieda T, Kitagawa H: Pedigree analysis of factor XI deficiency in Japanese Black cattle. *J Vet Med Sci*, 70 (3): 297-299, 2008.

16. Watanabe D, Hirano T, Sugimoto Y, Ogata Y, Abe S, Ando T, Ohtsuka H, Kunieda T, Kawamura S: Carrier rate of factor XI deficiency in stunted Japanese Black cattle. *J Vet Med Sci,* 68 (12): 1251-1255, 2006.

17. Azad MS, Gupta ID, Archana V, Bohra V, Kumar SR, Kour K: Factor XI gene (plasma thromboplastin antecedent) deficiency in Karan Fries cattle. *Current Trends in Biotechnology and Pharmacy,* 5 (1): 1060-1063, 2011.

18. Eydivandi C, Amirinia C, EmamJomeh-Kashan N, Chamani M, Fayazi J: Study of factor XI deficiency in Khuzestan cattle population of Iran. *Afr J Biotechnol*, 10 (4): 718-721, 2011.

19. Dochi O, Takahashi K, Hirai T, Hayakawa H, Tanisawa M, Yamamoto Y, Koyama H: The use of embryo transfer to produce pregnancies in repeat-breeding dairy cattle. *Theriogenology,* 69, 124-128, 2008.

20. Akyüz B, Sarıözkan S, Bayram D: Factor XI mutation in normally fertile and repeat breeding Holstein cows in the Middle Anatolian region of Turkey: A financial approach. *Anim Prod Sci,* 52, 1042-1045, 2012.

21. Yaşar G, Akyüz B: Kayseri civarında yetiştirilen Holştayn ineklerde kalıtsal faktör XI yetmezliği geninin allel frekansının belirlenmesi. *Erciyes Üniv Vet Fak Derg*, 9 (1): 7-12, 2012.

22. Karslı T, Şahin E, Karslı BA, Alkan S, Balcıoğlu MS: Identification of alleles for factor XI (FXID) and uridine monophosphate synthase (DUMPS) deficiencies in Holstein cows reared in Antalya. *Kafkas Univ Vet Fak Derg,* 17(3): 503-505, 2011.

23. Oner Y, Keskin A, Elmaci C: Identification of BLAD, DUMPS, citrullinaemia and factor XI deficiency in Holstein cattle in Turkey. *Asian J Anim Vet Adv,* 5 (1): 60-65, 2010.

24. Akyüz B, Ertuğrul O, Ağaoğlu ÖK: Detection of bovine leukocyte adhesion deficiency (BLAD) allele in Holstein cows reared in Kayseri vicinity. *Kafkas Univ Vet Fak Derg*, 16 (3): 519-521, 2010.

25. Akyüz B, Kul BÇ: Türkiye'de Holştayn ırkı ineklerde üridin monofosfat senteaz eksikliğinin (DUMPS) belirlenmesi. *Ankara Üniv Vet Fak Derg*, 56, 231-232, 2009.

26. Kulaklı GN, Akyüz B: Kayseri bölgesinde yetiştirilen Holştayn sığırlarında kompleks vertebral malformasyon hastalığı geninin allel frekansının belirlenmesi. *Erciyes Üniv Vet Fak Derg*, 8 (2): 69-74, 2011.

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RESEARCH ARTICLE

Etanersept - Endotoksemi Tedavisinde Kullanılabilir mi? [1] [2]

Ayse ER * Burak DIK * Gul CETIN ** Feray ALTAN ***
Kamil UNEY * Muammer ELMAS * Enver YAZAR *

[1] *Bu araştırma SÜBAPK (11401096) tarafından desteklenmiştir*
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* Selçuk Üniversitesi, Veteriner Fakültesi, Farmakoloji ve Toksikoloji Anabilim Dalı, TR-42075 Konya - TÜRKİYE
** Mehmet Akif Ersoy Üniversitesi, Veteriner Fakültesi, Farmakoloji ve Toksikoloji Anabilim Dalı, TR-15030 Burdur - TÜRKİYE
*** Dicle Üniversitesi, Veteriner Fakültesi, Farmakoloji ve Toksikoloji Anabilim Dalı, TR-21280 Diyarbakır - TÜRKİYE

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Summary

Araştırmanın amacı endotoksemide etanersept uygulamasının kan sitokinler, fibrinojen, antitrombin, 13,14-dihidro-15-keto prostaglandin $F_{2\alpha}$ ve biyokimyasal parametrelere etkisini araştırmaktır. Erişkin 126 adet Sprague Dawley ırkı erkek rat 3 gruba ayrılarak; 1. Gruba lipopolisakkarit (4 mg, IP), 2. Gruba etanersept (8 mg/kg, IP) ve 3. Gruba lipopolisakkarit + etanersept uygulamaları yapıldı. Uygulamalardan sonra 0., 1., 2., 4., 8., 12. ve 24. saatlerde kan örnekleri alındı. Serum tümör nekrozis faktör-α, interlöykin-1β, interlöykin-10 ve plazma 13,14-dihidro-15-keto-prostaglandin $F_{2\alpha}$ düzeyleri ELISA okuyucusunda; sitratlı plazma antitrombin ve fibrinojen düzeyleri koagulometrede; serum biyokimyasal parametreleri otoanalizörde belirlendi. Etanerseptin fibrinojen düzeyinde düzensiz değişimlere ve 13,14-dihidro-15-keto-prostaglandin $F_{2\alpha}$, alkalen fosfataz ile alanin aminotransferaz düzeyinde yükselmelere neden olduğu belirlendi. Lipopolisakkarit uygulaması sitokinler, 13,14-dihidro-15-keto-prostaglandin $F_{2\alpha}$, fibrinojen, organ hasar belirteçleri ve trigliserit düzeylerinde yükselmelere neden olurken, antitrombin seviyesinde düzensiz değişimlere neden oldu. Lipopolisakkarit + etanersept uygulanan grupta sitokinler, 13,14-dihidro-15-keto-prostaglandin $F_{2\alpha}$ ve fibrinojen düzeyinde yükselmeler, antirombin düzeyinde düzensiz değişimler gözlendi. Lipopolisakkarit uygulaması ile yükselen kreatin kinaz-MB düzeyinin etanersept tarafından tamamen, tümör nekrozis faktör-α yükselmesinin kısmen engellendiği ancak kanda kalış süresini uzattığı ve interlöykin-10 düzeyini daha fazla yükselttiği belirlendi. Sonuç olarak endotoksemide etanerseptin kalp üzerindeki koruyucu etkisi ve interlöykin-10 düzeyini yükseltmesi nedeni ile tek doz uygulamasının veteriner sahada faydalı olabileceği belirlendi.

Keywords: *Endotoksemi, Etanersept, Sitokinler, Pıhtılaşma, Prostaglandin*

Etanercept - Can It be Used in the Treatment of Endotoxemia?

Özet

Aim of this study was to investigate that effect of etanercept administration on the concentrations of cytokines, fibrinogen, antithrombin, 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ and biochemical values in the endotoxemia. A totally male 126 Sprague Dawley rats were divided 3 equal groups; lipopolysaccharide (4 mg, IP), etanercept (8 mg/kg, IP) and lipopolysaccharide + etanercept were administered to Group 1, 2, and 3, respectively. Blood samples were collected at 0, 1, 2, 4, 8, 12 and 24th hours after treatments. Serum tumor necrosis factor-α, interleukin-1β, interleukin-10 and plasma 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ levels were measured with ELISA reader, while antithrombin and fibrinogen levels were measured with coagulometer. Serum biochemical parameters were determined with auto-analyzer. Etanercept had irregular effect on the fibrinogen levels and it increased PGM, alkaline phosphatase and alanine aminotransferase activities. Lipopolysaccharide increased cytokines, PGM, fibrinogen, organ damage markers and triglyceride levels, and it had irregular effect on the antithrombin levels. Increased cytokines, PGM and fibrinogen levels were determined in lipopolysaccharide + etanercept group, but irregular changes were observed in antithrombin levels. Etanercept exactly depressed the increased creatine kinase-MB level and relatively depressed the increased tumor necrosis factor-α while it extended the presence of tumor necrosis factor-α, and it increased the elevated interleukin-10 levels by lipopolysaccharide. In conclusion, when protective effect of etanercept on the heart and increasing effect on the interleukin-10 levels in the endotoxemia is considered, single dose etanercept may be beneficial in the veterinary medicine.

Anahtar sözcükler: *Endotoxemia, Etanercept, Cytokines, Coagulation, Prostaglandin*

İletişim (Correspondence)
+90 332 2232733
aer@selcuk.edu.tr

GİRİŞ

Mikroorganizmaların steril canlı dokusunda bulunması sonrasında canlının gösterdiği reaksiyona enfeksiyon adı verilir. Canlının sistemik dolaşımında bakteri bulunmasına bakteriyemi, enfeksiyonun sistemik duruma gelmesine ise sepsis adı verilir. Gram (-) bakteri endotoksinin dolaşımda bulunmasına endotoksemi, gerekli sıvı ve sempatomimetik tedavisi yapılmasına rağmen düzeltilemeyen hipotansiyonun görüldüğü sistemik yangısal cevaba ise septik şok adı verilir. Bakteri, virüs, mantar ile parazitler septik şoka neden olabilir ve ölüm oranı %20-80 civarındadır [1-3].

Gram (-) bakteri hücre duvarının bir parçası olan lipopolisakkarit (LPS), endotoksin olarak da adlandırılır. LPS'nin septik şokların oluşumunda önemli rolü bulunmaktadır [4,5]. Deneysel araştırmalarda uygulanan doz ve uygulama şekline göre lokal yangıları modellemeden, septik şoku modellemeye kadar değişen aralıkta kullanılmaktadır [3,6,7]. LPS, immunolojide tanımlanan en iyi antijendir. Dolaşıma yeterince LPS geçtiğinde, savunma hücrelerince yangısal cevap tetiklenir. Özellikle monosit ve makrofajlarca ilk cevap olarak tümör nekrozis faktör alfa (TNF-α) ve interlöykin-1beta (IL-1β) gibi proinflamatuar sitokinler devamında ise diğer interlöykinler salınır [2,8]. Salgılanan TNF-α'nın konakçıda hemodinamik değişiklikler, doku hasarı, hipotansiyon ve organ yetmezliklerine neden olduğu bildirilmiştir. TNF-α gibi bir diğer proinflamatuar sitokin olan IL-1β düzeyi sepsisli hastalarda yüksek tespit edilmiştir. Salgılanan IL-1β'da, canlıda ateş, hipotansiyon ve anoreksi gibi sepsisin bulgularına neden olmaktadır. Septik şoklarda TNF-α ile IL-1β birlikte sinerjik hareket ederek daha ağır klinik belirtiler görülmesine neden olmaktadır. Sitokin salınımı bir enfeksiyonda normal immun cevap olarak gelişir. Ancak çok fazla sitokin üretimi ise dolaşım bozukluğu ve ölüme neden olabilmektedir. Deneysel araştırmalarda sistemik TNF-α uygulamalarının ölüme neden olabileceği bildirilmiştir [3,8,9]. Septik şoklarda proinflamatuar sitokin (TNF-α, IL-1β) salınımından daha sonra antiinflamatuar sitokin olan IL-10 salgılanır. Deneysel araştırmalarda endotoksemik canlılara IL-10 uygulamasının TNF-α düzeyini düşürdüğü ve hayatta kalmayı artırdığı belirlenmiştir [9].

Sağlıklı canlıda kanda pıhtılaşma yapıcılar ile pıhtılaşmayı engelleyiciler denge halinde olduğundan pıhtılaşma şekillenmez. Ancak endotelde oluşan hasar veya kandaki değişiklikler, karaciğerden üretilen protrombini trombine dönüştürür. Trombin de karaciğerde üretilen fibrinojeni fibrine dönüştürerek pıhtının oluşmasını sağlar. Karaciğerde sentezlenerek dolaşıma salınan antitrombin III (AT) ise trombine bağlanarak antikoagulant etkinlik gösterir. Septik şoklarda koagulasyon uyarıldığı için genellikle kandaki miktarı azalır [9,10]. Salgılanan proinflamatuar sitokinler, konakçıda hücre düzeyinde birçok vasküler ve immunolojik değişikliklere neden olmaktadır. Etkilenen endotel sistem bir yandan pıhtılaşmayı başlatırken diğer yandan proinflamatuar sitokin sentezi yapar. Sitokinler ise pıhtılaşmayı uyarır. Pıhtılaşma mekanizmasının başlaması, mikrotrombuslar, yaygın damariçi pıhtılaşma (YDP), hipoperfüzyon, hipoksi ve ölümle sonuçlanan çoklu organ yetmezliklerinin (ÇOY) gelişmesini sağlar. Septik şokun son döneminde pıhtılaşma faktörlerinin yetersizliği nedeni ile kanamalar oluşur [1-3].

Yangılı durumlar ve enfeksiyonlarda araşidonik asitten siklooksijenaz (COX) aracılığı ile prostaglandinler sentezlenir. COX'ın, COX1 ve COX2 olmak üzere iki tipi bulunur. COX1 canlıda sürekli aktiftir ve biyolojik olarak varlığına ihtiyaç duyulur. COX2 ise sitokinler ve yangı etkenlerince uyarılarak prostaglandin sentezini sağlarlar. Üretilen prostaglandinler ise ağrı, ateş, vazodilatasyon ve ödem gibi klinik belirtilere neden olurlar [11]. Enfeksiyonlarda salgılanan TNF-α'nın prostaglandin sentezini uyarabildiği, LPS uygulaması sonrasında kan prostaglandin $F_{2\alpha}$ ana metaboliti kabul edilen 13,14-dihidro-15-keto-prostaglandin $F_{2\alpha}$ (PGM) yükseldiği ve endotoksemilerde oluşan serbest radikallerin PGM sentezine aracılık ettiği bildirilmiştir [6,9,12-14].

Septik şoklarda ölüm, genellikle ÇOY'den kaynaklanmaktadır. ÇOY ise oluşan generalize yangı ve YDP'dan kaynaklanmaktadır [2]. Serum kreatin kinaz-MB (Ck-MB) düzeyi kalp hasarı belirteci, alkalen fosfotaz (ALP), alanin aminotransferaz (ALT), aspartat aminotransferaz (AST), gamma glutamiltransferaz (GGT) düzeyi karaciğer hasarı belirteci olarak kabul edilirken, kreatinin ile kan üre nitrojen (BUN) düzeyi böbrek hasarı belirteci olarak kabul edilir [15]. Endotoksemilerde serum organ hasar belirteçlerinin artabileceği ve trigliserit, düşük yoğunluklu lipoprotein (LDL) ile yüksek yoğunluklu lipoprotein (HDL) düzeylerinde değişimler olabileceği ifade edilmiştir [14,16].

Septik şokun tedavisinde birçok farklı tedavi protokolleri uygulanmasına rağmen hayatta kalma oranı kabul edilemeyecek düzeyde yüksektir [9]. Etanersept (ETA) bir rekombinant füzyon proteinidir. Önemli proinflamatuar sitokinlerden olan TNF-α ve TNFβ'nın her ikisinin de etkisini engelleyerek anti-TNF etkinlik gösterir. ETA beşeri hekimlikte, ankilozan spondilitis, Crohn hastalığı, romatoid artritis, juvenil ve psoriatik artritis gibi otoimmun hastalıkların tedavisinde kullanılır [8,17].

Deneysel endotoksemilerde tedavi amaçlı kullanılan TNF antagonistlerinin hayatta kalmayı artırabildiği bildirilmiştir [9,18]. Mevcut araştırmada endotoksemilerde oluşan aşırı proinflamatuar sitokin sentezi [19] ve TNF'nın ölümcül etkilere neden [3,18] olduğu dikkate alındığında, glukokortikoid kullanımının tedavide önerildiği gibi bir diğer immun depresant olan ETA [8] uygulamasının da tedavide etkili olabileceği hipoteze edildi.

Araştırmanın amacı, endotoksemide ETA uygulamasının, sitokinler, fibrinojen, AT, PGM, organ hasar belirteçleri ve bazı biyokimyasal parametrelere etkisini araştırmaktır.

MATERYAL ve METOT

Araştırmada 126 adet erişkin Sprague Dawley ırkı erkek rat (170-220 g, Kobay Deney Hayvanları AŞ, Ankara, Türkiye) kullanıldı. Araştırma prosedürü Selçuk Üniversitesi Veteriner

Fakültesi Etik Kurulunca onaylandı (No:2010/054). Ratlar 3 eşit gruba ayrılarak; 1. Gruba LPS (4 mg, periton içi, *Escherichia coli* 0111:B4, Sigma-Aldrich Chemical, Almanya) [14], 2. Gruba ETA (8 mg/kg, periton içi, Enbrel® flk., Wheth İlaçları A.Ş., İstanbul) [20] ve 3.Gruba LPS (4 mg, periton içi) + ETA (8 mg/kg, periton içi) uygulamaları yapıldı. Uygulamalardan önce 0. saat ve sonrasında 1., 2., 4., 8., 12. ve 24. saatlerde ratlardan tiopental sodyum (70 mg/kg, periton içi, Pental® Sodyum 1 g Enj. Sol., İ. E. Ulagay İlaç Sanayi Türk A. Ş., Topkapı, İstanbul) anestezisi altında kalplerinden kan alındı. Serum TNF-α (eBioscience Rat TNF-α kit, San Diego, CA, USA), IL-1β (eBioscience Rat IL-1β kit), IL-10 (eBioscience Rat IL-10kit) ve plazma PGM (13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ EIA kit, Cayman Chemical, Michigan) düzeyleri ticari kit prospektüslerine uygun olarak ELISA/spektrofotometre okuyucusunda (MWGt Lambda Scan 200, USA) belirlendi. Sitratlı plazma antitrombin III ve fibrinojen düzeyleri koagulometre (Siemens Sysmex CA 1500 Model, Japonya) ile belirlendi. Serum Ck-MB, ALP, AST, ALT, GGT, BUN, kreatinin, albümin, trigliserit, yüksek dansiteli lipoprotein (HDL) ve düşük dansiteli lipoprotein (LDL) düzeyleri otoanalizörde (Tokyo Boeki Prestige 24i, Japonya) belirlendi.

Örneklemenin her zamanlamasında 5 adet rattan elde edilen veriler ANOVA ve Tukey testi ile değerlendirildi (SPSS 10.0). P<0.05 değeri istatistiki açıdan önem sınırı olarak kabul edildi.

BULGULAR

Serum sitokin, plasma koagulasyon ve PGM parametreleri *Tablo 1*'de, organ hasar belirteçleri ile diğer biyokimyasal parametreler *Tablo 2*'de sunuldu. LPS grubunda toplam 3 rat ve LPS + ETA grubunda 2 rat öldü.

ETA uygulamasının sitokin ve AT düzeylerine etkisinin olmadığı, fibrinojen düzeyinde düzensiz değişimlere neden olduğu ve uygulama sonrası 1. saatte PGM düzeyinde yükselmeye neden olduğu belirlendi. LPS uygulamasının sitokin, PGM ve fibrinojen düzeylerinde yükselmelere neden olduğu ve AT seviyesinde düzensiz değişimlere neden olduğu tespit edildi. LPS + ETA uygulamasının serum sitokin, PGM ve fibrinojen düzeyinde yükselmelere, AT düzeyinde düzensiz değişimlere neden olduğu tespit edildi. ETA uygulamasının, LPS'nin neden olduğu TNF-α yükselmesini kısmen engellediği ancak TNF-α'nın kanda kalış süresini uzattığı ve IL-10 düzeyini daha fazla yükselttiği belirlendi *(Tablo 1)*.

ETA uygulamasının ALP ve ALT düzeyini yükselttiği belirlendi. LPS uygulamasının kalp, karaciğer ile böbrek hasar belirteçleri ve trigliserit düzeyinde yükselmelere neden olduğu, Ck-MB düzeyinde oluşan yükselmenin ETA tarafından engellenirken, yükselen diğer biyokimyasal parametreler üzerine ETA'nın olumlu etkisinin bulunmadığı gözlendi *(Tablo 2)*. Albümin, kreatinin, HDL ve LDL düzeyinde belirlenen

Table 1. *Effect of etanercept on the cytokines, coagulation values and 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ levels*
Tablo 1. *Etanerseptin sitokinler, koagulasyon parametreleri ve 13,14-dihidro-15-keto-prostaglandin $F_{2\alpha}$ düzeylerine etkisi*

Parametreler	Gruplar	Örnekleme Zamanları						
		0. saat	1. saat	2. saat	4. saat	8. saat	12. saat	24. saat
TNFα pg/mL	ETA	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	LPS	BLD	5086±416^{a}	4217±343^{a}	351±55.3^{b}	BLD	BLD	BLD
	ETA+LPS	BLD	1230±368^{ab}	951±122^{ab}	1599±351^{a}	1059±263^{ab}	461±37.4^{b}	BLD
IL-1β pg/mL	ETA	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	LPS	BLD	BLD	812±177	1504±142	1347±310	644±433	BLD
	ETA+LPS	BLD	BLD	497±166^{ab}	1317±257^{a}	1258±334^{a}	789±198^{ab}	251±102^{b}
IL-10 pg/mL	ETA	BLD	BLD	BLD	BLD	BLD	BLD	BLD
	LPS	BLD	146±58.2	552±537	544±186	1007±498	865±280	376±141
	ETA+LPS	BLD	BLD	1757±376^{ab}	126±126^{c}	608±411^{bc}	3344±304^{a}	1263±550^{bc}
AT %	ETA	141±10.3	156±0.74	146±12.2	431±282	151±8.32	169±2.71	156±8.29
	LPS	152±10.1^{ab}	126±26.7^{ab}	160±4.34^{a}	141±7.66^{ab}	96.5±15.8^{b}	122±7.27^{ab}	169±2.71^{a}
	ETA+LPS	157±7.35^{bc}	160±3.06^{b}	167±1.35^{b}	110±13.0^{d}	286±13.3^{a}	115±11.6^{cd}	147±12.4^{bcd}
Fibrinogen mg/dL	ETA	108±9.45^{b}	198±53.0^{ab}	576±235^{a}	323±9.72^{ab}	123±76.3^{b}	358±40.1^{ab}	342±17.4^{ab}
	LPS	112±15.9^{de}	73.8±22.7^{e}	465±116^{b}	920±26.3^{a}	206±52.0^{cde}	322±18.6^{bcd}	358±40.1^{bc}
	ETA+LPS	97.2±13.8^{c}	98.7±25.9^{c}	908±36.5^{a}	457±127^{b}	94.4±19.9^{c}	65.1±65.1^{c}	293±10.5^{bc}
PGM pg/mL	ETA	62.1±23.1^{b}	773±157^{a}	282±55.9^{b}	109±24.5^{b}	37.6±15.5^{b}	86.4±22.6^{b}	102±14.1^{b}
	LPS	66.3±16.2^{b}	235±50.9^{b}	380±178^{b}	270±114^{b}	1956±685^{a}	656±269^{ab}	116±46.0^{b}
	ETA+LPS	60.1±18.7^{b}	184±70.7^{b}	389±161^{ab}	283±87.1^{ab}	860±383^{ab}	1571±559^{a}	824±396^{ab}

$^{a\text{-}e}$ *Aynı satırdaki farklı harfler istatistiki açıdan önem arz eder (P<0.05),* ***BLD;*** *belirlenemedi*

Table 2. *Effect of etanercept on the serum organ damage markers and some biochemical values*
Tablo 2. *Etanerseptin serum organ hasar belirteçleri ve bazı biyokimyasal parametrelere etkisi*

Parametreler	Gruplar	Örnekleme Zamanları						
		0. saat	1. saat	2. saat	4. saat	8. saat	12. saat	24. saat
Ck-MB (U/L)	ETA	171±13.9^{ab}	252±62.9^{ab}	294±37.7^{a}	203±21.4^{ab}	209±30.3^{ab}	131±13.4^{b}	231±30.6^{ab}
	LPS	643±190^{ab}	335±80.0^{b}	491±153^{b}	580±86.9^{ab}	1246±230^{a}	643±97.1^{ab}	390±150^{b}
	ETA+LPS	182±17.8	274±50.3	580±±138	512±69.4	469±66.4	542±60.2	577±168
ALP (U/L)	ETA	143±8.62^{b}	2455±110^{a}	281±50.8^{b}	208±51.8^{b}	165±20.8^{b}	206±39.4^{b}	114±18.3^{b}
	LPS	139±27.5^{b}	379±70.3^{ab}	668±183^{a}	350±81.1^{ab}	177±34.8^{b}	186±19.1^{b}	221±27.6^{b}
	ETA+LPS	112±18.1^{b}	399±77.6^{b}	1565±346^{a}	613±429^{ab}	204±32.2^{b}	328±61.9^{b}	307±63.7^{b}
ALT (U/L)	ETA	92.0±5.44^{ab}	120±13.4^{a}	92.6±15.9^{ab}	78.0±10.1^{abc}	48.4±6.45^{bc}	40.8±6.25^{c}	45.8±6.66^{c}
	LPS	128±20.2^{b}	106±21.1^{b}	94.4±6.61^{b}	425±70.9^{b}	953±246^{a}	321±48.2^{b}	121±47.2^{b}
	ETA+LPS	71.8±14.7	104±6.07	104±21.5	321±90.3	251±109	314±58.2	176±51.5
AST (U/L)	ETA	113±9.69^{ab}	159±15.2^{a}	165±19.1^{a}	136±11.6^{a}	81.6±1.43^{b}	75.0±7.12^{b}	79.6±11.4^{b}
	LPS	186±32.8^{c}	146±12.8^{c}	175±12.9^{c}	645±124^{ab}	888±139^{a}	347±76.6^{bc}	224±112^{c}
	ETA+LPS	94.8±11.4^{b}	204±32.8^{ab}	171±20.4^{ab}	403±72.1^{ab}	300±102^{ab}	488±68.3^{a}	427±116^{a}
GGT (U/L)	ETA	0.20±0.20	0.00±0.00	0.80±0.48	0.00±0.00	0.40±0.40	0.20±0.20	0.00±0.00
	LPS	0.20±0.20^{b}	0.20±0.20^{b}	1.00±0.31^{ab}	1.40±0.40^{ab}	3.80±1.01^{ab}	13.4±6.42^{a}	7.40±4.23^{ab}
	ETA+LPS	0.20±0.20^{b}	0.40±0.24^{b}	0.60±0.24^{b}	0.80±0.48^{b}	3.60±0.74^{ab}	12.0±3.68^{a}	11.8±4.06^{a}
Albümin (g/dL)	ETA	4.16±0.08	4.08±0.09	4.08±0.34	3.96±0.08	3.46±0.24	3.92±0.04	3.44±0.16
	LPS	4.66±0.19^{a}	3.48±0.08^{b}	3.84±0.08^{b}	3.34±0.06^{b}	3.34±0.10^{b}	3.44±0.18^{b}	3.54±0.20^{b}
	ETA+LPS	4.32±0.12^{a}	3.60±0.12^{ab}	3.96±0.16^{ab}	3.46±0.15^{b}	3.18±0.28^{b}	3.60±0.16^{ab}	3.16±0.19^{b}
Kreatinin (mg/dL)	ETA	0.39±0.01^{ab}	0.40±0.01^{ab}	0.39±0.02^{ab}	0.43±0.04^{a}	0.44±0.03^{a}	0.32±0.03^{ab}	0.28±0.01^{b}
	LPS	0.59±0.08	0.52±0.03	0.40±0.02	0.45±0.04	0.72±0.11	0.45±0.04	0.50±0.16
	ETA+LPS	0.34±0.03^{b}	0.40±0.02^{b}	0.39±0.04^{b}	0.37±0.02^{b}	0.47±0.08^{ab}	0.46±0.05^{b}	0.88±0.20^{a}
BUN (mg/dL)	ETA	53.8±1.88^{bc}	69.4±2.97^{a}	65.0±3.28^{ab}	57.8±4.21^{abc}	49.8±2.22^{c}	58.8±2.13^{abc}	49.4±3.41^{c}
	LPS	59.2±3.58^{d}	72.6±3.29^{d}	66.2±6.52^{d}	97.2±6.41^{cd}	140±17.2^{bc}	171±12.2^{b}	264±26.6^{a}
	ETA+LPS	55.4±3.29^{c}	68.6±3.66^{c}	83.4±5.50^{bc}	94.0±4.06^{bc}	109±11.4^{abc}	164±8.53^{ab}	195±52.8^{a}
Trigliserit (mg/dL)	ETA	39.0±5.54^{ab}	39.8±9.10^{ab}	19.8±2.72^{b}	32.0±5.81^{b}	41.0±7.50^{ab}	68.0±2.56^{a}	52.0±13.7^{ab}
	LPS	86.2±16.4^{c}	42.8±7.29^{c}	101±11.7^{c}	120±15.5^{bc}	141±45.0^{bc}	403±70.6^{a}	295±66.6^{ab}
	ETA+LPS	30.2±7.13^{b}	38.6±7.60^{b}	64.6±6.48^{b}	121±26.5^{ab}	172±33.8^{ab}	278±84.3^{a}	142±41.8^{ab}
HDL (mg/dL)	ETA	49.8±4.75^{a}	47.0±2.68^{a}	45.2±2.03^{ab}	37.2±4.37^{ab}	41.4±4.09^{ab}	37.8±3.38^{ab}	29.6±4.76^{b}
	LPS	49.2±2.92^{a}	39.4±2.58^{abc}	40.8±2.37^{ab}	30.4±2.13^{bc}	36.6±6.85^{abc}	25.4±2.48^{bc}	23.2±3.81^{c}
	ETA+LPS	47.4±4.87^{a}	41.8±2.33^{ab}	42.2±3.27^{ab}	32.0±4.00^{abc}	28.8±5.08^{bc}	32.0±3.87^{abc}	16.2±3.13^{c}
LDL (mg/dL)	ETA	8.00±1.92^{a}	6.40±0.50^{ab}	8.00±0.63^{a}	6.60±0.40^{ab}	5.00±0.44^{ab}	4.40±0.50^{ab}	3.20±0.37^{b}
	LPS	8.00±0.70	6.60±0.74	11.0±3.03	6.40±0.81	11.0±2.30	11.8±1.39	15.6±3.62
	ETA+LPS	5.20±0.37^{b}	6.20±0.96^{ab}	8.00±1.14^{ab}	6.60±1.07^{ab}	7.60±1.02^{ab}	10.8±1.24^{a}	10.2±1.85^{ab}

$^{a-d}$*: Aynı satırdaki farklı harfler istatistiki açıdan önem arz eder (P<0.05)*

istatistiki değişimlerin ise sağlıklı ratlar için bildirilen referans değerler arasında olduğu tespit edildi [16,21-23].

TARTIŞMA ve SONUÇ

Deneysel modellemelerde, sistemik LPS uygulaması sonrasında, septik şokta gözlenen klinik ve laboratuar bulgularının tamamı gözlenebilmektedir. Buzağılara LPS uygulaması sonrasında klinik olarak ateş yükselmesi, solunum sayısında artış, taşikardi, diyare, depresyon ve iştahsızlık gibi belirtiler gözlendiği bildirilmiştir [7]. Ayrıca doza bağlı olarak %70'in üzerinde ölümler gözlenebilmektedir [24]. Laboratuar bulguları olarak serum heptaglobulin, serum amiloid A [7], sitokin, adenozin deaminaz [13,19], nitrik oksit [25] kalp, karaciğer, böbrek hasar belirteçlerinde artış [19,26,27], oksidatif stres [28-30] ve koagulasyon parametrelerinde değişimler [19] gözlenmektedir. Yeni birçok tedavi protokolleri uygulanmasına rağmen, ölüm oranı ise hala çok yüksek düzeylerdedir [9,31].

ETA uygulamasının sitokinler üzerine etkisi belirlenmezken, LPS uygulamasının serum TNF-α, IL-1β ve IL-10 düzeylerini yükselttiği belirlendi *(Tablo 1)*. Gram negatif bakterilerin hücre duvarının bir komponenti olan LPS'nin sistemik uygulaması sonrasında serum sitokin düzeylerinde artışlar gözlendiği bildirilmiştir [32-34]. Endotoksemilerde önce proinflamatuar sitokinlerin (TNF-α, IL-1β) salgılandığı, hastalığın şiddeti ile TNF-α arasında korelasyon olduğu ve enfeksiyonun devamında ise tepki olarak antiiflamatuar sitokinlerin (IL-10) salgılandığı bilinmektedir. Septik şoklarda anti-TNF uygulamalarının faydalı olabileceği ifade edilmiştir [9]. LPS uygulaması sonrası serum düzeyi 1. saatte 5086 pg/mL çıkan TNF-α konsantrasyonunun, ETA uygulaması ile 1230 pg/mL'ye düştüğü belirlendi *(Tablo 1)*. ETA'nın TNF-α üzerindeki baskılayıcı etkisi anti-TNF-α etkinliğinden kaynaklanabilir [17]. Mevcut araştırmada ETA uygulamasının, LPS tarafından sentezi uyarılan TNF-α'nın daha uzun süre (4 saatten 12 saate kadar) dolaşımda kalmasına neden olduğu belirlendi *(Tablo 1)*. Benzer şekilde endotoksemik ratlara ETA uygulaması sonrası serum TNF-α düzeyinin deney süresince genellikle LPS grubundan daha yüksek düzeylerde kalmasına neden olduğu bildirilmiştir. Guo ve ark.[18] tarafından yapılan bu araştırmada yüksek düzeylerde ölçümü yapılan TNF-α'nın ise biyolojik olarak aktif olmadığı bildirilmiştir. Endotoksemilerde ilk dönemde TNF-α ile birlikte yükselen IL-1β düzeyi üzerine, ETA'nın belirgin etkisinin olmadığı gözlendi *(Tablo 1)*. ETA uygulamasının TNF-α'nın neden olduğu nitrik oksit sentezini engellerken, IL-1β'nın neden olduğu nitrik oksit sentezini engelleyemediğinin bildirilmesi [35], ETA'nın seçici anti-TNF-α etkinliğinin bir sonucu olarak IL-1β sentezi üzerine etkisinin olmadığını da açıklayabilir. Endotoksemilerde ilk salgılanan proinflamatuar sitokinlere tepki olarak sonradan salgılanan antiinflamatuar sitokin IL-10 düzeyini ETA uygulamasının bazı örnekleme zamanlarında daha da artırdığı belirlendi *(Tablo 1)*. Doku kültüründe LPS ile uyarılan monositlerden IL-10 sentezi üzerine ETA'nın etkisinin olmadığı bildirilmiştir [36]. *In vivo* ve *in vitro* araştırmalardan her zaman birbiri ile uyumlu sonuçlar elde edilemeyeceği iyi bilinen bir gerçektir. Özellikle endotoksemi gibi birçok endojen maddenin katıldığı sistemik reaksiyon düşünüldüğünde, endotoksemiyi *in vitro* testlerle değerlendirilerek sonuca varmak oldukça zordur. ETA'nın IL-10 sentezini artırıcı etkisi dolaylı olmakla birlikte antiinflamatuar etkinliğe sahip olduğunu göstermektedir. Septik şoklu insanlarda anti-TNF-α etkili ürünün hayatta kalma üzerine etkisinin incelendiği bir araştırmada, hayatta kalmayı artırmadığı, 28 gün sonunda ölüm oranını doza bağlı olarak artırabileceği bildirilmiştir [37]. Doza bağlı ölümün muhtemel nedeni ise immun sistemin baskılanmasıdır. Beşeri hekimlikte yoğun bakım ünitelerinin veteriner hekimliğe göre daha donanımlı olması ve medikal tedavinin daha güçlü yapılabilmesi nedeni ile hastalar 28 gün kadar yaşatılabilmektedir. Ancak veteriner hekimlikte septik şoklu hastaların 28 gün süresince yoğun bakım ünitelerinde bulundurulması pratikte mümkün değildir. Genellikle bir hafta içinde vakalarda iyileşme veya ölüm gözlenmektedir. Veteriner hekimlik alanında, beşeri hekimlikteki kadar uzun süreli ilaç kullanımı mümkün olmadığı için beşeri hekimlikte gözlenen immun sistemin baskılanması ile olumsuz etkiler veteriner hekimlikte gözlenmeyebilir. Ratlarda yapılan araştırmada [18] septik şokta tek doz ETA uygulamasının hayatta kalmayı artırdığının belirlenmesi bu düşünceyi desteklemektedir.

LPS uygulamasının AT düzeyinde düzensiz değişimlere, LPS ile birlikte ETA uygulamasının ise AT düzeyinde 4. saatte düşmeye ($P<0.05$), 8. saatte yükselmeye ($P<0.05$) ve deney sonunda normal düzeylere düşmesine neden olduğu belirlendi *(Tablo 1)*. Septik şoklu hastalarda koagulasyonun uyarılması sonucu AT düzeyinin genellikle azaldığı [9] ve deneysel endotoksemide AT düzeyinin ilk uygulama sonrasında düştüğü devamında normal değerler düzeyine yükseldiği bildirilmiştir [19]. ETA uygulaması sonrasında fibrinojen düzeyinde düzensiz değişimler gözlenirken, LPS uygulaması sonrası önce düşme ($P>0.05$), sonrasında yükselmelere ($P<0.05$) neden olduğu ve LPS ile birlikte ETA uygulamasının düşme olmaksızın yükselmelere neden olduğu belirlendi *(Tablo 1)*. LPS uygulaması sonrasında fibrinojen düzeyinde önce düşme sonra yükselmeler gözlendiği bildirilmiştir [19]. Fibrinojen ve AT düzeyinde gözlenen önce düşme ve sonrasında yükselmeler, LPS uygulaması sonrasında başlayan YDP ile tüketiminin artmasından veya hasara uğrayan karaciğer nedeni ile sentezlerinin azalmasından kaynaklanabilir. Mevcut araştırmada ETA uygulaması ile LPS'nin neden olduğu fibrinojen düzeyindeki düşme engellendi *(Tablo 1)*. Bu durum ETA uygulamasının kısmen de olsa YDP engellemesinden kaynaklanabilir. İlerleyen örnekleme zamanlarında LPS ile LPS+ETA gruplarında gözlenen fibrinojen yükselmeleri *(Tablo 1)* ise akut faz proteinleri olarak da kabul edilen pıhtılaşma faktörlerinin enfeksiyona karşı verdiği cevaptan kaynaklanabilir. Enfeksiyon durumunda karaciğerden akut faz proteinlerinin sentezinin uyarıldığı ve fibrinojen düzeyinin normal düzeyinin 2-10 katına kadar yükselebildiği bilinmektedir [1,38].

Bakteriyel etkenlerce sentezi uyarılabilen PGM'nin, ETA grubunda 1. saat, LPS grubunda 8. saat ve LPS+ETA grubunda 12. saatte yüksek tespit edildi *(Tablo 1)*. Kan PGM düzeyi endotoksemi [6,12-14] ve genital sistem enfeksiyonlarında [39,40] yüksek tespit edilmiştir. Endotoksemide oluşan serbest radikallerin COX2 üzerinden katalizlediği bir reaksiyon sonucu PGM sentezinin oluşabileceği bildirilmiştir [12]. Antiinflamatuar etkinliği kabul edilen ETA'nın, sağlıklı ratlarda 1. saatte PGM miktarının artırdığı tespit edildi *(Tablo 1)*. ETA'nın sağlıklı ve enfekte canlıların PGM değerleri üzerine etkisi ile ilgili kaynağa ulaşılamadı. Ancak ETA'nın anti-TNF etkinliği düşünüldüğünde, PGM düzeyini artırıcı etkisini açıklamak mümkün görünmemektedir. Sağlıklı ratlarda 1. saat örnekleme zamanında tespit edilen PGM yükselmesinin oluşan bireysel farklılıktan kaynaklanabileceği düşünüldü. ETA uygulamasının COX ekspresyonunu engelleyici etkisinin varlığı bulunmasına [41,42] rağmen, LPS ile sentezi uyarılan PGM düzeyinin ETA tarafından baskılanamadığı belirlendi *(Tablo 1)*. Yapılan kaynak taramalarında ETA'nın PGM konsantrasyonu üzerine doğrudan etkisi ile bir kaynağa ulaşılamadı. Ancak mevcut ve diğer araştırmaların [41,42] sonuçları birlikte değerlendiril-

diğinde, mevcut araştırmada ETA'nın LPS'nin neden olduğu PGM artışı engelleyememesinin nedeni, ETA'nın dozuna bağlı olabileceği gibi ETA'nın antiprostagalandin etkinliğinden söz etmek için sadece ekspresyon çalışmalarının yeterli olamayabileceği ifade edilebilir.

Mevcut araştırmada kalp hasarı belirteci kabul edilen serum Ck-MB düzeyinin, ETA grubunda sağlıklı ratlar için ifade edilen değerler arasında kaldığı, LPS uygulamasının düzeyini yükselttiği ($P<0.05$) ve ETA uygulamasının LPS'nin neden olduğu artışı engellediği belirlendi *(Tablo 2)*. Septik şoklu hastalarda miyokardial disfonksiyon oluştuğu [2,3] ve serum kalp hasarı belirteçlerinin yükseldiği bildirilmiştir [14,43]. Septik şokta oluşan kalp hasarının nedeni tam olarak bilinmemekle birlikte, üretilen sitokinlerin kalpte doğrudan depresör etki göstererek ve apoptozise neden olarak miyokardial depresyona neden olabileceği bildirilmiştir [2,44]. Mevcut araştırmada kullanılan ETA'nın anti-TNF etkinliği düşünüldüğünde *(Tablo 1)*, endotoksemilerde salgılanan sitokinlerin neden olduğu kalp hasarını engellemede TNF antagonisti ürünlerin faydalı olabileceği ifade edilebilir.

Mevcut araştırmada serum karaciğer hasar belirteçleri ALP, AST, ALT ve GGT düzeylerinin LPS uygulaması sonrasında yükseldiği belirlendi *(Tablo 2)*. Yapılan deneysel modellemelerde de serum karaciğer hasar belirteçlerinin yükseldiği bildirilmiştir [45,46]. LPS uygulaması sonrasında karaciğerin oksidatif hasara, kalp ve böbreğe göre daha duyarlı olduğu [29] ve karaciğer Kupffer hücreleri ile hepatositlerin dolaşımda bulunan LPS'yi kendi içinde hızlı bir şekilde biriktirip eliminasyonunu sağladığı bildirilmiştir. Detoksifikasyon esnasında oluşan nekrozlar ise karaciğer hasarına neden olabilmektedir [3,4]. Endotoksemide ETA'nın kalpte gösterdiği koruyucu etkiyi, karaciğer hasarını engellemede gösteremediği belirlendi *(Tablo 2)*. Karaciğer LPS'nin vücuttan uzaklaştırılmasında rol alan en önemli organdır ve safra ile atılımını sağlar. Ayrıca karaciğer Kupffer hücrelerinin de dolaşımdaki LPS'nin uzaklaştırılmasında rol aldığı ve bu esnada karaciğerde yangı mediatörleri ürettiği bildirilmiştir [1]. Karaciğer hasarının engellenmemesi sitokinlerden bağımsız olarak, LPS'nin doğrudan ve Kupffer hücrelerinin ürettiği yangı mediatörleri üretmesinden kaynaklanabilir.

Mevcut araştırmada LPS uygulamasının böbrek hasar belirteci BUN düzeyini yükselttiği belirlendi *(Tablo 2)*. Proinflamatuar sitokinlerin (TNF-α, IL-1β) böbrek epitelinde apoptozis ile nekroza neden olduğu [2] ve endotoksemide gelişen hipoperfüzyon, mikrotrombüsler ile lokal nekrozların böbrek hasarına neden olabileceği bildirilmiştir [3]. Deneysel LPS uygulamalarında serum böbrek hasar belirteçlerinin yükseldiği ifade edilmiştir [45,46]. LPS tarafından yükseltilen BUN düzeyinin, ETA tarafından kısmen düşürüldüğü belirlenmesine rağmen, sağlıklı ratlar için bildirilen düzeylere indiremediği belirlendi *(Tablo 2)*. İşemi-reperfüzyon sonrası yükselen BUN düzeyinin, ETA tarafından düşürüldüğü, ancak kontrol değerlere inmediği bildirilmiştir [47]. ETA'nın böbrek koruyucu etkisi, TNF-α'nın böbreklerde neden olduğu apoptozis ve nekrozları engellemesinden kaynaklanabilir.

LPS uygulaması sonrasında trigliserit düzeyinin yükseldiği, ETA uygulamasının ise artışı kısmen engellediği ve araştırma sonunda sağlıklı değerlere düşürdüğü belirlendi *(Tablo 2)*. Endotoksemilerde yükselen trigliserit düzeyinin [43,48,49], hepatik lipogenezisin artması ve trigliserit klerensinin azalmasından kaynaklanabileceği bildirilmiştir [50]. Farklı hastalıklarda ETA uygulaması sonrasında trigliserit düzeyinde etkilenmenin olmadığı [51] veya yükselmeler [52] gözlendiği bildirilmiştir. Bu sonuç ETA'nın trigliserit üzerindeki etkisinin hastalığın tipine göre farklılık gösterebileceğini göstermektedir.

Sonuç olarak veteriner hekimlik alanında septik şoklu vakalarda tek doz ETA uygulamasının faydalı olabileceği ifade edilebilir. Ancak hayvan türlerine göre dozaj rejimi üzerine araştırmalar yapılmalıdır.

KAYNAKLAR

1. Van Amersfoort ES, Van Berkel TJ, Kuiper J: Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. *Clin Microbiol Rev*, 16, 379-414, 2003.

2. Lopez-Bojorquez LN, Dehesa AZ, Reyes-Teran G: Molecular mechanisms involved in the pathogenesis of septic shock. *Arch Med Res,* 35, 465-479, 2004.

3. Opal SM: The host response to endotoxin, antilipopolysaccharide strategies, and the management of severe sepsis. *Int J Med Microbiol*, 297, 365-377, 2007.

4. Buttenschoen K, Radermacher P, Bracht H: Endotoxin elimination in sepsis: Physiology and therapeutic application. *Langenbecks Arch Surg*, 395, 597-605, 2010.

5. Davies B, Cohen J: Endotoxin removal devices for the treatment of sepsis and septic shock. *Lancet Infect Dis*, 11, 65-71, 2011.

6. Er A, Yazar E: Effects of macrolide antibiotics on blood inflammatory mediators and organ damage markers in lipopolysaccharide-induced pulmonary damage rats. *Eurasian J Vet Sci,* 26, 7-13, 2010.

7. Coskun A, Sen I: Acute phase response and clinical changes in calves with lipopolysaccharide induced endotoxemia. *Eurasian J Vet Sci*, 28, 21-26, 2012.

8. Reimold AM: New indications for treatment of chronic inflammation by TNF-alpha blockade. *Am J Med Sci,* 325, 75-92, 2003.

9. Karabacak A, Yazar E: Current approaches in the treatment of septic shock. *Eurasian J Vet Sci*, 22, 95-103, 2006.

10. Ganidagli S, Gedik R, Koruk S, Mizrak A: Yoğun bakımda koagülasyon. *TAD*, 6, 36-44, 2008.

11. Kaya S: Narkotik olmayan ağrı kesiciler. **In,** Kaya S, Pirinçci İ, Bilgili A (Eds): Veteriner Hekimliğinde Farmakoloji. 2. Baskı, s. 373-400, Medisan Yayınları, Ankara, 2002.

12. Basu S, Eriksson M. Oxidative injury and survival during endotoxemia. *FEBS Lett*, 438, 159-160, 1998.

13. Altan F, Elmas M, Er A, Uney K, Cetin G, Tras B, Yazar E: Effects of drugs on kinetic values of cytokines, adenosine deaminase and 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ in endotoxemia: A different approach. *Eurasian J Vet Sci*, 26, 15-19, 2010.

14. Yazar E, Er A, Uney K, Bulbul A, Avci GE, Elmas M, Tras B: Effects of drugs used in endotoxic shock on oxidative stress and organ damage markers. *Free Radic Res*, 44, 397-402, 2010.

15. Turgut K: Karaciğer hastalıkları ve testleri-Endokrin, metabolik ve lipid bozuklukları ve testleri. **In,** Veteriner Klinik Laboratuar Teşhis. 2. Baskı. s. 202-486, Bahçıvanlar Basım Sanayi, Konya, 2000.

16. Yurt AO: Effect of meloxicam on serum vitamin and cytokine levels during endotoxemia. *Eurasian J Vet Sci*, 28, 47-53, 2012.

17. Taban M, Dupps WJ, Mandell B, Perez VL: Etanercept (enbrel)-associated inflammatory eye disease: Case report and review of the literature. *Ocul Immunol Inflamm,* 14, 145-50, 2006.

18. Guo Z, Wang S, Jiao Q, Xu M, Xu Z: Soluble TNFR II/IgG1 Fc fusion protein treatment in the LPS-mediated septic shock of rats. *Biomed Pharmacother*, 63, 537-542, 2009.

19. Yazar E, Bulbul A, Avci GE, Er A, Uney K, Elmas M, Tras B: Effects of enrofloxacin, flunixin meglumine and dexamethasone on disseminated intravascular coagulation, cytokine levels and adenosine deaminase activity in endotoxaemia in rats. *Acta Vet Hung*, 58, 357-367, 2010.

20. Tukov FF, Luyendyk JP, Ganey PE, Roth RA: The role of tumor necrosis factor alpha in lipopolysaccharide/ranitidine-induced inflammatory liver injury. *Toxicol Sci*, 100, 267-280, 2007.

21. Ness RD: Rodents. **In,** Carpenter JW (Ed): Exotic Animal Formulary. Third edition, p. 377-410, Elsevier Saunders, Pennsylvania, USA, 2004.

22. Tasgin E, Lok S, Demir N: Combined usage of testosterone and nandrolone may cause heart damage. *African J Biotechnol*, 10, 3766-3768, 2011.

23. Buyukleblebici O, Karagul H: Streptozotosin ile deneysel olarak diyabet oluşturulan ratlarda kromun biyokimyasal etkisi. *Kafkas Univ Vet Fak Derg*, 18, 21-26, 2012.

24. Er A, Uney K, Altan F, Cetin G, Yazar E, Elmas M: Effects of different doses of dexamethasone plus flunixin meglumine on survival rate in lethal endotoxemia. *Acta Vet Beograd*, 59, 47-51, 2009.

25. Irmak K, Civelek T: Septik şok şüpheli neonatal buzağılarda serum nitrik oksit (NO) konsantrasyonları. *Kafkas Univ Vet Fak Derg*, 10, 65-67, 2004.

26. Citil M, Karapehlivan M, Gunes V, Atakisi E, Uzlu E: Septisemi şüpheli buzağılarda serum sialik asit ve bazı biyokimyasal parametre düzeyleri. *Kafkas Univ Vet Fak Derg*, 10, 19-22, 2004.

27. Demirel MA, Kuplulu Ş: Investigation on the antiendotoxic effect of the combination of polymyxine and ampicillin in dogs with endotoxic pyometra. *Kafkas Univ Vet Fak Derg*, 16, 313-318, 2010.

28. Yazar E, Konyalioglu S, Col R, Birdane YO, Bas AI, Elmas M: Effects of vitamin E and prednisolone on some oxidative stress markers in endotoxemic rabbits. *Revue Med Vet*, 155, 538-542, 2004.

29. Keskin E, Oztekin E, Col R, Sivrikaya A, Uney K, Yazar E: Effect of pentoxifylline on antioxidant status of healthy and endotoxemic New Zealand white rabbits. *Acta Vet Brno,* 74, 17-21, 2005.

30. Konyalioglu S, Er A, Uney K, Elmas M, Yazar E: Effect of flunixin meglumin on the antioxidant status in endotoxemia. *Acta Vet Beograd*, 57, 241-246, 2007.

31. Er A, Altan F, Cetin G, Uney K, Tras B, Elmas M, Yazar E: Effects of enrofloxacin, flunixin and dexamethasone on indicators of oxidative and organ damage in lipopolysaccharide induced endotoxemia. *J Anim Vet Adv*, 9, 1495-1500, 2010.

32. Yazar E, Er A, Uney K, Altunok V, Elmas M: Effect of flunixin meglumine on cytokine levels in experimental endotoxemia in mice. *J Vet Med A Physiol Pathol Clin Med*, 54, 352-325, 2007.

33. Uney K, Er A, Avci GE, Bulbul A, Elmas M, Yazar E: Effect of tilmicosin on serum cytokine levels in the endotoxemia. *J Anim Vet Adv*, 8, 1021-1024, 2009.

34. Er A, Yazar E, Uney K, Elmas M, Altan F, Cetin G: Effects of tylosin on serum cytokine levels in healthy and lipopolysaccharide-treated mice, *Acta Vet Hung*, 58, 75-81, 2010.

35. Vuolteenaho K, Moilanen T, Hamalainen M, Moilanen E: Effects of TNFalpha-antagonists on nitric oxide production in human cartilage. *Osteoarthritis Cartilage*, 10 (4): 327-32, 2002.

36. Shen C, Assche GV, Colpaert S, Maerten P, Geboes K, Rutgeerts P, Ceuppens JL, Adalimumab induces apoptosis of human monocytes: A comparative study with infliximab and etanercept. *Aliment Pharm Ther*, 21, 251-258, 2005.

37. Fisher CJ Jr, Agosti JM, Opal SM, Lowry SF, Balk RA, Sadoff JC, Abraham E, Schein RM, Benjamin E: Treatment of septic shock with the tumor necrosis factor receptor: Fc fusion protein. The Soluble TNF Receptor Sepsis Study Group. *N Engl J Med*, 334, 1697-1702, 1996.

38. Redman CM, Xia H: A review of the expression, assembly, secretion and intracellular degradation of fibrinogen. *Fibrinolysis and Proteolysis,* 14 (2-3): 198-205, 2000.

39. Mateus L, Lopes da Costa L, Diniz P, Ziecik AJ: Relationship between endotoxin and prostaglandin (PGE2 and PGFM) concentrations and ovarian function in dairy cows with puerperal endometritis. *Anim Reprod Sci*, 76, 143-154, 2003.

40. Hagman R, Kindahl H, Fransson BA, Bergstrom A, Holst BS, Lagerstedt AS: Differentiation between pyometra and cystic endometrial hyperplasia/mucometra in bitches by prostaglandin F2alpha metabolite analysis. *Theriogenology*, 15, 198-206, 2006.

41. Bawolak MT, Touzin K, Moreau ME, Desormeaux A, Adam A, Marceau F: Cardiovascular expression of inflammatory signaling molecules, the kinin B1 receptor and COX2, in the rabbit: Effects of LPS, anti-inflammatory and anti-hypertensive drugs. *Regul Pept,* 146, 157-168, 2008.

42. Paiotti AP, Ribeiro DA, Silva RM, Marchi P, Oshima CT, Neto RA, Miszputen SJ, Franco M: Effect of COX-2 inhibitor lumiracoxib and the TNF-α antagonist etanercept on TNBS-induced colitis in Wistar rats. *J Mol Histol*, 2012. DOI 10.1007/s10735-012-9400-8

43. Yazar E, Col R, Konyalioglu S, Birdane YO, Elmas M, Bas AI: Effects of vitamin E and prednisolone on biochemical and haematological parameters in endotoxaemic New Zeland white rabbits. *Bull Vet Ins Pulawy*, 48, 105-108, 2004.

44. Makwana N, Baines PB: Myocardial dysfunction in meningococcal septic shock. *Curr Opin Crit Care,* 11, 418-423, 2005.

45. Elmas M, Yazar E, Uney K, Er Karabacak A: Influence of *Escherichia coli* endotoxin-induced endotoxaemia on the pharmacokinetics of enrofloxacin after intravenous administration in rabbits. *J Vet Med A Physiol Pathol Clin Med*, 53, 410-414, 2006.

46. Elmas M, Yazar E, Uney K, Er Karabacak A, Tras B: Pharmacokinetics of enrofloxacin and flunixin meglumine and interactions between both drugs after intravenous co-administration in healthy and endotoxaemic rabbits. *Vet J,* 177, 418-424, 2008.

47. Choi DE, Jeong JY, Lim BJ, Na KR, Shin YT, Lee KW: Pretreatment with the tumor nerosis factor-alpha blocker etanercept attenuated ischemia-reperfusion renal injury. *Transplant Proc*, 41, 3590-3696, 2009.

48. Yazar E, Col R, Uney K, Atalay B, Elmas M, Tras B: Effects of pentoxifylline on biochemical parameters in endotoxemic New Zeland white rabbits. *Bull Vet Ins Pulawy*, 48, 105-108, 2004.

49. Elmas M, Yazar E, Uney K, Karabacak A: Pharmacokinetics of flunixin after intravenous administration in healthy and endotoxaemic rabbits. *Vet Res Commun*, 30, 73-81, 2006.

50. Khovidhunkit W, Memon RA, Feingold KR, Grunfeld C: Infection and inflammation-induced proatherogenic changes of lipoproteins. *J Infect Dis,* 181, 462-472, 2000.

51. Mathieu S, Dubost JJ, Tournadre A, Malochet-Guinamand S, Ristori JM: Soubrier M.Effects of 14 weeks of TNF alpha blockade treatment on lipid profile in ankylosing spondylitis. *Joint Bone Spine,* 77, 50-52, 2010.

52. Gisondi, P, Cazzaniga S, Chimenti, S, Giannetti A, Maccarone M, Picardo M, Girolomoni G, Naldi L and Psocare Study Group: Metabolic abnormalities associated with initiation of systemic treatment for psoriasis: evidence from the Italian Psocare Registry. *J Eur Acad Dermatol Venereol*, 2012 Feb 7. doi: 10.1111/j.1468-3083.2012.04450.x.

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RESEARCH ARTICLE

Türkiye'de Kültürü Yapılan Gökkuşağı Alabalıklarında (*Oncorhynchus mykiss* Walbaum, 1792) İnfeksiyöz Pankreatik Nekrozis Virus Varlığının Tespiti Üzerine Bir Araştırma [1]

Metin GÜRÇAY * Turhan TURAN * Ayşe PARMAKSIZ *

[1] *Bu çalışma Gıda Tarım ve Hayvancılık Bakanlığı, Tarımsal Araştırmalar ve Politikalar Genel Müdürlüğü (Proje no: TAGEM/HS/11/09/02/189) tarafından desteklenmiştir*

* Gıda Tarım ve Hayvancılık Bakanlığı, Veteriner Kontrol Enstitüsü, Viroloji Laboratuvarı TR-23200 Elazığ - TÜRKİYE

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Özet

Bu çalışmada, Türkiye'nin farklı bölgelerindeki gökkuşağı alabalık çiftliklerinde infeksiyöz pankreatik nekroz virusunun (IPNV) varlığı araştırılmıştır. Bu amaçla, Aralık 2010 ile Mart 2011 tarihleri arasında, 30 alabalık çiftliğindeki anaçlardan 47 seminal sıvı, 62 ovarial sıvı alınmış ve 134 yavru alabalıktan oluşan toplam 243 izolasyon materyali temin edilmiştir. Virus izolasyonu için izolasyon materyallerinin BF-2 (Bluegill fry-2) hücresinde iki pasajı yapılmıştır. Sitopatik effekt (SPE) gösteren hücre kültür süpernatantları, IPNV identifikasyonu için antijen enzyme linked immunosorbent assay (Ag-ELİSA) metodu ile analize tabi tutulmuştur. Bütün izolasyon materyallerine, hücre kültürü izolasyonu testiyle karşılaştırmak amacıyla Ters transkriptaz-polimeraz zincir reaksiyonu (RT-PZR) testi de uygulanmıştır. Toplam 243 izolasyon materyalinin 26'sından (%10.7) IPNV izole edilmiştir. İzolasyon materyali alınan 11 ilin 7'sinde (%63.6) ve toplam 30 çitliğin 17'sinde (%56.6) IPNV varlığı tespit edildi. Ayrıca, virus izolasyon yöntemine göre daha ucuz ve daha hızlı bir metot olan RT-PZR testinin, IPNV'nun direkt dokudan tanısında alternatif bir metot olarak kullanılabileceği belirlenmiştir.

Anahtar sözcükler: *İnfeksiyöz pankreatik nekroz virüsü (IPNV), Gökkuşağı alabalığı, Virus izolasyonu, RT-PZR, ELİSA*

A Study on the Presence of Infectious Pancreatic Necrosis Virus Infections in Farmed Rainbow Trout (*Oncorhynchus mykiss* Walbaum, 1792) in Turkey

Summary

In this study, the presence of infectious pancreatic necrosis virus (IPNV) was investigated in farmed rainbow trout in different regions of Turkey. For this purpose, total of 243 isolation materials (47 seminal fluids, 62 ovarian fluids and 134 fingerling) were collected from 30 farms in the period of December and March 2010-2011. Isolation materials were passaged twice in BF-2 (Bluegill fry-2) cell cultures for virus isolation. The cell culture supernatants showed cytopathic effect (CPE) were tested by antigen-capture enzyme linked immunosorbent assay (Ag-ELISA) for IPNV identification. All isolation materials were tested also by reverse transcriptase polymerase cahin reaction (RT-PCR) in order to compare with the test of the virüs isolation. IPNV was detected from 26 (10.7%) of total 243 isolation materials obtained from 17 (56.6%) of 30 farms in 7 (63.6%) of 11 provinces. In addition, that RT-PCR test can be used as an alternative method in the IPNV-diagnosis by directly tissue-testing due to less expensive and faster than the virus isolation method was determined.

Keywords: *Infectious Pancreatic Necrosis Virus (IPNV), Rainbow Trout, Virus isolation, RT-PCR, ELISA*

GİRİŞ

Son yıllarda Türkiye'de kültür balıkçılığı hızlı bir gelişme göstermektedir. İstatistik rakamlarına göre, Türkiye'deki iç su alabalık üretimi, toplam balık üretiminin %46'sını oluşturmaktadır. İç su alabalık üretiminin yaklaşık %17'si ise, Doğu

İletişim (Correspondence)
+90 424 2181834
mgurcay2000@yahoo.com

ve İç Anadolu bölgelerinde yapılmaktadır [1].

İnfeksiyöz pankreatik nekrozis virusu (IPNV), *Birnaviridae* familyasında *Aquabirnavirus* genusunda yer almakta olup, ortalama 60 nm çapında, ikosahedral simetrili, zarfsız, iki segmentli ve çift iplikçikli RNA'ya sahiptir [2,3]. Virus, değişik balık türlerinde pankreas nekrozu ile karakterize olan bulaşıcı ve sistemik bir hastalık oluşturmaktadır. Hastalık, özellikle yavru ve genç salmonid balıklarda yüksek mortalite ile seyretmektedir [2]. İnfeksiyöz pankreatik nekrozis virüs (IPNV) enfeksiyonu, klinik veya subklinik enfeksiyon şeklinde görülmektedir. Her iki durumunda enfeksiyonu atlatıp sağ kalan balıklar, hayat boyu enfeksiyonu taşıyıcı olarak kalmaktadır [3]. Enfeksiyonun ortaya çıkmasında suyun sıcaklığı, oldukça önemli bir etkendir. Enfeksiyon, genellikle suyun sıcaklığının 12°C'nin altına düştüğü zamanlarda ortaya çıkmaktadır [4]. IPNV'nin teşhisinde virus izolasyonu, enzyme linked immunosorbent assay (ELİSA) ve moleküler teknikler gibi pek çok metot kullanılmaktadır [5,6].

Üretimi yapılan balıklarda IPNV 'nin varlığı, dünyanın birçok ülkesinde [7-10] olduğu gibi Türkiye'de de bildirilmiştir [4,11-14]. Değirmenci ve ark.[12] tarafından, 2003-2008 yılları arasında Ege, Marmara, İç ve Güneydoğu Anadolu bölgelerinden, enfeksiyonun salgın halde görüldüğü işletmelerden alınan hasta yavru gökkuşağı alabalık örneklerinin virolojik muayenesi sonucunda toplam 21 IPNV suşu izole edilmiştir. Toplu ve ark.[13], bir alabalık işletmesinde iştahsızlık ve yüzme bozukluğu gösteren balıkların nekropsisinde herhangi bir belirtiye rastlamamalarına rağmen, RT-PZR ile IPNV varlığını tespit etmişlerdir. Tatlı sulardaki gökkuşağı alabalıklarında IPNV izolasyonu ile ilgili yapılan bir çalışmada [14] ise, Orta ve Doğu Karadeniz Bölgesi'ndeki alabalığı çiftliklerinden temin edilen yavru ve porsiyon (3-500 g) balıkları analize tabi tutulmuş ve IPNV izole edilmiştir.

IPNV enfeksiyonu, ortaya çıktığı alabalık işletmelerinde büyük ekonomik kayıplara yol açtığı gibi, aynı zamanda uluslararası ticarette de bazı kısıtlamaların uygulanmasına neden olmaktadır. Bu konuda OIE ve Avrupa Birliği direktifleri ile Gıda Tarım ve Hayyvancılık Bakanlığı tarafından hazırlanan Hayvan Hastalıkları ile Mücadele ve Hayvan Hareketleri Kontrolü genelgesinde ihbarı mecburi balık hastalıkları ve IPNV enfeksiyonlarına duyarlı türler arasında *Oncorhynchus mykiss*' in olduğu belirtilmiş ve hastalıklarla mücadele ve kontrol altına alınmasında belirleyici kurallar konulmuştur [15].

Yapılan literatür taramasında, bugüne kadar Türkiye'de gökkuşağı alabalık çiftliklerinde IPNV enfeksiyonunun varlığı ve yaygınlığını vaka bazında veya bölgesel olarak araştıran çalışmalar [4,11-14] yapılmış, izolasyon materyali olarak da sadece balıklar kullanılmıştır. Ancak, farklı bölgeleri ele alarak Türkiye'deki genel durumu ortaya koyan, aynı zamanda yavru alabalıkların yanında, anaç balıklara ait seminal ve ovarial sıvıların izolasyon materyali olarak kullanıldığı herhangi bir çalışmaya rastlanılmamıştır. Bu çalışmada, bir taraftan Türkiye'nin farklı bölgelerinde bulunan gökkuşağı alabalık çiftliklerindeki IPNV enfeksiyonunun varlığının ve yaygınlığının araştırılması, diğer taraftan da izolasyon materyali olarak yavru alabalıkların yanı sıra, anaç alabalıklara ait seminal ve ovarial sıvılarının kullanılması amaçlanmıştır.

MATERYAL ve METOT

Yavru Alabalık, Alabalık Seminal ve Ovarial Sıvılar

Çalışmada kullanılan yavru alabalık, anaç alabalığı seminal ve ovarial sıvıları, Doğu, Güneydoğu, Akdeniz ve İç Anadolu bölgelerinde bulunan toplam 11 ilde (Elazığ, Malatya, Sivas, Muş, Bitlis, Erzincan, Tunceli, Şanlıurfa, Kahramanmaraş, Erzurum, Mardin) tatlı su kaynakları üzerinde kurulmuş toplam 30 gökkuşağı alabalık çiftliğinden temin edilmiştir. Bu çiftliklerden, izolasyon materyali elde etmek amacıyla, su sıcaklığının 12°C'nin altına düştüğü Aralık 2010 - Mart 2011 döneminde 670 yavru alabalık (1-5 g), 235 seminal sıvı ve 310 ovarial sıvıdan oluşan toplam 1215 numune alınmıştır *(Tablo 1)*.

Tablo 1. *Gökkuşağı alabalık çiftliklerinden alınan izolasyon materyallerinin illere göre dağılımı*
Table 1. *Distribution of the isolation materials obtained from the rainbow trout farms according to the provinces*

İller	İşletme Sayısı	İzolasyon Materyallerinin Sayıları			Toplam İzolasyon Materyali
		Yavru Alabalık	Seminal Sıvı	Ovarial Sıvı	
Elazığ	4	24	6	8	38
Malatya	4	37	6	9	52
Sivas	6	44	10	11	65
Muş	1	-	2	4	6
Bitlis	2	-	3	6	9
Erzincan	2	4	4	6	14
Tunceli	1	2	2	4	8
Şanlıurfa	2	4	3	-	7
Kahramanmaraş	3	7	5	7	19
Erzurum	4	9	6	7	22
Mardin	1	3	-	-	3
Toplam	30	134	47	62	243

İzolasyon Materyalleri

Yavru balıkların kuyrukları kesildikten sonra tüm balık izolasyon materyali olarak kullanılmıştır. Bu yavru balıklardan 5'er adedi ile birer izolasyon havuzu oluşturulmuştur. Aynı şekilde alabalık işletmelerine ait anaç balıklarından alınan seminal ve ovarial sıvıların 5'er adedi ile de izolasyon havuzları oluşturulmuş, böylece 134 yavru alabalık izolasyon materyali, 47 seminal sıvı izolasyon materyali ve 62 ovarial sıvı izolasyon materyalinden oluşan toplam 243 izolasyon materyali elde edilmiştir *(Tablo 1)*. Bu doku materyalleri antibiyotikli PBS içinde 4°C'de parçalayıcı yardımıyla homojenize (MagNa Lyser (Roche, Mannheim, Germany) edildikten sonra 3.000 rpm'de 15 dk. santrifüj edilmiştir. Elde edilen süpernatantlar, 0.45 µm çaplı enjektör filtreden geçirilmiştir. Ayrıca, süpernatantlardan sterilite kontrolü için de örnekler alınmıştır. Örnekler, daha sonra hücre kültürü inokulasyonu ve RT-PZR aşamalarında kullanılmak üzere -80°C'de muhafaza edilmiştir.

Hücre ve Virus

Materyallerin izolasyonunda kullanılan BF-2 (Bluegill fry-2) doku kültürü hücreleri ve kontrol virusları ulusal referans laboratuar olan Bornova Veteriner Kontrol Enstitüsü'nden temin edilmiştir. Hücre ve virus üretiminde %1 Penisilin (10.000 U/ml) - Streptomisin (10 mg/ml) - Amfoterisin B (0.025 mg/ml) solüsyonu ve 1 M Hepes içeren DMEM (Dulbecco's Modified Eagle's Medium) ile %10 fötal dana serumu kullanılmıştır.

Virus İzolasyonu

BF-2 hücreleriyle kaplanmış 24 kuyucuklu makropleytlere izolasyon materyallerinin 1/10 ve 1/100'lik dilüsyonları 100 µl hacimde inoküle ederek %5 CO_2'li etüvde 15°C'de 7 gün boyunca sitopatik efekt (SPE) [5,16] yönünden her gün kontrol edilmiştir. IPNV-pozitif olan materyaller hücre kültüründe 24 saat içerisinde çekirdek piknozislerine ve gözle görülebilen küçük plakların oluşmasıyla karakterize sitopatik efektlere rastlanmıştır. Total hücre yıkımı 2-3 gün sonra gerçekleşmiştir. Hücre kültüründe SPE meydana gelen kuyucuklardan elde edilen süpernatantlara dondur-çöz yapılıp 3.000 rpm'de 10 dk süreyle santrifüj edildikten sonra virusun idendifikasyonu yapılmak üzere -80°C'de stoklanmıştır. SPE oluşturmayan materyallerin bulunduğu makropleytler ise 7 gün boyunca doku kültürü mikroskobunda (Nikon ECLİPSE TS 100) kontrol edilmiş ve BF-2 hücresinde en az iki pasajı yapılmıştır.

Ag-ELİSA

Hücre kültürü ekimlerinde SPE oluşturan izolatların, immünolojik identifikasyonu antijen enzyme linked immunosorbent assay (Ag-ELİSA) (BIO K 282 IPNV ELISA test kit, Bio X Diagnostics S.P.R.L. Belgium) testi ile üretici firmanın önerdiği prosedüre göre yapılmıştır. Test pleytleri, ELİSA okuyucuda 450 nm filtre absorbans değerlerinde okunarak, sonuçlar hesaplanmıştır [5,7].

RNA Ekstraksiyonu ve Ters Transkriptaz-Polimeraz Zincir Reaksiyonu (RT-PZR)

Anaç balıklara ait ovarial-seminal sıvılar ve yavru balıkların organ homojenatlarından elde edilen izolasyon materyalleri RT- PZR testine tabi tutulmuştur [5,6]. Virüs RNA'sı ekstraksiyonu, tek adım reçine-tabanlı ekstraksiyon kitleri (RNAeasy Mini Kit, Qiagen) ile yapılmıştır *(Tablo 2)* [17]. Test kiti içine RNA ekstraktı katılmadan önce 2 µl ekstrakte edilmiş RNA 1 µl deiyonize formamide ile karıştırılarak 100°C'de 40 sn bırakılarak denatürasyonu sağlandı. RT-PZR işlemi, Qiagen one-step RT-PCR kit ile üretici firmanın prosedürüne göre [18] gerçekleştirilmiştir. Kısaca, toplam hacim 50 µl olacak şekilde 10.0 µl buffer, 2.0 µl dNTP mix, 2.0 µl enzyme mix, 10.0 µl 5x Q-Solution ve primerleden [19] *(Tablo 2)* oluşan ana karışıma kalıp RNA ilave edilerek elde edilen karışım, 55°C'de 30 dk., 94°C'de 2 dk, daha sonra 45 siklus 94°C'de 45 sn, 45°C'de 45 dk, 68°C'de 2 dk ve sonunda 68°C'de 7 dk bir siklus olacak şekilde programlanarak thermal cycler'a konulmuştur. Amplifiye olmuş DNA (206 bp) agaroz jelde tespit edilmiştir.

BULGULAR

Virus İzolasyonu

Türkiye'nin Doğu, Güneydoğu, Akdeniz ve İç Anadolu bölgelerinde bulunan 11 ildeki toplam 30 gökkuşağı alabalık çiftliğinden elde edilen ve bu çalışmada kullanılan 47 seminal sıvı izolasyon materyalinin sadece birinde (%2.12), 62 ovarial sıvısı izolasyon materyalinin 6'sında (%9.67) ve 134 yavru alabalık izolasyon materyalinin 19'unda (%14.17) virus bulunduğu saptanmıştır. Daha sonra uygulanan Ag-ELISA testleri sonucunda, bu viruslar İPNV olarak tanımlanmıştır *(Tablo 3)*. Toplam 243 izolasyon materyalinin 26'sında (%10.69) IPNV izolasyonu sağlanmıştır. Bu sonuçlara göre virus izolasyoın materyallerinin temin edildiği 11 ilden 7'sinde (Elazığ, Malatya, Sivas, Erzincan, Şanlıurfa, Kahramanmaraş ve Erzurum) (%63.6) bulunan toplam 17 alabalık çiftliğinde (% 56.6) İPNV izole edilmiştir.

Ters Transkriptaz-Polimeraz Zincir Reaksiyonu (RT-PZR)

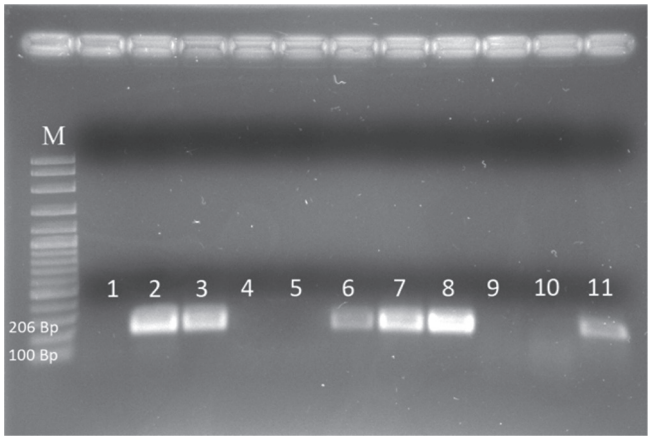
Alabalık çiftliklerinden alınan izolasyon materyallerine, hücre kültürü izolasyonu testiyle karşılaştırmak amacıyla Ters

***Tablo 2**. IPNV izolasyonunda uygulanan RT-PCR'da kullanılan primerlerin özellikleri*
***Table 2.** Characteristics of the primers used in RT-PCR applied to the IPNV isolation*

Primer (5' 3')	Proteini Kodlayan Gen	Ürün Büyüklüğü	Referans
WB1, CGCAACTTACTTGAGATCCATTATGC WB2, GTCTGGTTCAGATTCCACCTGTAGTG	VP2	206 (bp)	19

***Tablo 3.** İzolasyon materyallerinin ve IPNV-pozitif izolasyon materyallerinin sayıları*
***Table 3.** The counts of isolation materials and IPN-positive isolation materials*

Numune (n)	İzolasyon Materyali	IPN-Pozitif İzolasyon Materyali (%)
Seminal sıvı (235)	47	1 (% 2,12)
Ovarial sıvı (310)	62	6 (%9,67)
Yavru balık (670)	134	19 (%14,17)
Toplam (1215)	243	26 (%10,69)



Şekil 1. IPNV'nin kontroller ve numunelerin pozitif-negatif agaroz gel elektroforez görüntüsü. 1: Negatif kontrol, 2: Pozitif kontrol 3, 6, 7, 8 ve 11: Pozitif saha örneği, 4, 5, 9 ve 10: Negatif saha örneği

Fig 1. Agarose gel elctrophoresis of IPNV positive-negative specimens and controls. 1: Negative control, 2: Positive control, 3, 6, 7, 8 and 11: Positive field of samples, 4, 5, 9 and 10: Negative field of samples

transkriptaz-polimeraz zincir reaksiyonu (RT-PZR) testi uygulanmıştır. RT-PZR testi sonucunda, toplam 243 izolasyon materyalinin 26'sında (%10.7) IPN viral nükleik asidin varlığı gösterilmiştir *(Şekil 1)*. Hücrede virus izolasyonunu takiben uygulanan Ag-ELİSA testinin sonuçları ile direkt dokudan yapılan RT-PZR sonuçları karşılaştırıldığında %100 uyumlu olduğu tespit edilmiştir.

TARTIŞMA ve SONUÇ

Türkiye'nin çeşitli bölgelerinde özellikle tatlı sularda gökkuşağı alabalık üretimi oldukça yoğun yapılmaktadır. Yapılan araştırmalarda, 2010 yılı verilerine göre avlanan deniz balığı miktarı toplam 120 bin ton iken, deniz ve iç sularda üretimi yapılan balık miktarı 170 bin ton düzeyinde gerçekleşmiştir. Üretimi yapılan balıkların yaklaşık yarısına yakın bir kısmını, iç sularda yetiştiriciliği yapılan alabalıklar oluşturmaktadır [1].

IPNV taşıyıcısı olan balıkların tespiti için, üreme (sağım) döneminde seminal ve ovarial sıvıları ile yavru balıklarda kuyruk ve solungaçlar kesildikten sonra balığın tamamının izolasyon materyali olarak kullanılabileceği bildirilmektedir [5,20]. IPNV izolasyonu işlemlerinde de genellikle BF-2 (bluegill fry, *Lepomis macrochirus*), RTG (rainbow trout, *Oncorhynchus mykiss* gonad), CHSE-214 (chinook salmon, - *Oncorhynchus tschawytscha* embryo), FHM (fathead minnow, - *Pimephales promelas* epitheloid cell) ve EPC (epithelioma papillosum of carp, - *Cyprinus carpio*) hücre hatları kullanılmaktadır. Bu hücreler arasında IPNV'a en duyarlı hücre hatlarının BF-2 hücreleri olduğu bildirilmektedir [2,5,16]. Bundan dolayı bu çalışmada, IPNV izolasyonunda BF-2 hücreleri kullanılmıştır.

Gökkuşağı alabalıklarında IPNV enfeksiyonları ile ilgili olarak Türkiye'de yapılan çalışmalar [4,11-13] vaka bildirimi veya lokal [14] olup, hem enfeksiyonun Türkiye'deki yaygınlığı hakkında bir veri içermemekte, hem de IPNV izolasyon materyali olarak sadece balıklar kullanılmıştır. Bu çalışmada ise, klinik olarak hastalığın ortaya çıkmasına bakılmaksızın, belli bir dönemde Türkiye'nin farklı bölgelerinden alınan alabalık izolasyon materyalleri (yavru alabalık, ovarial ve seminal sıvılar) IPNV bakımından analize tabi tutulmuş ve IPNV izole edilmiştir. Gökkuşağı alabalıklarında IPNV enfeksiyonlarının varlığını ve yaygınlığını Orta ve Doğu Karadeniz Bölgesi'nde araştıran çalışmada [14] ise, toplam 32 alabalık çiftliğinin 10'undaki (%31.25) yavru ve porsiyon balıklardan elde edilen toplam 229 izolasyon materyallerinin 10'unda (%4.36) IPNV izole edildiği bildirilmektedir. Çalışmamızda, alabalık çiftliklerinden alınan toplam 243 izolasyon materyalinin 26'sında (%10.69) IPNV saptanırken *(Tablo 3)*, izolasyon materyali alınan alabalık çiftliklerinin bulunduğu illerin %63.6'sında ve çitliklerin de %56.6'sında IPNV bulunduğu tespit edilmiştir. Her iki araştırmanın sonuçları karşılaştırıldığında, çalışmamızda elde edilen IPNV pozitif izolasyon materyallerinin ve çiftlik sayılarının daha yüksek olduğu görülmektedir. Bu durum, çiftliklerden sağlanan örnek sayılarının fazlalığından veya enfeksiyonun yoğunluğundan kaynaklanmış olabileceği düşünülmektedir. Bu çalışmada kullanılan izolasyon materyallerinden elde edilen %10.69 oranındaki IPNV izolasyonu, gökkuşağı alabalıklarında IPNV enfeksiyonunun Türkiye'de yaygınlığının yoğun olduğunu göstermektedir. Meksika'da ise, bir eyalette bulunan 10 yerleşim biriminin 8'inde (%80), ayrıca numune alınan 29 gökkuşağı alabalık çiftliğinin de 25'inde (%86.2) IPNV izole edilmiştir [7]. Araştırıcılar, bu oranın yüksek olmasını, farklı büyüklükteki balıkların çiftlikler arası yoğun transfer işlemlerine, bazı çiftliklerin su kaynaklarının aynı olmasına, bazı çiftliklerde ise üretimde kullanılan suyun tekrar üretimde kullanılmasına bağlamaktadırlar. Çalışmamızda, IPNV enfeksiyonunun yüksek oranda bulunmasındaki en önemli faktörün, kontrolsüz yapılan yumurta, yavru ve porsiyon balık nakillerinin olduğu düşünülmektedir.

Smail ve ark.'ları [20] tarafından IPNV izolasyonu amacıyla 2003-2005 yılları arasında yapılan bir çalışmada, izolasyon materyali olarak 8 farklı Atlantik somon balığı populasyonundan temin edilen ovarial ve seminal sıvılar kullanılmıştır. Araştırma sonucunda ovarial sıvılardaki izolasyon oranlarının (2003 yılı için %9.13, 2004 yılı için %4.30, 2005 yılı için %0.009), seminal sıvılardaki izolasyon oranlarından (2003 yılı için %1.18, 2004 yılı için %0.76, 2005 yılı için %0.002) yüksek olduğu bulunmuştur. Bu çalışmamızda kullanılan ovarial izolasyon materyallerinden de, seminal izolasyon materyallerine göre daha fazla sayıda IPNV izole edilmiştir (sıra-

sıyla %9.67 - %2.22, *Tablo 3*). Elde edilen bu sonuç, Smail ve ark.'larının [20] elde ettikleri sonuçla uyumluluk göstermektedir. Ovarial sıvılarda IPNV izolasyon oranının seminal sıvılardan daha yüksek olması, ovarial sıvıların IPNV'nu daha fazla absorbe etmesinden kaynaklanmış olabileceğini düşündürmektedir [20]. IPNV balık yumurtası kabuğuna affinitesinin (ilgisinin) olduğu, yumurtanın içine girdiği ve gözlenmiş yumurtadan dezenfeksiyonla virüsün giderilemediği bildirilmiştir [21,22]. IPNV'nin seminal ve ovarial sıvıların içeriğinde bulunması ve bu şekilde taşınması, genellikle intra ovarial veya vertikal taşınma şeklinde değerlendirilmektedir [23]. Balık üretimi entegre yetiştiricilik şeklinde yapıldığından, üretim aşamasında biyolojik ürünlerin bir yerden başka bir yere nakledilmesiyle enfeksiyoz ajanların yayılması da kolaylaştırmaktadır. Dolayısıyla seminal ve ovarial sıvılarda ve yavru balıklarda virüsün varlığı, hastalığın yaygınlığını artırmaktadır [2,3,20-25].

IPNV enfeksiyonunun teşhisinde standart teşhis metodu, IPN virusunun duyarlı hücre kültüründe izolasyonudur [5,26]. Hücrede izolasyonunu takiben, IPNV identifikasyonunda en yaygın olarak kullanılan serolojik metotlar nötralizasyon testi, floresans antikor tekniği (FAT), immunoperoksidaz ve enzyme linked immunosorbent assay (ELİSA) olduğu rapor edilmiştir [5,7,27,28]. Ayrıca, daha az sıklıkla kullanılanlar immunoperoksidaz, komplement fikzasyon tekniği, immunoblot ve Polimeraz zincir reaksiyonu gibi teknikler çeşitli araştırmalar tarafından kullanılmıştır [2,29,30]. IPNV'nun dokudan direkt olarak tespit etmek amacıyla RT-PZR test metodu da kullanılmış ve bu metot birçok araştırıcılar tarafından daha hassas bulunmuştur [6,7,14,17]. Bu araştırmada hücre kültüründe izole edilen virus uygulanan Ag-ELİSA testi sonuçları ile direkt dokudan yapılan RT-PZR testi sonuçlarının her ikisinin de %100 uyumlu olduğu tespit edilmiştir. Bu nedenle, virus izolasyon yöntemine göre daha ucuz ve daha hızlı bir metot olan RT-PZR testinin, IPNV'nin direkt dokudan tanısında alternatif bir metot olarak kullanılabileceği tespit edilmiştir.

Sonuç olarak, Doğu ve Orta Anadolu bölgelerinde tatlı sularda gökkuşağı alabalık üretimi yapılan çiftliklerde, seminal ve ovarial sıvılarda ve yavru balıklarda yüksek oranda IPNV' nin bulunduğu tespit edilmiştir. IPNV'nin, daha fazla su kaynağına ve alabalık çiftliklerine bulaşmasını engellemek veya minimize etmek için, özellikle yavru alabalık nakillerinde IPNV kontaminasyonuna karşı dikkatli olunması gerekmektedir. Bunun yanında kontamine olmamış balıklarla ve balık transfer malzemeleriyle üretim yapılmamasına özen gösterilmelidir.

KAYNAKLAR

1. Anonim: Su ürünleri istatistikleri 2010 yılı raporu, Türkiye İstatistik Kurumu, 2010.

2. Wolf K: Infectious pancreatic necrosis. **In**, Wolf K (Ed): Fish viruses and fish viraldiseases. pp. 115-157, Cornell University Press, Ithaca, NY, 1988.

3. Smail DA, Munro ALS: Infectious pancreatic necrosis virus persistence in farmed Atlantic salmon *Salmo salar* L. **In,** Ellis AE (Ed): Fish and Shellfish Pathology. pp. 277-288, Academic Press, New York, 1985.

4. Ogut H, Altuntas C: Occurrence and prevalence of infectious pancreatic necrosis virus in rainbow trout *(Oncorhynchus mykiss)* cultured in cages in the Black Sea. *Aquacult Res*, 2011, doi: 10.1111/j.1365-2109.2011.02959.x.

5. Anonim: Infectious pancreatic necrosis. Manual of Diagnostic Tests for Aquatic Animals. Office International des Epizooties, 2003.

6. Taksdal T, Dannevig BH, Rimstad E: Detection of infectious pancreatic necrosis (IPN)-virus in experimentally infected Atlantic salmon parr by RT-PCR and cell culture isolation. *Bull Eur Ass Fish Pathol,* 21, 214-219, 2001.

7. Ortega C, Vega F Enríquez R: Occurrence of the ınfectious pancreatic necrosis virus in rainbow trout *(Oncorhynchus mykiss)* farms in Hidalgo State, Mexico. *Bull Eur Ass Fish Pathol,* 27(3): 100-107 2007

8. Varvarigos P, Way K: First isolation and identification of the infectious pancreatic necrosis (IPN) virus from rainbow trout *Onchorhynchus mykiss* fingerlings farmed in Greece. *Bull Eur Ass Fish Pathol,* 22 (3): 195-199, 2002.

9. Crane MSJ, Hardy-Smith P, Williams LM, Hyatt AD, Eaton LM, Gould A, Handlinger J, Kattenbelt J, Gudkovs N: First isolation of an aquatic birnavirus from farmed and wild fish species in Australia. *Dis Aquat Org,* 43, 1-14, 2000.

10. Zhao ZF, Ke F, Li Z, Gui J, Zhang O: Isolation, characterization and genome sequence of a birnavirus strain from flounder *Paralichthys olivaceus* in China. *Arch Virol,* 153, 1143-1148, 2008.

11. Candan A: First report on the diagnosis of infectious pancreatic necrosis (IPN) based on reverse transcription polymerase chain reaction (RT-PCR) in Turkey. *Bull Eur Ass Fish Pathol,* 22 (1): 45, 2002.

12. Değirmenci U, Nemli E, Çağırgan H: Türkiye'nin değişik bölgelerinden infeksiyöz pankreatik necrozis virüsü izolasyonu. *I. Ulusal Alabalık Sempozyumu, 14-16 Ekim, Isparta,* p. 35, 2008.

13. Toplu N, Albayrak H, Aydoğan A, Epikmen ET, Metin N: Gökkuşağı alabalıklarında (*Oncorhynchus mykiss* Walbaum, 1792) enfeksiyöz pankreatik nekrozun patogenezisinde apoptozisin rolü. *Ankara Üniv Vet Fak Derg*, 57, 191-196, 2010.

14. Albayrak H, Özan E: Gökkuşağı alabalıklarında (*Oncorhynchus mykiss* Walbaum, 1792) infeksiyöz pankreatik nekrozis ve infeksiyöz hematopoietik nekrozis virus enfeksiyonlarının varlığının araştırılması. *Ankara Üniv Vet Fak Derg,* 57, 125-129, 2010.

15. Anonim: Hayvan hastalıkları ile mücadele ve hayvan hareketleri kontrolü genelgesi. Gıda ve Kontrol Genel Müdürlüğü, 2012.

16. Lorenzen E, Carstensen B, Olesen NJ: Interlaboratory comparison of cell lines for susceptibility to three viruses: VHSV, IHNV. *Dis Aquat Org*, 37, 81-88, 1999.

17. Dopoza CP, Barja JL: Diagnosis and identification of IPNV in salmonids by molecular methods. **In,** Cunningham CO (Ed): Molecular Diagnosis of Salmonid Disease. pp. 23-48, Kluwer Academic Publishers, Notherlans, 2002.

18. Anon: Qiagen one step RT-PCR kit handbook. http://www.qiagen.com. *Accessed:* 02.11.2010.

19. Williams K, Blake S, Sweeny A, Singer JT, Nicholson BL: Multiplex reverse transcriptase PCR assay for simultaneus detection of three fish viruses. *J Clin Microbiol*, 37, 4139-4141, 1999.

20. Smail DA, Munro ES: Isolation and quantification of infectious pancreatic necrosis virus from ovarian and seminal fluids of Atlantic salmon, *Salmo salar* L. *J Fish Dis*, 31 (1): 49-58, 2008.

21. Snieszko SF, Wood EM, Yasutake WT: Infectious pancreatic necrosis in trout. A.M.A. *Archives of Pathology* 63, 229-233, 1957.

22. Ahne W, Negele RD: Studies on the transmission of infectious pancreatic necrosis virus via eyed eggs and sexual products of salmonid fish. **In,** Ellis AE (Ed): Fish and Shellfish Pathology. Academic Press, pp. 261-269, 1985.

23. Mulcahy D, Pascho RJ: Adsorption of fish sperm to vertically transmitted fish viruses. *Sci Magazine*, 225, 333-335, 1984.

24. Ahne W, Kelly RK, Schlotfeldt HJ: Factors affecting the transmission and outbreak of infectious pancreatic necrosis (IPN). **In,** Lillelund K, Rosenthal H (Eds: Fish Health Protection Strategies. Federal Ministry for Research and Technology, Hamburg/Bonn, pp. 19-67, 1989.

25. Dorson MCT: Experimental transmission of infectious pancreatic

necrosis virus via the sexual products. **In,** Ellis AE (Ed): Fish and Shellfish Pathology. pp. 251-260, Academic Press, London, 1985.

26. Agius C, Mangunwiryo H, Johnson RH, Smail DA: A more sensitive technique for isolating infectious pancreatic necrosis virus from asymptomatic carrier rainbow trout, *Salmo gairdneri* Richardson. *J Fish Dis,* 5, 285-292, 1982.

27. Hill BJ, Way K: Serological classification of infectious pancreatic necrosis (IPN) virüs and other aquatic birnaviruses, *Ann Rev Fish Dis,* 5, 55-77, 1995.

28. Dixon PF, Hill BJ: Rapid detection of infectious pancreatic necrosis virus (IPNV) by the enzyme-linked immunosorbent assay. *J Gen Virol,* 64, 321-330, 1983.

29. McAllister, PE, Schill, WB: Immunoblot assay: A rapid and sensitive method for identification of salmonid fish viruses. *J Wildl Dis,* 22, 468-474, 98, 1986.

30. Lopez-Lastra M, Gonzalez M, Jashes M, Sandino AM: A detection method for infectious pancreatic necrosis virus (IPNV) based on reverse transcription (RT)-polymerase chain-reaction (PCR). *J Fish Dis,* 17, 269-282, 1994.

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RESEARCH ARTICLE

Genetic Variability of *CSN1S1* Gene in Goat Populations Raised in Southeastern Region of Turkey

Faruk BOZKAYA * Şükrü GÜRLER * Muğdat YERTÜRK **

* Harran University, Faculty of Veterinary Medicine, Department of Genetics, TR-63200 Sanliurfa - TURKEY
** Yuzuncuyil University, Faculty of Veterinary Medicine, Department of Zootechnics, TR-65080 Van - TURKEY

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Summary

The objective of this study was to investigate genetic variability of *CSN1S1* gene coding for alpha-s1-casein in goat populations raised in Southeastern Region of Turkey. Blood samples were collected from goats raised in Kilis(n=60), Sanliurfa (n=56), and Siirt (n=55) provinces of Turkey. From the blood samples DNA was isolated by using phenol-chloroform extraction. Genotypes of animals were determined by using polymerase chain reaction (PCR), allele specific PCR or PCR and restriction fragment length polymorphism methods. In Kilis and Sanliurfa populations *CSN1S1* A*, B*, F and N alleles were observed, while in Siirt population only A* and B* alleles were found. Frequencies of A*, B*, F and N alleles were 0.375, 0.367, 0.017 and 0.242 in Kilis, 0.632, 0.208, 0.009 and 0.151in Sanliurfa and 0.782, 0.218, 0.000 and 0.000 in Siirt populations, respectively. *CSN1S1* E and 01 alleles were not observed among the populations studied. Observed and expected genotype frequencies did not differ significantly (P>0.05). The results of this study suggested that there were sufficient genetic variability of *CSN1S1* gene especially in Sanliurfa and Kilis populations in order to select individuals for different breeding purposes.

Keywords: *Goat, Casein, CSN1S1, Polymorphism*

Güneydoğu Anadolu Bölgesi'nde Yetiştirilen Keçilerde Alfa-s1-Kazein (*CSN1S1*) Genindeki Genetik Çeşitlilik

Özet

Bu çalışmanın amacı güneydoğu anadolu bölgesinde yetiştirilen keçilerde alfa-s1-kazeini kodlayan *CSN1S1* genindeki çeşitliliğin araştırılmasıdır. Türkiye'nin Kilis (n=60) Şanlıurfa (n=56) ve Siirt (n=55) illerinde yetiştirilen keçilerden kan örnekleri toplanmıştır. Kan örneklerinden fenol-kloroform yöntemi ile DNA izolasyonu yapılmıştır. Keçilerin genotipleri polimeraz zincir reaksiyonu (PCR), allel spesifik PCR ve kesim bölgesi polimorfizmi yöntemleri ile belirlenmiştir. Kilis ve Şanlıurfa populasyonlarında A*, B*, F ve N allelleri gözlenirken Siirt populasyonunda sadece A* ve B* allelleri bulunmuştur. A*, B*, F and N allellerinin frekansları sırasıyla Kilis keçilerinde 0.375, 0.367, 0.017 ve 0.242, Şanlıurfa populasyonunda 0.632, 0.208, 0.009 ve 0.151, Siirt populasyonunda ise 0.782, 0.218, 0.000 ve 0.000, rolarak hesaplanmıştır. İncelenen populasyonlarda *CSN1S1* E ve 01 allelleri gözlenmemiştir. Beklenen ve gözlenen genotip frekansları arasında önemli bir farklılık bulunmamıştır (P>0.05). Çalışma sonucunda özellikle Kilis ve Şanlıurfa populasyonlarında değişik yetiştirme hedefleri açısından seleksiyon yapılabilecek düzeyde genetik çeşitliliğin bulunduğu kanaatine varılmıştır.

Anahtar sözcükler: *Keçi, Kazein, CSN1S1, Polimorfizm*

INTRODUCTION

Casein genes polymorphism in goats has been extensively investigated due to the less allergenicity of goat's milk than that of cows [1]. Therefore a considerable amount of data on the structure and diversity of casein genes in goat has accumulated in the literature [2,3]. The structure of the *CSN1S1* gene coding for the alfa-S1-Casein (αs1-Cn) has been studied in detail by several researchers [3-5]. *CSN1S1* gene is highly polymorphic and at least 17 alleles of this gene have been detected to date. The alleles are characterized by single nucleotide substitutions, and insertions or deletions [6]. This high poly-

İletişim (Correspondence)
+90 414 3183911
farukbozkaya@yahoo.com

morphism might be originated partly by inter allelic recombination events [3,7]. The polymorphism of this gene affects casein and protein content of goat milk. Goat *CSN1S1* gene represents an excellent model for demonstrating that the major part of the variability observed in the αs1-Cn content in the goat milk is due to the presence of autosomal alleles at a single structural locus [3]. Based on the content of αs1-Cn in goat milk the *CSN1S1* variants can be classified into four groups: strong alleles (A, B_1, B_2, B_3, B_4, C, H, L and M) each producing nearly 3.5 g/L; intermediate alleles (E and I) each producing 1.1 g/L; weak alleles (D, F and G; 0.45 g/L); and null alleles (0_1, 0_2 and N) producing no αs1-Cn [3-5,7-10]. Content of αs1-Cn in goat milk is highly correlated with individual milk components, thereby with the coagulation properties of the milk. Milk containing higher amount of αs1-Cn has higher total solid and protein and better cheese making properties such as shorter coagulation time and firmer curd [11,12].

Goat and goat milk production still plays an important role for the economy of especially south-eastern region of Turkey. In this region goat milk is produced for cheese making and for producing ice-cream. Breeding of goats producing milk for special consumer needs, i.e. for infant nutrition or nutrition of individuals allergic to cow's milk, would contribute to economical development of the region and to maintain genetic resources of goat in Turkey. Therefore determining the genetic variability of casein genes in goat populations raising in this region might help develop breeding or conservation strategies [13]. There are some reports on the genetic variability of casein genes in goat populations in Turkey. Some research groups have investigated the genetic variability of casein genes (*CSN1S1, CSN1S2, CSN2* and *CSN3*) in Angora and Hair goats raised in Turkey [14,15]. Another research group has investigated the presence and distribution of O and D alleles of *CSN1S2* gene (αs2-casein) in goat populations raised in southeastern region of Turkey [16]. However there is no report on genetic variability of *CSN1S1* gene in goat populations raised in southeastern region of Turkey.

The objective of this study was to investigate genetic variability of *CSN1S1* locus in goat populations raised in Sanliurfa, Kilis and Siirt provinces of Turkey.

MATERIAL and METHODS

Animal Material

Blood samples were collected from goats raised in Kilis (n=60), Sanliurfa (n=56), and Siirt (n=55) provinces of Turkey. Kilis goat has been developed by crossing native Hair goats with Aleppo goats and by subsequent inter-breeding among the crossbred generations. This goat has been considered a separate breed. Kilis goats are distributed especially in the Kilis province and also raised in the provinces along the Syrian border of Turkey. They are kept in small flocks of 2 to 10 goats for particularly milk production. The samples collected from the Sanliurfa province included Aleppo goats, Hair goats and the crossbred animals of these two breeds. The samples from the Siirt province included native Hair goats and their crossbred animals with Angora goats.

Genotyping of the Animals

Genomic DNA was isolated using proteinase-K digestion and phenol-chloroform extraction method [17]. Concentration of the DNA samples were measured by using spectrophotometer and diluted to an end concentration of 100 ng/μL. Sequences of the primers used for amplification of the target region of *CSN1S1* locus were shown in *Table 1*.

The *CSN1S1* F allele is characterized by a deletion of cytosine at the 23th nucleotide of the 9th exon and two insertions of 11 and 3 bp length in the subsequent intron [3,4]. In order to detect A* (A, G, I, H and 02), B* (B_1, B_2, B_3, B_4 C and D), F and N alleles a polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method was used [9]. PCR was carried out in 25 μL volume containing: 100 ng of genomic DNA, 10 pmol of each primer, dNTPs each 0.2 mM, 1.25 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 3 mM $MgCl_2$ and 2.5 μl of 10X reaction buffer contained 100 mM Tris- HCl (pH 8.8) 500 mM KCl and 0.8% Nonidet P40. The amplification protocol consisted of an initial denaturing step of 94°C for 5 min, followed by 10 cycles of 94°C for 30 s, 60°C for 30 s decreasing 1°C in each cycle and 72°C for 30 s and 25 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s. In the final cycle, the extension step was carried out at 72°C for 10 min.

A 10 μL PCR product was digested with the enzyme *Xmn*I according to instructions of the manufacturers (Fermentas, Vilnius, Lithuania). Restriction products were examined on 4% agarose gels stained with ethidium bromide.

The *CSN1S1* E allele carries a truncated long interspersed repeated element (LINE) of 475 bp length within the 19th exon [5]. A direct PCR was used for detecting 475 bp LINE element characterizing *CSN1S1* E allele by using the primer pair given in *Table 1* [18]. For *CSN1S1* E allele a 662 bp PCR product was expected while for other alleles a 205 bp PCR product was amplified. The protocol consisted of an initial

Table 1. *Primer sequences used for detecting different alleles*
Tablo 1. *Farklı allelleri tespit etmek için kullanılan primerlerin baz dizileri*

Allele	Primer Name	Sequence (5'-3')	Reference
A, B, F, N	CSN1S1-F	TTCTAAAAGTCTCAGAGGCAG	9
	CSN1S1-R	GGGTTGATAGCCTTGTATGT	
E and non E	CSN1S1E-F	ATGGGATTGAAAATTCCATGC	18
	CSN1S1E-R	ATACTACTGGAATTTAGGTA	
01 and non 01	AS1a	CCCCAGCTGGTAATGTTTTA	19
	AS1b	GGTCCATCAATTCCCTGTGT	
	AS1c	TGTATGGATCCCTGATTCCTTC	

step of 94°C for 3 min; followed for 30 cycles of 94°C for 30 sec, 59°C 30 sec and 72°C for 30 sec and a final step of 72°C for 3 min. The PCR products were examined on 2% agarose gels stained with ethidium bromide.

The *CSN1S1* 01 allele is characterized by a 8.5 kb deletion spanning from the 12th to 19th exons [10]. For detection of the *CSN1S1* 01 allele an allele specific PCR reaction protocol was used [19]. By using these primers a PCR product of 249 bp fragment length was expected for *CSN1S1* 01 allele while a 281 bp PCR product for alleles other than *CSN1S1* 01 was amplified. The amplification protocol consisted of an initial denaturation step at 94°C for 3 min followed by 40 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min. The final extension step was carried out at 72°C for 3 min. The PCR products were examined on 2% agarose gels stained with ethidium bromide.

Data Analysis

Direct counting was used to estimate allele, haplotype and genotype frequencies. Chi-square statistic (χ^2) was used to check whether the populations were Hardy-Weinberg equilibrium. All statistical analyses were performed using GENALEX 6. software package [20].

RESULTS

Restriction products of some samples after *Xmn*I digestion on agarose gel were shown in *Fig. 1*. Allele and haplotype frequencies after *Xmn*I digestion of PCR products were shown in *Table 2*. All four haplotypes were present in Kilis and Sanliurfa populations, while only D^-I^+ and D^-I^- haplotypes were observed in Siirt population. PCR amplification for detecting *CSN1S1* E allele yielded only a single band of 205 bp fragment size *(Fig. 2)* while PCR amplification for detecting *CSN1S1* 01 allele resulted in a single 281 bp fragment *(Fig. 3)*. Therefore CSN1S1 E and 01 alleles were not found in the three populations studied. In Kilis and Sanliurfa populations *CSN1S1* A* (A, G, I, H and 02) , B* (B_1, B_2, B_3 B_4 C and D), F and N alleles were observed, while in Siirt population only A* and B* alleles were found. Allele frequencies in the three populations were shown in *Table 3*. Observed genotype frequencies were given in *Table 4*. Observed and expected genotype frequencies did not differ significantly (P>0.05).

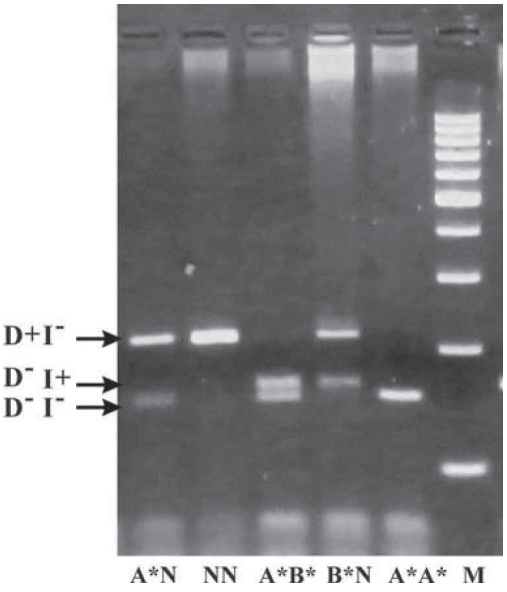

Fig 1. *Xmn*I restriction products of some samples from Sanliurfa population. The haplotypes assigned were shown at the left side of the figure. The genotypes of the individuals assigned were shown at the bottom of the figure. M: Molecular size marker (100 bp ladder)

Şekil 1. Şanlıurfa populasyonundan bazı örneklere ait *Xmn*I enzimi ile kesim ürünleri. Tepit edilen haplotipler resmin sol tarafında gösterilmiştir. Her bir birey için tespit edilen genotipler resmin alt kısmında gösterilmiştir. M: Moleküler markör (100 bp ladder)

DISCUSSION

There are some reports on the genetic variability of casein genes in goat populations in Turkey. The genetic variability of casein genes (*CSN1S1*, *CSN1S2*, *CSN2* and *CSN3*) in Angora and Hair goats raised in Turkey were studied by

Table 2. *Allele and haplotype frequencies after XmnI digestion of PCR products*
Tablo 2. *XmnI ile kesimin ardından tespit edilen allel ve haplotiplerin frekansları*

Locus	Kilis (n=60)	Sanliurfa (n=56)	Siirt (n=55)	All Populations (N=171)
D^+	0.258	0.161	0.000	0.143
D^-	0.742	0.839	1.000	0.857
I^+	0.383	0.214	0.218	0.275
I^-	0.617	0.786	0.782	0.725
Haplotype				
D^+I^+	0.017	0.009	0.000	0.009
D^+I^-	0.241	0.152	0.000	0.135
D^-I^+	0.367	0.205	0.218	0.266
D^-I^-	0.375	0.634	0.782	0.590

D^+ or D^-: *Presence or absence of the cytosine deletion at 23th nucleotide of the 9th exon,* ***I^+ or I^-:*** *Presence or absence of 11 bp insertion at in the 9th intron*

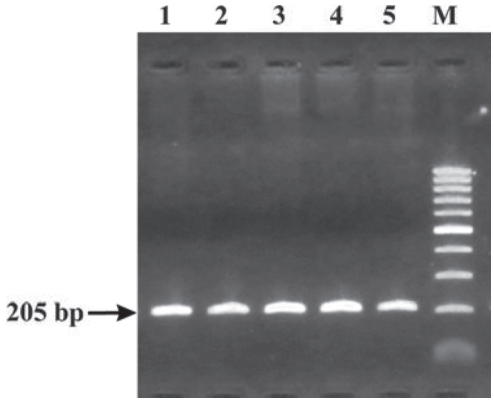

Fig 2. PCR products of some samples from Kilis population for detecting *CSN1S1* E allele (1-5). M: Molecular size marker (100 bp ladder)

Şekil 2. Kilis populasyonundan bazı örneklere ait *CSN1S1* E allelini tespit etmek için uygulanan PCR işlemine ait ürünler M: Moleküler markör (100 bp ladder)

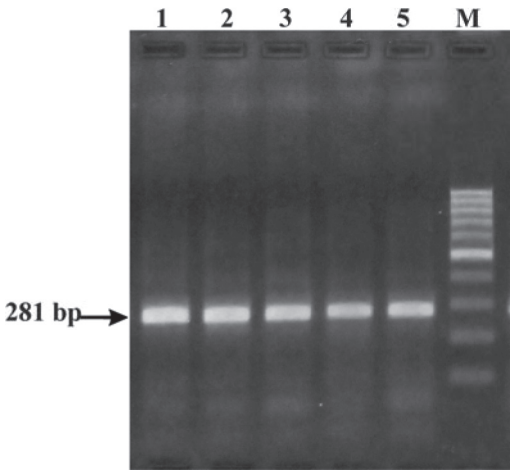

Fig 3. PCR products of some samples (1-5) from Kilis population for detecting 01 allele. M: Molecular size marker (100 bp ladder)

Şekil 3. Kilis populasyonundan bazı örneklerin *CSN1S1* 01 allelini tespit etmek için uygulanan PCR işlemine ait ürünleri M: Moleküler markör (100 bp ladder)

Table 3. *Allele frequencies of CSN1S1 gene in Kilis, Sanliurfa and Siirt goat populations*
Tablo 3. *Kilis, Şanlıurfa ve Siirt'te yetiştirilen keçilerde CSN1S1 geninin allel frekansları*

Allele	Kilis (n=60)	Sanliurfa (n=56)	Siirt (n=55)	All Populations (N=171)
A*	0.375	0.634	0.782	0.590
B*	0.367	0.205	0.218	0.266
E	0.000	0.000	0.000	0.000
F	0.017	0.009	0.000	0.009
N	0.241	0.152	0.000	0.135
01	0.000	0.000	0.000	0.000

Table 4. *Genotype frequencies of CSN1S1 gene in Kilis, Sanliurfa and Siirt populations*
Tablo 4. *CSN1S1 geninin Kilis Şanlıurfa ve Siirt populasyonlarındaki genotip frekansları*

Genotip	Kilis (n=60)	Sanliurfa (n=56)	Siirt (n=55)	All Populations (N=171)
A*A*	0.182	0.429	0.582	0.391
A*B*	0.232	0.286	0.400	0.304
A*F	0.000	0.000	0.000	0.000
A*N	0.150	0.125	0.000	0.094
B*B*	0.170	0,036	0.018	0.077
B*F	0.017	0.000	0.000	0.006
B*N	0.150	0.053	0.000	0.070
FF	0.000	0.000	0.000	0.000
FN	0.017	0.018	0.000	0.012
NN	0.082	0.053	0.000	0.046

several research groups [14,15]. The presence and distribution of O and D alleles of *CSN1S2* gene in goat populations raised in southeastern region of Turkey was also investigated [16]. Another research group studied *Sac*II restriction polymorphism of *CSN3* gene in Honamli, Hair and Saanen goats raised in Burdur vicinity [21]. This is the first report on the genetic variability of *CSN1S1* gene in goat populations raised in southeastern region of Turkey.

The PCR-RFLP method used in this study has also been used by other research groups [2,14]. Haplotype $D^{-}I^{-}$ is associated with *CSN1S1* A* and 01 alleles, haplotype $D^{-}I^{+}$ is associated with B* and E alleles, haplotype $D^{+}I^{+}$ is associated with F allele and haplotype $D^{+}I^{-}$ is associated with N allele [2,3]. Since no *CSN1S1* 01 allele was detected haplotype $D^{-}I^{-}$ was accepted as *CSN1S1* A* allele. On the other hand haplotype $D^{-}I^{+}$ was considered B* allele, as no E allele was detected.

Different allele frequencies were observed among the populations studied. *CSN1S1* F and N alleles were found with different frequencies in Kilis and Sanliurfa populations, while these alleles were not detected in Siirt population. The results found for Siirt population were similar to those reported for Angora and Hair goats raised in Turkey [14]. In contrast to the present study the *CSN1S1* E and F alleles have been found in higher frequencies in other goat breeds raised in different European countries [2,22].

In the present study *CSN1S1* E and 01 alleles were not observed among the populations examined. These alleles have been observed with different frequencies in other goat populations [2,18,22-28].

The *CSN1S1* F allele is characterized by a deletion of cytosine at the 23th nucleotide of the 9th exon and two insertions of 11 and 3 bp length in the subsequent intron [3,4]. The single nucleotide deletion in exon 9 and the two insertions in the ninth intron might be responsible for the alternative skipping of the exons 9, 10, 11 which reduces mRNA level transcribed from F allele [4]. In a study the lowest αs1-casein content has been found in Saanen breed in which *CSN1S1* E and F alleles segregate with a high frequency [29].

The *CSN1S1* N allele is characterized by a deletion of cytosine at the 23th nucleotide of the 9th exon without the insertion of 11 and 3 bp in the subsequent intron, which is present on *CSN1S1* F allele. The one nucleotide deletion results in a frame shift leading to a stop codon at 12th exon. On the other hand this deletion might also affect the splicing mechanism and thereby reduce the mRNA level transcribed from *CSN1S1* N allele [3,4]. The amount of mRNA transcribed from *CSN1S1* N allele is 1/3 of that transcribed from *CSN1S1* F allele and might explain the apparent absence of αs1-Cn in the milk samples of goats homozygote for the *CSN1S1* N allele [3].

These results indicated that both "strong" and "weak" or "null" alleles of *CSN1S1* in terms of *α*s1-Cn content in goat milk were segregating in Kilis and Sanliurfa goat populations. The results of this study suggested that there were sufficient genetic variability of *CSN1S1* gene especially in Sanliurfa and Kilis populations in order to select individuals for different breeding purposes. Goat's milk containing low level of αs1-Cn might reduce intestinal and systemic sensitization to β-lactoglobulin [30]. Human milk contains low level of αs1-Cn [31]. Therefore goats' milk containing low level of αs1-Cn (homozygotes for *CSN1S1* N allele) might be used for preparing "humanized" milk for infant nutrition. On the other hand goats' milk containing higher level of αs1-Cn (ie. *CSN1S1* A*A* or B*B*) might be preferred for cheese production [12]. Further studies are required in order to assess the effect of *CSN1S1* variants on the milk casein content of goat populations raised in Kilis and Sanliurfa goat populations. However CSN1S1 locus is closely linked to, casein genes *CSN1S2*, *CSN2* and *CSN3* and alleles of these loci are inherited together as allele groups called haplotype [32,33]. Therefore future studies should consider not *CSN1S1* gene alone but also other casein genes.

REFERENCES

1. Haenlein GFW: Goat milk in human nutrition. *Small Rumin Res,* 51, 155-163, 2004.

2. Cosenza G, Paciullo A, Colimono L, D'Avino A, Mancusi A, Ramunno L: Genotyping at the CSN1S1 locus by PCR-RFLP and AS-PCR in a Neapolitan goat population. *Small Rumin Res*, 74, 84-90, 2008.

3. Ramunno L, Cosenza G, Rando A, Pauciullo A, Illario R, Gallo D, Berardino D, Masina P: Comparative analysis of gene sequence of goat CSN1S1 F and N alleles and characterization of CSN1S1 transcript variants in mammary gland. *Gene*, 345, 289-299, 2005.

4. Leroux C, Mazure N, Martin P: Mutations away from splice site recognition sequences might cis-modulate alternative splicing of goat αs1-casein transcripts. *J Biol Chem*, 267, 6147-6157, 1992.

5. Jansa-Perez M, Leroux C, Sanchez A, Martin P: Occurence of a LINE sequence in the 3' UTR of the goat αs1-casein E- encoding allele associated with reduced protein synthesis level. *Gene*, 147, 179-187, 1994.

6. Marletta D, Criscione A, Bordonaro S, Guastella AM, D'Urso G: Casein polymorphism in goat's milk. *Lait*, 87, 491-504, 2007.

7. Bevilacqua C, Ferranti P, Garro G, Veltri C, Lagonigro R, Leroux C, Pietrola E, Addeo F, Pilla F, Chianese L, Martin P: Interallelic recombination is probably responsible for the occurrence of a new αs1-casein variant found in the goat species. *Eur J Biochem*, 269, 1293-1303, 2002.

8. Grosclaude F, Mahe MF, Brignon G, DiStasio L, Jeunet R: A Mendelian polymorphism underlying quantitative variations of goat as1-casein. *Genet Sel Evol*, 19, 399-412, 1987.

9. Ramunno L, Cosenza G, Pappalardo M, Pastore N, Gallo D, Digregorio P, Masina P: Identification of the goat CSN1S1^{F} allele by means of PCR-RFLP method. *Anim Genet,* 31, 342-343, 2000.

10. Cosenza G, Illario R, Rando A, Digregorio P, Masina P, Ramunno L: Molecular characterization of the goat CSN1S1^{01} allele. *J Dairy Res*, 70, 237-240, 2003.

11. Ambrosoli R, DiStasio L, Mazzocco P: Content of alphas1-casein and coagulation properties in goat milk. *J Dairy Sci*, 71, 24-28, 1988.

12. Clark S, Sherbon JW: Alpha$_{s1}$-CN, milk composition and coagulation properties of goat milk. *Small Rumin Res*, 38, 123-134, 2000.

13. Serradilla JM: The goat αs1-casein gene: A paradigm of the use of a major gene to improve milk quality? Options Mediteraneennes, *Seminaires Mediteraneens*, *Serie A,* 55, 99-106, 2002.

14. Chessa S, Chiatti F, Rignanese D, Ibeagha-Awemu EM, Özbeyaz C, Hassan YA, Baig MM, Erhardt G, Caroli A: The casein genes in goat breeds from different Continents: analysis by Polymerase Chain Reaction - Single Strand Conformation Polymorphism (PCR-SSCP): *Ital J Anim.Sci.* 6 (Suppl. 1): 73-75, 2007.

15. Prinzberg EM, Gutscher K, Chessa S, Caroli A, Erhardt G: Caprine k-casein (CSN3) polymorphism: New developments in molecular knowledge. *J Dairy Sci*, 88, 1490-1498, 2005.

16. Bozkaya F, Mundan D, Karabulut O, Yerturk M, Gurler S, Aral F: An investigation on the distribution of O and D alleles of the CSN1S2 gene in goat populations raised in southeastern region of Turkey. *Small Rumin Res*, 78, 193-196, 2008.

17. Sambrook J, Fritsch EF, Maniatis T: Maloecular Cloning- A Laboratory Manual. Second ed., Cold Spring Harbor Laboratory Press, New York, 1989.

18. Torres-Vazquez JA, Flores FV, Montaldo HH, Ulloa-Arvizu R, Posadas MV, Vazquez AG, Morales RAA: Genetic polymorphism of the αs1-casein locus in five populations of goats from Mexico. *Electron J Biotechn*, 11, 1-11, 2008.

19. Sztankoova Z, Kott T, Czernekova V, Dudkova G, Matlova V, Soldat J: A new allele specific polymerase chain reaction method (AS-PCR) for detection of the goat CSN1S1^{01} Allele. *Small Rumin Res*, 66, 282-285, 2006.

20. Peakall R, Smouse PE: GENALEX 6: genetic analysis in Excel. Population

genetic software for teaching and research. *Mol Ecol Notes*, 6, 288-295, 2006.

21. Korkmaz Agaoglu O, Cinar Kul B, Akyuz B, Elmaz O, Ozcelik Metin M, Saatci M, Ertugrul O: Identification of β-Lactoglobulin gene SacII polymorphism in Honamli, Hair and Saanen goat breeds reared in Burdur vicinity. *Kafkas Univ Vet Fak Derg,* 18 (3): 385-388, 2012.

22. Caroli A, Chiatti F, Chessa C, Rignanese D, Bolla P, Pagnacco G: Focusing on the goat casein complex. *J Dairy Sci*, 89, 3178-3187, 2006.

23. Clark S, Sherbon JW: Genetic variants of alpha$_{s1}$-CN in goat milk: breed distribution and associations with milk composition and coagulation properties. *Small Rumin Res*, 38, 135-143, 2000.

24. Vacca GM, Chianses L, Ghibellini A, Carcangiu V, Mauriello R, Bini PP: AlphaS1-casein genetic variants in Sarda goat breed. *Proceedings of the ASPA 15th Congress, Parma, Italy18-20 June,* 2003.

25. Chessa S, Ceriotti G, Dario C, Erhardt G, Caroli A: Genetic polymorphism of alphaS1-, alphaS2- and k-casein in Maltese goat breed. *Proceedings of the ASPA 15th Congress, Parma, Italy18-20 June,* 2003.

26. Kusza S, Veress G, Kukovis S, Javor A, Sanches A, Angiolillo A, Bosze Z: Genetic polymorphism of αs1- and αs2-caseins in Hungarian Milking Goats. *Small Rumin Res*, 68, 329-332, 2007.

27. Sacchi P, Chessa S, Budelli E, Bolla P, Ceriotti G, Soglia D, Rasero R, Cauvin E, Caroli A: Casein haplotype structure in five italian goat breeds. *J Dairy Sci*, 88, 1561-1568, 2005.

28. Caroli A, Chiatti F, Chessa S, Rignanese D, Ibeagha-Awemu EM, Erhardt E: Characterization of the casein gene complex in west african goats and description of a new αs1-casein polymorphism. *J Dairy Sci*, 90, 2989-2996, 2007.

29. Moatsou G, Samolada M, Panagiotou P, Anifantakis E: Casein fraction of bulk milks from different caprine breeds. *Food Chem*, 87, 75-81, 2004.

30. Bevilacqua C, Martin P, Candalh C, Fauquant J, Piot M, Roucayrol AM, Pilla F, Heyman M: Goat's milk of defective α_{s1}-casein genotype decreases intestinal and systemic sensitization to β-lactoglobulin in guinea pigs. *J Dairy Sci*, 68, 217-227, 2001.

31. Martin P, Brignon G, Furet JP, Leroux C: The gene encoding αs1-casein is expressed in human mammary epithelial cells during lactation. *Lait*, 76, 523-535, 1996.

32. Hayes H, Petit E, Bouiniol C, Popescu P: Localization of the α-S2-casein gene (CASAS2) to the homoelogous cattle, sheep, and goat chromosomes 4 by in situ hybridization. *Cytogenet Cell Genet*, 64, 281-285, 1993.

33. Rijnkels M: Multispecies comparison of the casein gene loci and evolution of casein gene family. *J Mammary Gland Biol Neoplasia*, 7, 327-345, 2002.

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RESEARCH ARTICLE

İyonize Radyasyon Uygulaması Yapılmış Rat Pineal Bezi Üzerinde Melatoninin Koruyucu Etkisi Var mıdır? Kronobiyolojik ve Elektron Mikroskobik Çalışma

Çiğdem ELMAS * Deniz ERDOĞAN * Güleser GÖKTAŞ * Canan ULUOĞLU **
Yıldız GÜNEY *** Gül ÖZBEY ** Ayşe HİÇSÖNMEZ **** Berna YÜCEL **
Seçil ÖZKAN ***** Dila ŞENER *

* Gazi Üniversitesi, Tıp Fakültesi, Histoloji ve Embriyoloji Anabilim Dalı, TR-06490 Ankara - TÜRKİYE
** Gazi Üniversitesi, Tıp Fakültesi, Farmakoloji Anabilim Dalı, TR-06490 Ankara - TÜRKİYE
*** Ankara Üniversitesi, Radyasyon Onkolojisi Anabilim Dalı, TR-06100 Ankara - TÜRKİYE
**** Ankara Üniversitesi, Tıp Fakültesi, Radyasyon Onkolojisi Anabilim Dalı, TR-06100 Ankara - TÜRKİYE
***** Gazi Üniversitesi, Tıp Fakültesi, Halk Sağlığı Anabilim Dalı,TR-06490 Ankara - TÜRKİYE

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Özet

Çalışmamızda, tüm vücuda uygulanan iyonize radyasyonun, sıçan pineal bezinde yaptığı ince yapı düzeyindeki dejeneratif değişiklikler ve melatoninin (MLT) olası koruyucu etkisi, elektron mikroskobik ve kronobiyolojik olarak araştırılmıştır. Bu amaçla toplam 48 adet erkek Wistar albino sıçan kullanılmıştır. Araştırmamızda, kontrol (K), tüm vücut radyasyon (TVR) ve tüm vücut radyasyon+melatonin (TVR+MLT) grupları, sabah ve akşam olmak üzere günün iki farklı saatinde çalışılarak toplam 6 deney grubu oluşturulmuştur. TVR grubundaki deneklere 8 Gy subletal (öldürücü dozun altında) radyasyon, 60Co (sağaltıcı aygıt Chisostat, açığa çıkarma hızı 0.29 Gy.dak-1) kaynağı ile deneklerin derilerinden 80 cm uzaklıktan, akşam (21.00) ve sabah (09.00) uygulanmıştır. MLT ise radyasyon verilmesinden once (10 mg/kg), hemen sonra (20 mg/kg) ve 24 saat sonra (10 mg/kg) olmak üzere intraperitoneal olarak uygulanmıştır. İnce yapı düzeyinde mitokondriyon ve pinealosit çapları ölçülmüş ve organel özellikleri histolojik olarak değerlendirilmiştir. Radyasyon uygulamasından en çok akşam grubunun etkilendiği ve melatoninin bu dejeneratif etkileri ortadan kaldırdığı, çok büyük bir farklılık olmamakla beraber TVR+MLT sabah grubunun daha iyi durumda olduğu saptanmış ancak, iki melatonin grubu arasında anlamlı bir farklılık belirlenmemiştir. Farklı organeller açısından olsa da, sıçan pineal bezinin, iyonize radyasyonun neden olduğu zedelenmeye akşam daha duyarlı olduğu, buna karşın, MLT uygulamasının oluşturduğu sağaltıcı etkide, sabah ve akşam arasında bir fark olmadığı düşünülmüştür.

Anahtar sözcükler: *Melatonin, Radyasyon, Pineal bez, Elektron mikroskobu, Kronobiyoloji*

Has Melatonin Protective Effects on Irradiated Rat Pineal Gland? Cronobiologic and Electron Microscobic Study

Summary

In this study, effect of whole body ionizing irradiation on pineal gland in rats and possible protective effects of melatonin (MLT) were investigated by chronobiological and electron microscopic methods. For this purpose 48 male Wistar rats consisting of 6 group that were; control (morning and evening groups), whole body irradiation (WBI) (morning and evening groups), whole-body irradiation+melatonin (WBI + MLT) (morning and evening groups) were used. WBI group exposed to sublethal (below the lethal dose) irradiation dose of 8 Gy using 60Co source (Chisostat therapeutic device, revealing rate 0.29 Gy.dak-1) focusing of 80 cm away from the skin in morning (09.00 am) and evening (09.00 pm). Melatonin administered intraperitoneally immediately before (10 mg/kg), after (20 mg/kg) and 24 h after (10 mg/kg) whole body irradiated rats. Ultrastructurally, mitochondria and pinealocyte diameters measured and organelle properties were evaluated histologically. Evening groups were the most affected groups by application of irradiation. However, melatonin administration considerably inhibited these degenerative changes. Although there was not any significant differences between two melatonin groups, WBI + MLT morning group was better than that of evening group. We thought that, although in different organelles, rat pineal gland was more sensitive to irradiation in evening than morning. However, we did not find any significant difference between therapeutic effect of MLT administration in morning and evening groups.

Keywords: *Melatonin, Irradiation, Pineal gland, Electron microscopy, Chronobiology*

İletişim (Correspondence)
+90 312 2024648
00cigdem@gmail.com

GİRİŞ

Pineal bez başta endokrin sistem olmak üzere organizmadaki pek çok sistemin işlevlerini gündüz ve gece değişimlerine göre düzenleyen nöroendokrin bir dokudur [1]. Hipotalamusda bulunan suprakiyazmatik çekirdek (SKÇ) yolağı ile biyolojik bir saat gibi işlev görür [2,3]. Bu özelliği ile organizmayı oluşturan dokuların işlevsel zamanlamasını ölçer ve düzenler. Pineal bez vücudun diğer yapılarına sirkadyan ritimle ve bezin karanlıkta salgıladığı melatonin (MLT) hormonu aracılığı ile zamanlama sinyalleri gönderir. Bu şekilde üreme başta olmak üzere yılın mevsimsel farklılıklarına ve gündüz ile gece değişimlerine göre vücuttaki fizyolojik olaylar düzenlenir [3]. Pineal bezin işlev görebilmesinde nörolojik uyarım, aydınlık ve karanlık önemli rol oynar. Aydınlık ve karanlık, bezden melatonin hormonunun salgılanmasında son derece önemlidir [4]. Karanlık melatonin sentezini uyarır, aydınlık ise baskılar. Işık uyarıları retinanın fotoreseptörleri ile algılanarak retinohipotalamik yol ile hipotalamusdaki SKÇ'ye ulaşır [5]. Melatonin salgılanmasının sirkadyan ritmi, bu çekirdek tarafından düzenlenmektedir [3,4]. SKÇ, algılanan ışığın miktarına göre melatonin sentezini baskılar [5].

Radyoterapi hedef dokular dışında çevredeki normal dokularda da inflamatuar bir süreci başlatmakta, bölgeye lökosit infiltrasyonu olmakta ve radyasyonla indüklenen lipid peroksidasyonu sonucunda serbest radikaller oluşmaktadır [6-8]. Organizmanın bu süreçle savaşta, antioksidan sistemleri devreye soktuğu bilinmektedir [9]. Yapılan araştırmalar melatoninin, antioksidan defans sistemlerinden biri olduğunu göstermektedir [10-12]. Diğer yandan, organizmadaki birçok fonksiyon düzenli olarak tekrarlayan bir organizasyon içinde olup, bu değişikler "biyolojik ritimler" olarak adlandırılmaktadır. Melatoninin diurnal (gün içi) salgılanma ritmi de bunlardan biridir. Lökositler tarafından serbest radikal oluşumunun [13] ya da sıçanlarda pineal bez tarafından da salgılanan ve antioksidan bir enzim olan süperoksit dismutaz aktivitesinin diurnal bir ritmi olduğu gösterilmiştir [14].

Melatonin, pineal bezde triptofan aminoasidinden sentezlenen ve bezin işlev görmesine aracılık eden son derece güçlü bir antioksidandır. Sadece suda çözünen C vitamini ya da yağda çözünen E vitamininin aksine her iki ortamda da çözünebilir ve yine bu iki antioksidandan ayrıcalıklı olarak kan beyin bariyerini geçebilir. Melatoninin tavsiye edilen günlük dozu olmamakla birlikte diger antioksidanların aksine, çok yüksek dozlarda (300 mg/gün) ve 5 yıl gibi uzun süre kullanımda bile toksik bir etki göstermemektedir [15]. Yiyeceklerden direkt olarak alınamaz ancak hindi gibi triptofan aminoasidinden zengin besin maddeleri tüketildiğinde vücuttaki sentezi artar. Sentetik melatonin kaynakları da güvenle tüketilmektedir [15]. Uyku bozuklukları, otizim, alzheimer, AIDS, epilepsi, kardiovasküler hastalıklar ve hatta kanser tedavisinde etkili olabileceğini gösteren çalışmalar bulunmaktadır. Güçlü antioksidan özelliğinin yanı sıra melatoninin radioprotektif (ışıma-koruyucu) etkisi olduğu da bilinmektedir. Daha önce sıçan ince bağırsağında, akciğerinde ve testisinde yapmış olduğumuz çalışmalarda iyonize radyasyonun ve melatoninin etkileri araştırılmıştır [16-19]. Literatürde radyasyonun pineal bez üzerine ince yapı düzeyinde neden olabileceği zedelenme ya da pineal bezden üretilen melatonin miktarındaki değişimler kısmen araştırılmış olmakla birlikte [15,20], radyasyonun oluşturduğu zedelenmenin ve ekzojen uygulanan melatoninin bu zedelenme üzerindeki etkisinin araştırıldığı çalışmalar henüz netlik kazanmamıştır. Ayrıca, sıçan pineal bezinin radyoterapiye verdiği yanıtın gün içinde nasıl değiştiği ve buna melatonin etkisi bilinmemektedir. Bu nedenle çalışmamızda tüm vücut radyasyon uygulaması yapılan deneklerde deneklerde, pineal bezde oluşabilecek histolojik değişimlere karşı, pineal bezin kendi salgısı olan, ancak araştırmamızda dışarıdan verilen melatoninin, olası koruyucu etkisi kronobiyolojik olarak elektron mikroskop düzeyinde araştırılmıştır.

MATERYAL ve METOT

Bu deney Ankara Üniversitesi Tıp Fakültesi Deney Hayvanları Etik Komitesi tarafından onaylandı. Deneyde ağırlıkları 250-300 g arasında değişen erkek Wistar albino cinsi sıçanlar kullanıldı. Standart sıçan diyeti ile beslenerek suya serbest ulaşımları sağlanmış, ışık, sıcaklık ve beslenme saatlerinin kontrol altında tutulduğu kafeslerde bakılmışlardır. Denekler, deneyin başlamasından iki hafta öncesinden itibaren sabah sekizden akşam sekize kadar aydınlıkta, geri kalan zaman diliminde ise karanlıkta bırakıldı. 48 denek, her bir grupta sekiz denek olacak şekilde (n=8) altı gruba ayrıldı *(Tablo 1)*. G1 sabah kontrol grubu, G2 akşam kontrol grubu, G3 sabah TVR, G4 akşam TVR, G5 sabah TVR+melatonin, G6 akşam TVR+Melatonin gruplarıdır. Mevsimsel ritimin bulgularımızı etkilememesi için bütün deney protokolü kış mevsiminde iki ay içinde tamamlandı [18].

İyonize Radyasyon Uygulaması

Radyasyon grubundaki deneklerin bir grubuna sabah 09.00'da, diğer grubuna akşam 21.00'de olacak şekilde ketamin anestezisi altında (100 mg/kg), 60Co kaynağı ile 8 Gy radyasyon deneklerin derilerinden 80 cm uzaklıktan, uygulandı [6].

Melatonin Uygulaması

Melatonin (MLT) ya da çözücü (%20 etanol), radyasyon uygulamasından hemen önce, hemen sonra ve 24 saat sonra (melatonin dozu: 10, 20, and 10 mg/kg, ip) intraperitoneal olarak verildi. Radyasyondan 48 saat sonra tüm denekler xylazine HCl sedasyonunu izleyerek ketamine HCl ile anestezi altına alınarak, %2.5'lik fosfat tamponlu gluteraldehit sol ventriküllerine enjekte edilmeye başlanıp tespit tüm vücut kasılmaları sona erdiğinde ve denekler katılaştığında sonlandırıldı [18].

Elektron Mikroskobik İzleme Yöntemi

Tespit işlemi tamamlandıktan sonra deneklerin pineal bezleri çıkartılarak %2.5lik fosfat tamponlu gluteraldehit

(Sigma-Aldrich, USA) solüsyonunda ayrı şişelerde 2-3 saat bekletildi. Dokular fosfat tamponunda 2-3 kez yıkandıktan sonra 1/15 M fosfat tamponlu %1'lik osmium tetroxide (OsO_4) (Sigma-Aldrich) ile +4°C'de ikinci tespitleri yapıldı. Bu aşamadan sonra tekrar fosfat tamponu ile yıkanarak, artan eter alkol serilerinden geçirilerek dokular sudan kurtarıldı. Son olarak propilen oksitten (Sigma-Aldrich) geçirilen dokular Araldite CY 212 (Ciba-Geigy, USA), (2-dodecen-1-yl) succinic anhydride (Sigma-Aldrich), benzyldimethyl amine (Poly-Sciences Inc., USA) ve dibutylphtalate (Sigma-Aldrich) karışımına gömüldüler. Polimerizasyon işlemi dokuların 24 saat 40°C'de ve 48 saat 60°C'de etüvde bırakılmasıyla tamamlandı. Yarı ince kesitler (Leica EM UC7 Ultramicrotom) toluidin mavisiyle boyanarak (Sigma-Aldrich) foto-ışık mikroskopta (Leica DM4000, Germany) değerlendirildiler. İnce kesitler ise uranil asetat (ProSciTech, Australia) ve kurşun sitrat (Sigma-Aldrich) ile boyanarak Carl Zeiss EVO LS 10 elektron mikroskopunda incelenerek değerlendirildiler [6].

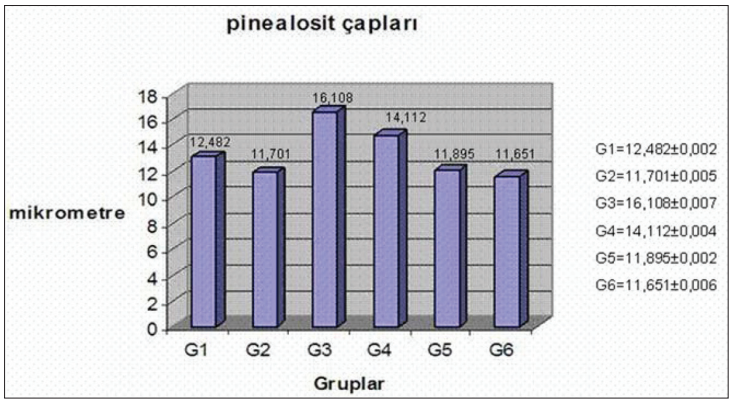
İstatistiksel Analiz

Pinealosit mitokondriyon çapları ölçülürken, her gruptan 8 ayrı pinealositte, 1 merkez 5 perifer alanda aynı büyültmede (10.09 KX), rasgele seçilen mitokondriyonlar kullanıldı *(Şekil 1, Şekil 2)*. Ayrıca her gruptan 8 farklı pinealositin çapları ölçüldü. Ölçümler için Carl Zeiss EVO LS 10 elektron mikroskobunun programı kullanıldı. İstatistiksel analiz Steel ve arkadaşlarının çalışmasında belirtildiği gibi yapıldı [19]. Gruplararası karşılaştırma yapılabilmesi için ANOVA, Mann Whithey U testi uygulandı.

BULGULAR

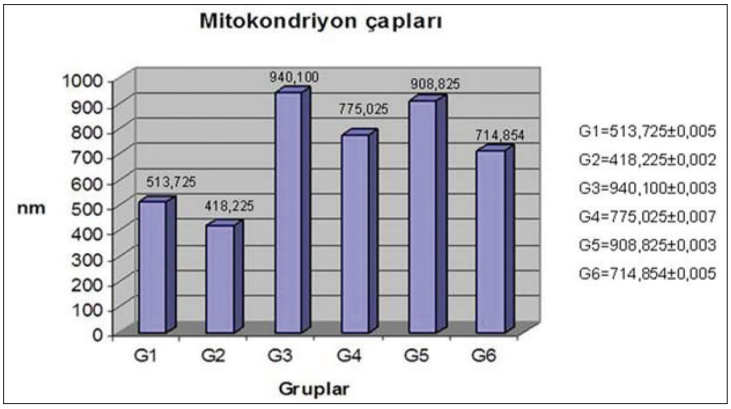
Kontrol grubunun, hem sabah (G1) *(Şekil 3. A, B)* hem de akşam (G2) *(Şekil 3. C, D)* olan alt grubunda pinealositler, glia hücreleri normal ince yapılarında izlendiler. Sabah ve akşam gruplarının arasında anlamlı bir farklılık görülmedi (P>0.05) *(Tablo 1)*.

TVR uygulaması yapılan sabah grubunda (G3) *(Şekil 4. A, B)* pineal bezde, kontrol grubuna göre son derece önemli ve anlamlı dejeneratif değişimler izlendi (P<0.05). Pinealositler oldukça dilate görünümdeydi *(Şekil 1)*. Yaygın nekroz görüntüsündeki pinealositler patlamış bir izlenim verirken mitokondriyon dışında diğer organeller net bir şekilde ayırt edilemiyordu. Mitokondriyonlar oldukça dilate ve kristolizis son derece belirgindi. Lipid damlalarında artma, damar duvarında belirgin ödem, çekirdek zarında balonlaşma (Resimde görülmüyor), pinealositlerde şişme saptandı. Bu grupta, akşam radyasyon uygulaması yapılan gruptan (G4) *(Şekil 4. C, D)* ayrıcalıklı olarak glia hücrelerinde de belirgin bir dejenerasyon izlendi. Tüm glia hücreleri hücresel bütünlüklerini kaybetmiş görünümdeydi. TVR akşam grubunda da (G4), kontrol grubu ile karşılaştırıldığında oldukça ciddi dejeneratif değişimler görüldü (P<0.05). Sabah (G3) grubunda nekroz ön plandayken akşam grubunda (G4) son derece artmış apopitotik hücrelere rastlandı. Ancak bu gruptaki pinealositler G3 grubundan daha az dilateydiler *(Şekil 1)*. Damar duvarında ciddi ödem, pinealositlerin çekirdek zarı bütünlüğünde bozulma, lipid damlacıklarında kontrole göre azalma, çekirdekcikte bölünme ve dejenerasyon, ayrıca



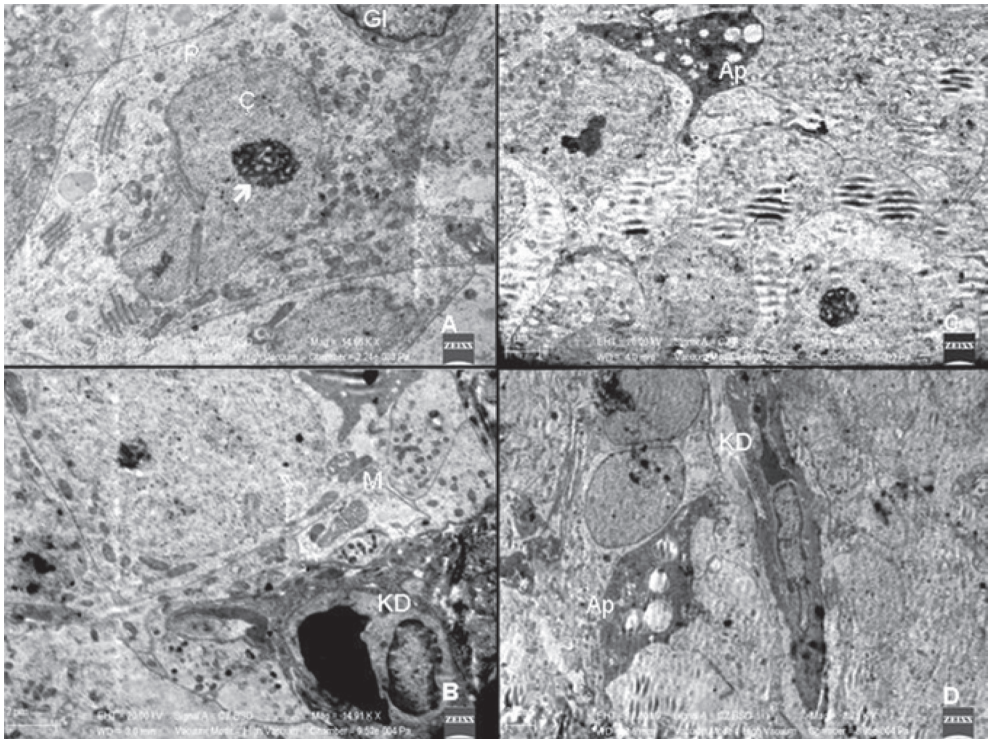
Şekil 1. Pinealosit çap ölçümleri

Fig 1. Measurement of pinealocyte diameter



Şekil 2. Pinealosit mitokondriyon çaplarının ölçümleri

Fig 2. Measurement of pinealocyte mitochondrion diameter



Şekil 3. Kontrole ait sabah **(A, B)** ve akşam **(C, D)** gruplarında pinealositler (P) ve glia (Gl) hücreleri normal ince yapılarında izleniyorlar. Ç: Çekirdek, è: Çekirdekcik, î: GER, M: Mitokondriyon, L: Lizozom, KD: Kan damarı, Ap: Apopitotik cisim (Uranil asetat-Kurşun sitrat)

Fig 3. Normal pinealocytes (P) and glial (GL) cells observed in morning **(A, B)** and evening **(C, D)** control group. N: Nucleus, è: Nucleolus, î: GER, M: Mitochondria, L: Lysosome, BV: Blood vessel, Ap: Apoptotic body (Uranyl acetate-lead citrate)

Tablo 1. *Çalışma gruplarının tedavi düzeneği*
Table 1. *Treatment composition of experimental groups*

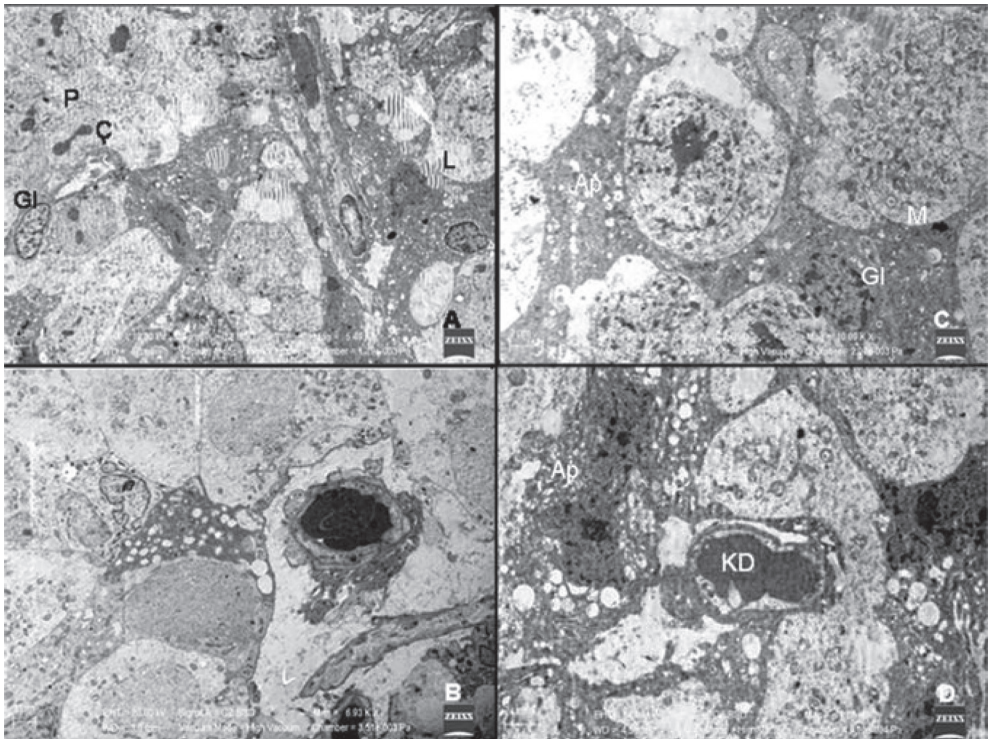
Gruplar	Radyasyon	Çözücü	Melatonin Tedavisi
G1 (Kontrol- Sabah)	-	-	-
G2 (Kontrol-Akşam)	-	-	-
G3 (TVR-Sabah)	+	+	-
G4 (TVR-Akşam)	+	+	-
G5 (TVR+MLT-Sabah)	+	+	+
G6 (TVR+MLT-Akşam)	+	+	+

sitoplazmalarında osmiyofilik cisimcikler izlendi. Pinealosit sitoplazmasında çok sayıda dev vakuoller dikkat çekiciydi (Resimde gösterilmiyor). Mitokondriyal kristolizis belirgindi, ayrıca krista kaybı nedeniyle vakuolleşen mitokondriyonlarda granüler oluşumlar izlendi. Mitokondriyonların çapları yine kontrole karşın artmıştı *(Şekil 2)*. Bu grubun hem sabah hem de akşam gruplarında GER tubulusları silinmiş gibiydi. Özellikle akşam grubunda GER tubuluslarının azalması melatonin sentezinde aksaklıkların olaylanabileceğini düşündürdü. İki radyasyon grubu karşılaştırıldığında ince yapı düzeyinde akşam grubunun uygulamadan daha fazla etkilendiği ancak pinealosit ve mitokondrial dilatasyonunun sabah grupların-

Tablo 2. *Gruplarda izlenen dejenerasyon kriterlerinin yoğunluğu*
Table 2. *The density of the degeneration criteria in experimental groups*

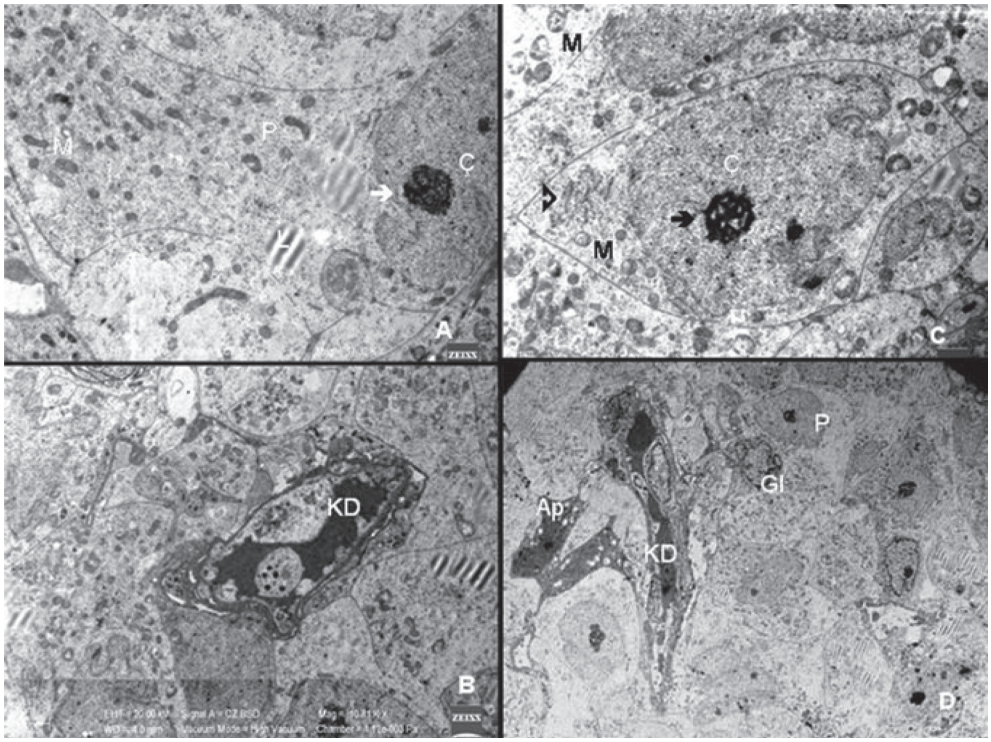
Dejenerasyon Kriterleri	G1	G2	G3	G4	G5	G6
Lipid damlacıkları	2	2	3	1	2	2
Vakuol	0	0	2	3	2	2
Kristalizis	1	1	2	2	2	2
Apopitoz	1	2	0	3	2	1
Vasküler ödem	0	0	3	3	2	2
Miyelin figürler	0	0	1	2	0	1
Çekirdek zarında balonlaşma/ayrışma	0	0	3	3	0	1
Çekirdekçikte bozulma	0	0	3	2	0	1
Osmiyofilik cisimcik	1	1	1	3	1	1
Lizozom	1	1	2	2	1	1
Nekroz	0	0	3	1	0	0

0: *Dejeneratif değişim yok;* ***1:*** *Az dejenerasyon;* ***2:*** *Orta dereceli dejenerasyon,* ***3:*** *Güçlü dejenerasyon*



Şekil 4. TVR uygulamasına ait sabah **(A, B)** ve akşam **(C, D)** gruplarında pinealositler (P) ve glia (Gl) hücrelerinde belirgin dejenerasyon ayırt ediliyor. Ç: Çekirdek, M: Mitokondriyon, L: Lipid, KD: Kan damarı, Ap: Apopitotik cisim (Uranil asetat-Kurşun sitrat)

Fig 4. Distinct degeneration of the pinealocytes (P) and glial (Gl) cells were recognized in WBI morning **(A, B)** and evening **(C, D)** groups. N: Nucleus, M: Mitochondria, L: Lipid, BV: Blood vessel, Ap: Apoptotic body (Uranyl acetate-lead citrate)



Şekil 5. TVR+MLT uygulamasına ait sabah **(A, B)** ve akşam **(C, D)** gruplarında pinealositler (P) ve glia (Gl) hücrelerinde belirgin dejenerasyon ayırt ediliyor. Ç: Çekirdek, è: Çekirdekçik, M: Mitokondriyon, î: GER, L: Lizozom, KD: Kan damarı, Ap: Apopitotik cisim (Uranil asetat-Kurşun sitrat)

Fig 5. Distinct degeneration of the pinealocytes (P) and glial (Gl) cells were recognized in WBI+MLT morning **(A, B)** and evening **(C, D)** groups. N: Nucleus, è: Nucleolus, M: Mitochondria î: GER, L: Lysosome, BV: Blood vessel, Ap: Apoptotic body (Uranyl acetate-lead citrate)

da daha fazla olduğu anlaşıldı. İstatistiksel açıdan fark anlamlı bulundu (P<0.05).

TVR ile birlikte MLT uygulaması yapılan sabah grubunda (G5) *(Şekil 5. A, B)*, kan damarlarında minimal ödem, ender olarak apopitotik cisimcikler (Resimde izlenmiyor) izlenirken yapının kısmen normal ince yapısına döndüğü görüldü. Vakuoller azalmıştı, çekirdek zarı ve çekirdekcik normal ince yapısındaydı, minimal ve granüler kristolizis izlenirken, mitokondriyon çaplarının halen kontrole karşın son derece fazla olduğu izlendi. Mitokondriyal dilatasyondaki azalma oldukça azken melatonin uygulaması ile pinealosit çaplarının kontrole yakın bir değer kazandığı dikkati çekti *(Şekil 2)*. Melatoninin akşam uygulandığı grup (G6) *(Şekil 5. C, D)*, radyasyon akşam grubuyla (G4) karşılaştırıldığında birçok dejeneratif yapıda belirgin ve anlamlı düzelmeler saptandı (P<0.05). Ancak radyasyon uygulamasından en çok etkilenen grup akşam grubu olduğu için, G6 grubu G5 grubu

ile karşılaştırıldığında G5' in ince yapı düzeyinde kontrole daha yakın olduğu izlendi. Genelde iki melatonin grubu arasında anlamlı bir farklılık görülmedi (P>0.05). Minimal vasküler ödem, tek tük apopitotik cisimcikler izlenirken dev vakuollerin oldukça azaldığı dikkati çekti. Çekirdek ve çekirdekcik yapıları normaldi ancak mitokondriyon çapları hala kontrole karşın oldukça yüksek ve mitokondriyal granüller hala belirgindi. Bununla birlikte pinealosit çapları radyasyon grubuna göre oldukça azalmış ve kontrol grubu ile eşdeğer duruma gelmişti *(Şekil 2)*.

Genel olarak radyasyon uygulaması ile pineal bezin, özellikle pinealositler başta olmak üzere doku genelinde oldukça dejeneratif histolojik değişimler sergilediği görülmüştür. Radyasyon uygulamasının pinealosit ve mitokondriyon çaplarında belirgin dilatasyona neden olduğu ve melatonin uygulamasının pinelositlerin dilatasyonunu önleyebildiği, ancak mitokondriyal dilatasyon üzerinde kısmen etkili olduğu izlenmiştir. Bu durum melatoninin hücre zarı bütünlüğünün korunmasında daha etkin ancak mitokonri gibi organel mebranlarının korunmasında ise daha az etkin olabileceğini düşündürmüştür. Belkide organel zarlarının korunabilmesi için daha uzun süreli melatonin uygulaması gerekmektedir. Melatonin uygulama sürelerinin arttırılacağı ek çalışmalara gereksinim olduğu kanısındayız. Ek olarak çalışmamızda tüm vücut radyasyon uygulamasından en çok akşam grubunun etkilendiği anlaşılmıştır. Melatonin uygulamasının ince yapı düzeyinde pineal bez üzerinde koruyucu etkisi olduğu görülmüştür.

TARTIŞMA ve SONUÇ

Radyasyon uygulaması uzun yıllardır kanser tedavisinde önemli bir yer tutmaktadır. Radiosensitif tümörler üzerine olumlu etkilerinden dolayı yaygın kullanılıyor olmasına karşın sağlıklı hücreler üzerine olan olumsuz etkileri de bilinmektedir. Bu çalışmada sağlıklı sıçanlarda TVR uygulaması sonrası, endojen melatoninin üretiminden sorumlu yapı olan pineal bezde ve özellikle ince yapı düzeyinde mitokondriyonda oluşabilecek değişimler, radyasyon uygulaması öncesi ve sonrasında ekzojen verilen melatoninin etkisi elektron mikroskobik ve kronobiyolojik yöntemlerle araştırılmıştır. Melatoninin vücudumuzda günlük metabolik ritme koşut olarak oluşan serbest radikallerin zararlı etkilerini ortadan kaldırmak yolu ile güçlü bir antioksidan olduğu uzun yıllardan beri bilinmektedir [21]. Biyolojik membranlardan kolaylıkla geçebilecek kadar küçük moleküler ağırlıkta olması nedeniyle, hücrenin tüm organellerine kolaylıkla ulaşabilmekte ve DNA zedelenmesi de dahil olmak üzere hücre tarafından sentezlenmekte olan lipid ve proteinlerde oluşabilecek zedelenmeleri de kolaylıkla engelleyebilmektedir. Melatoninin radyoprotektif etkisi de gösterilmiştir [22]. Radyasyon uygulaması sonucunda ise tekli oksijen moleküllerinin ve serbest radikallerin oluşmasıyla apopitozis de dahil olmak üzere hücreyi ölüme götüren birçok zincirleme olayın geliştiği bildirilmiştir. Radyasyon uygulaması sonucunda oluşan serbest radikallerden, kendisi de serbest radikaller üretmesine rağmen en çok mitokondriyonun etkilendiği söylenmektedir [23].

Çalışmamızın bazı parametrelerine benzer bir araştırma makalesinde, Popichiev ve ark.[20] γ-radyasyon ışınımının pineal bez üzerinde yapabileceği olası zedelenmeyi elektron mikroskobik olarak araştırmışlardır. Pinealosit, glia hücreleri ve bezin damarlarında son derece ciddi ince yapı zedelenmesi olduğunu belirten araştırmacılar, adrenokortikal işlevlerin inhibisyonunun da, γ-radyasyon ışınımının neden olduğu zedelenmeyi büyük ölçüde geri çevirebildiğini vurgulamışlardır [20]. Bizim çalışmamızda da benzer olarak, pinealositlerde radyasyon uygulamasından sonra ince yapı düzeyinde önemli dejeneratif bulgular gözlemlenmiştir. Özellikle pinealosit ve mitokondriyon çaplarında belirgin artış, mitokondriyal kristolizis belirlenmiştir. Bu çalışmadan ayrıcalıklı olarak kronobiyolojik kurgulanan çalışmamızda, sabah uygulanan radyasyonun ardından glia hücrelerinde belirgin dejenerasyon olduğu saptanmıştır.

Kurgu olarak bizim çalışmamıza benzeyen Khavinson ve ark.'nın [24] yaptığı çalışmada, γ-ışınımına etkin kalan sıçanların pineal bezlerinde ince yapı düzeyinde oluşabilecek değişimler ve epithalonun'un olası koruyucu etkisi araştırılmıştır. Bu çalışmada da bizim çalışmamıza benzer olarak, ince yapı düzeyinde pinealositlerde, glia hücrelerinde ve damar yapılarında belirgin dejeneratif değişimler saptanmıştır. Bizim çalışmamızda pineal bezin salgısı olan melatoninin koruyucu etkisi araştırılırken, bu çalışmada yine pineal bezde sentezlenen epithalamin'in korucu etkisi incelenmiş ve ince yapı düzeyinde epithalamin uygulanan grupta belirgin düzelmeler rapor edilmiştir [24]. Bizim çalışmamızda bu çalışmadan ayrıcalıklı olarak mitokondriyon ve pinealosit çapları da ölçülmüş ve istatistiksel olarak gruplar arası farklılıklar değerlendirilmiştir *(Şekil 1, Şekil 2)*.

Başka bir çalışmada tedavi amacı ile uygulanan UV-B ışınımının, U937 promonositik lösemi hücrelerinde neden olduğu apopitizis üzerinde melatoninin koruyucu olumlu etkileri gösterilmiştir. Bu çalışmada apoptozisin birkaç yolaktan süregelebileceği ancak mitokondriyal yolağın son derece önemli olduğu vurgulanmıştır. Radyasyon uygulanması öncesinde verilen melatoninin ise apopitotik hücre sayısında önemli ölçüde düşüşe neden olduğu belirtilmiştir [23]. Bizim çalışmamızda etkilerin gözlemlendiği yapı pineal bezin bütünüdür ve çalışmamızın amacı apopitozis de dahil olmak üzere bezin genelinde olaylanabilecek değişimlerin saptanmasıdır. Çalışmamızın sonunda, radyasyon uygulanmasının ardından özellikle akşam grubunda sabah grubuna karşın anlamlı fark olacak şekilde, bezin genelinde apoptotik cisimciklerde önemli ölçüde artma ve melatonin uygulanmasından sonra ise azalma gözlemledik.

Biyolojik işlevlerin, hücre içi organellerden organizmanın bütününe varıncaya dek, her seviyede, zamana bağlı değişiklikler gösterdiği bilinmektedir [24]. Radyoterapinin yarattığı oksidatif stresin yol açtığı enzim aktivitesi ve

bunlara karşı savaş veren antioksidan sistemler de bu ritmik özelliğe sahiptirler [25]. Bu durum, organizmanın zararlı uyaranlara karşı, günün farklı zamanlarında, farklı durumlarda olması anlamına gelmektedir ki bu da uyaranın etkisi ya da yan etkilerinde farklılıklar oluşturabilmektedir. Kronobiyolojinin, bu konuyu araştıran alt bilim dalı kronotoksisite olarak adlandırılmaktadır. Radyoterapinin, sıçanlarda uygulanma zamanına bağlı olarak farklı etkiler oluşturabileceği daha önceki araştırmalarımız sonucunda sunulmuştur [16-18]. Literatürde de, 12 saatlik aydınlık-karanlık döngüsünde bırakılan fare ve sıçanlarda, tüm vücuda uygulanan iyonize radyasyonun kronobiyolojik etkisi detaylı olarak derlenmiştir [25,26]. Memelilerde hücre bölünmesi rastgele olmamakta, sirkadian bir ritm izlemektedir. Bu nedenle dokudan dokuya değişmekle birlikte hücrelerin, bulundukları siklusa göre belli sirkadian zamanlarda radiosensitif ve radiorezistan olduğu dönemler bulunmaktadır [8,27-29]. Örneğin farelerde ve sıçanlarda, tüm vücut radyoterapisine bağlı mortalite ve toksik etkiler en çok akşam uygulamaları sonrasında meydana gelmektedir [25-27,30]. Bu bulgular bizim çalışmamızın sonuçları ile paralellik göstermektedir. Kemirgenler bilindiği gibi, insanlardan farklı olarak biyolojik açıdan gece aktif olan canlılardır. Aktif oldukları bu dönemde, pineal bez radyoterapiye daha duyarlı gibi görünmektedir. Bunun bir nedeni, pineal bezdeki zedelenme sonucunda, karanlıkta salıverilen melatonin sekresyonundaki azalma; bir başka nedeni ise oksidadif stres yanıtının aktivite döneminde fazla olması olabilir. Bunları ancak hipotetik olarak söylememiz mümkündür çünkü çalışmamızın bir eksiği, melatonin düzeylerini ve oksidatif enzimleri ölçememiş olmamızdır.

Sonuç olarak oluşturduğumuz bu deney modelinde, ekzojen uygulanan melatoninin sitotoksik olmayan, antiapoptotik bir ajan olduğu ve radyasyon uygulamasının oluşturabileceği ince yapı düzeyindeki pineal bez zedelenmesi üzerine de koruyucu bir etkisinin olabileceği düşüncesindeyiz. Bu koruyucu özellik hem sabah hem de akşam gruplarında ince yapı düzeyinde izlenebilmektedir. Melatoninin etkisi diurnal bir fark göstermemekle birlikte, akşam uygulanan iyonize radyasyon anlamlı olarak daha fazla zedelenmeye neden olmaktadır. Hücresel dejenerasyonun gösterilmesine yönelik yeni antikorlar ile yapılacak immunohistokimyasal çalışmalar ile çalışmamız desteklenebilir.

KAYNAKLAR

1. Beyer CE, Steketee JD, Saphier D: Antioxidant properties of melatonin-an emerging mystery. *Biochem Pharmacol,* 56 (10): 1265-1272, 1998.

2. Buijs RM, Wortel J, Van Heerikhuize JJ, Feenstra MG, Ter Horst GJ, Romijn HJ, Kalsbeek A: Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. *Eur J Neurosci,* 11 (5): 1535-1544, 1999.

3. Pevet P, Challet E: Melatonin: both master clock output and internal time-giver in the circadian clocksnetwork. *J Physiol Paris,* 105 (4-6): 170-182, 2011.

4. Challet E: Interactions between light, mealtime and calorie restriction to control daily timing in mammals. *J Comp Physiol B,* 180 (5): 631-644, 2010.

5. Perreau-Lenz S, Kalsbeek A, Van Der Vliet J, Pévet P, Buijs RM: *In vivo* evidence for a controlled offset of melatonin synthesis at dawn by thesuprachiasmatic nucleus in the rat. *Neuroscience,* 130 (3): 797-803, 2005.

6. Mansour HH: Protective role of carnitine ester against radiation-induced oxidative stress in rats. *Pharmacol Res,* 54 (3): 165-171, 2006.

7. Sonmez OF, Odaci E, Bas O, Kaplan S: Purkinje cell number decreases in the adult female rat cerebellum following exposure to 900 MHz electromagnetic field. *Brain Res*, 1356, 95-101, 2010.

8. Çenesiz M, Atakişi O, Akar A, Önbilgin G, Ormancı N: Eff ects of 900 and 1800 MHz electromagnetic field application on electrocardiogram, nitric oxide, total antioxidant capacity, total oxidant capacity, total protein, albumin and globulin levels in guinea pigs. *Kafkas Univ Vet Fak Derg*, 17 (3): 357-362, 2011.

9. Carocho M, Ferreira IC: A review on antioxidants, prooxidants and related controversy: natural and syntheticcompounds, screening and analysis methodologies and future perspectives. *Food Chem Toxicol*, 51, 15-25, 2012.

10. Bharti VK, Srivastava RS, Subramaian P, Warren Spence D, Pandi-Perumal SR, Brown GM: Cerebral epiphyseal proteins and melatonin modulate the hepatic and renalantioxidant defense of rats. *Int J Nephrol,* 2011:142896. Epub: May 26, 2011.

11. Odaci E, Kaplan S: Melatonin and nevre regeneration. *Int Rev Neurobiol*, 87, 317-335, 2009.

12. Turgut M, Uyanıkgil Y, Baka M, Tunç AT, Yavaşoğlu A, Yurtseven ME, Kaplan S: Pinealectomy exaggerates and melatonin treatment suppresses neuroma formation of transected sciatic nerve in rats: gross morphological, histological and stereological analysis. *J Pineal Res*, 38 (4): 284-291, 2005.

13. Brigaga MRPL, Barroso AS, Colepicolo P: Fluctuation of reactive oxygen species released by inflammatory cells. **In,** Vanden Driessche T, Guisset J-L, Petiau-de Vries G (Eds): The Redox State and Circadian Rhythms. 1st ed., pp. 177-191, Kluwer Academic Publishers, The Netherlands, 2000.

14. Hodoğlugil U, Ongun O, Güney Z, Altan N, Zengil H: Temporal variation in hepatic superoxide dismutase activity in mice. *Chronobiol Int*, 12 (2): 152-155, 1995.

15. Reiter RJ: Interactions of the pineal hormone melatonin with oxygen-centered free radicals: A brief review. *Braz J Med Biol Res,* 26 (11): 1141-1155, 1993.

16. Guney Y, Hicsonmez A, Uluoglu C, Guney HZ, Ozel Turkcu U, Take G, Yucel B, Caglar G, Bilgihan A, Erdogan D, Nalca Andrieu M, Kurtman C, Zengil H: Melatonin prevents inflammation and oxidative stress caused by abdominopelvic and total body irradiation of rat small Intestine. *Braz J Med Biol Res,* 40 (10): 1305-1314, 2007.

17. Guney Y, Uluoglu C, Hicsonmez A, Ozel Turkcu U, Ozbey G, Bilgihan A, Nalca Andrieu M, Guney HZ, Zengil H: Day and night effect of single dose of ionising radiation and melationin treatment of lung tissue of rats. *Turk J Biochem*, 33 (3): 104-110, 2008.

18. Take G, Erdogan D, Helvacioglu F, Göktas G, Ozbey G, Uluoglu C, Yücel B, Guney Y, Hicsonmez A, Ozkan S: Effect of melatonin and time of administration on irradiation-induced damage to rat testes. *Braz J Med Biol Res,* 42 (7): 621-628, 2009.

19. Steel RGD, Torrie JH, Dickey DA: Principles and Procedures of Statistics: A Biometrical Approach. 3rd ed., New York: Mc Graw-Hill, New York, 1997.

20. Popichiev VV, Yakovleva ND, Konoplyannikov AG, Kıvetnoi IM, Kita K, Kita I: Ultrastructure of pineal gland after γ-irradiation under conditions of inhibitions of adrenocortical function. *Bull Exp Biol Med,* 135, 706-710, 2002.

21. Reiter RJ, Tan DX, Osuna C, Gitto E: Actions of melatonin in the reduction of oxidative stress: A review. *J Biomed Sci*, 7 (6): 444-458, 2000.

22. Vijayalaxmi, Reiter RJ, Tan DX, Herman TS, Thomas CR: Melatonin as a radioprotective agent: A review. *Int J Radiat Oncol Biol Phys,* 59 (3): 639-653, 2004.

23. Luchetti F, Canonico B, Curci R, Battistelli M, Mannello F, Papa S, Tarzia G, Falcieri E: Melatonin prevents apoptosis induced by UV-B treatment in U937cell line. *J Pineal Res,* 40, 158-167, 2006.

24. Khavinson VKH, Yakovleva ND, Popuchiev VV, Kvetnoi IM, Manokhina RP: Reparative effect of epithalon on pineal gland ultrastructure in irradiated rats. *Bull Exp Biol Med,* 131, 98-103, 2001.

25. Haus E, Touitou Y: Principles of clinical chronobiology. **In,** Touitou Y,

Haus E (Eds): Biologic Rhythms in Clinical and Laboratory Medicine. 2nd ed., pp. 6-34, Springer-Verlag, Berlin, 1994.

26. Haus E: Chronobiology of the mammalian response to ionizing radiation potential applications in oncology. *Chronobiol Int*, 19 (1): 77-100, 2002.

27. Scheving LE: Circadian rhythms in cell proliferation: their importance when investigating the basic mechanism of normal versus abnormal growth. *Prog Clin Biol Res*, 59, 39-79, 1981.

28. Haus E, Halberg F, Loken MK, Kim YS: Space radiation biology and related topics. **In,** Tobias CS, Todd P (Eds): Circadian Rhythmometry of Mammalian Radiosensitivity. 1st ed., pp. 435-474, Academic Press, New York, 1974.

29. Pizzarello DJ, Usaak D, Chua KE, Rhyne AL: Circadian rhythmicity in the sensitivity of two strains of mice to whole-body radiation. *Science*, 17 (145): 286-291, 1964.

30. **Erdogan A, İpekçioğlu E:** Radyo frekans sinyalinin rat spinal ganglion hücrelerinde kalsiyum bağımlı klor kanallarına etkisi. *Kafkas Univ Vet Fak Derg*, 15 (5): 739-744, 2009.

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RESEARCH ARTICLE

Effects of Different Levels Distillers Dried Grains with Solubles on Growth Performance, Carcass Quality and Some Blood Parameters in Broilers [1]

Özlem KAYA * Tarkan ŞAHİN **

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* Department of Agriculture and Animal Production, Kars Vocational High School, University of Kafkas, TR-36100 Kars - TURKEY

** Department of Animal Nutrition and Nutritional Disease, Faculty of Veterinary Medicine, University of Kafkas, TR-36100 Kars - TURKEY

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Summary

The objective of this study was to investigate the effects of different levels dried distillers grains with solubles (DDGS) on growth performance, carcass quality and blood parameters in broilers. In 42 d feeding trial, 1 day old broilers were allocated to 4 experimental groups with 4 replicates (22/pen): control, treatment 1 (5% DDGS), treatment 2 (10% DDGS) and treatment 3 (15% DDGS). In experiment 352 chicks were used. Feed and water are provided ad libitum. On day 42, 32 (16 male and 16 female) chicks per treatment were randomly chosen and slaughtered for determining the carcass yield. Blood samples were taken 8 (4 male and 4 female) chicks per treatment. At the conclusion of the trial, differences in terms of body weight, body weight gain, feed intake and feed conversion ratio were found between trial groups fed DDGS in different amounts ($P<0.05$). As a result of the slaughtering process at the conclusion of the trial, differences ($P<0.05$) were found in terms of slaughter weight and cold and hot carcass weight in the trial groups. No difference was found in terms of the weight of the heart and liver ($P>0.05$), but gizzard weights did vary ($P<0.05$). Differences also emerged between the groups in terms of total protein and total triglyceride levels in the blood samples taken during the slaughtering process ($P<0.05$), but there was no difference between the trial groups in terms of total cholesterol levels ($P>0.05$).

Keywords: *Blood parameters, Broilers, Carcass yield, Distillers dried grains with solubles*

Kurutulmuş Damıtma Çözünürlü Tanelerinin Farklı Düzeylerde Broyler Rasyonlarında Kullanılmasının Besi Performansı, Karkas Özellikleri ve Bazı Kan Parametreleri Üzerine Etkisi

Özet

Bu çalışma, farklı düzeylerde kurutulmuş damıtma çözünürlü tanelerinin (DDGS) broylerlerde besi performansı, karkas kalitesi ve kan parametreleri üzerine etkisini belirlemek için yapılmıştır. 42 günlük deneme süresinde, 1 günlük yaştaki civcivler 4 deneme grubu ve 4 alt grup olarak ayrılmıştır (22/adet): Kontrol, grup 1 (%5 DDGS), grup 2 (%10 DDGS) ve grup 3 (%15 DDGS). Denemede 352 adet civciv kullanılmıştır. Yem ve su ad- libitum olarak sağlanmıştır. 42. günde, her gruptan 32 adet broyler (16 erkek ve 16 dişi) rastgele seçildi ve karkas veriminin belirlenmesi için kesim işlemi uygulanmıştır. Kan örnekleri her deneme grubundan 8 adet broylerden (4 erkek ve 4 dişi) alınmıştır. Deneme sonunda, farklı düzeylerde DDGS ile beslenen deneme gruplarında canlı ağırlık, canlı ağırlık artışı, yem tüketimi ve yemden yararlanma oranı bakımından farklılıklar bulunmuştur ($P<0.05$). Kesim işlemi sonunda, kesim ağırlığı, sıcak ve soğuk karkas ağırlıkları bakımından farklılıklar tespit edilmiştir ($P<0.05$). Karaciğer ve kalp ağırlıklarında farklılık bulunmazken ($P>0.05$), taşlık ağırlığında farklılık olduğu tespit edilmiştir ($P<0.05$). Gruplar arasında toplam protein ve toplam trigliserit düzeyleri bakımından farklılık saptanırken ($P<0.05$), toplam kolesterol bakımından farklılık saptanmamıştır ($P>0.05$).

Anahtar sözcükler: *Kan parametreleri, Broyler, Karkas verimi, Kurutulmuş damıtma çözünürlü taneleri*

İletişim (Correspondence)
+90 474 2123623/125
drozlemkaya@hotmail.com

INTRODUCTION

In recent years, the global production of ethanol and other bio-fuels has continued to increase rapidly. Distillers dried grains with solubles (DDGS) is defined as the product obtained after removal of ethyl alcohol by distillation from the yeast fermentation of a grain or grain mixture by condensing and drying at least 75% of resultant whole stillage by methods employed in the grain distilling industry [1]. The vast increase in ethanol production over the last 5 to 10 year has led to on increased supply of DDGS that is available for livestock feed [2,3].

Previous research has demonstrated that DDGS can be fed to poultry successfully [4-7]. Waldroup et al.[8] reported that when DDGS were included into broiler diets with the metabolisable energy (ME) content held constant up to 25% DDGS could be used without reduction in body weight (BW) or feed utilization. Dale and Batal [9] used to 0, 6, 12 and 18% DDGS in a 42 d grow out study and reported that 12% DDGS resulted in a slight decrease in performance during the starter period while 18% DDGS had a negative impact on BW and feed conversion ratio (FCR) over the 42 d period. Wang et al.[10] used 0, 5, 10, 15, 20 and 25% DDGS in broiler diets and found that 15-20% DDGS supplementation to be effective low level on performance. Similarly, Wang et al.[11] used similar levels in a 18 d and reported that DDGS can be used in broiler diets up to 30% levels. Lu and Chen [12] used 10-20% DDGS in 16 week growth study on domestic colored chickens and indicated that during the 14 week while 10-20% DDGS had no negative effects BW, body weight gain(BWG), FCR, carcass weight and yield, liver weight and plasma total cholesterol, protein and triglyceride.

Therefore, the objective of this study was to determine the effect of different levels DDGS on fattening performance, carcass yield and some blood parameters.

MATERIAL and METHODS

Experimental Diets

In 42 day feeding trial, one day old broilers were allocated to 4 experimental groups with 4 replicates (22/ pen): control, group 1 (5% DDGS), group 2 (10% DDGS) and group 3 (15% DDGS). The component of corn DDGS (CP Animal Feed Industry, Bursa, Karacabey, Turkey) were dry matter (DM) 89.77%, crude protein (CP) 23.70%, crude fiber 6.32%, ether extract 11.45%, crude ash 4.88% and ME 2310 kcal/kg. The diets based on corn and soybean meal and fed as mash throughout the experiment. The ME and CP levels of the diets from 0-14 d of age 3050 kcal/kg and 22%, 15-35 d of age 3150 kcal/kg and 20% and 36-42 d of age 3200 kcal/kg and 18%, respectively. The rations are formulated to meet NRC [13] nutrient requirements. All experimental rations were maintained isocaloric and isonitrogenous. The compositions of the basal rations are shown *Table 1*.

Table 1. *Composition and calculated analysis of experimental diets*
Tablo 1. *Deneme rasyonlarının bileşimi ve hesaplanan analiz değerleri*

Ingredients (%)	**Days 0-14**				**Days 15- 35**				**Days 36-42**			
Corn	53.1	51	48.6	46.6	57.6	55.3	53.1	50.9	62.6	60.4	58.1	55.9
Soybean meal	40.5	37.6	35	32	35	32.3	29.5	26.7	30	27.2	24.5	21.7
DDGS	-	5	10	15	-	5	10	15	-	5	10	15
Oil	4	4	4	4	4	4	4	4	4	4	4	4
Limestone	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
DCP	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Salt	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
DL-methionine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Premix[a]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Chemical Analyses (%)												
Dry matter	91.10	91.62	91.33	91.38	90.62	90.51	90.18	90.29	94.39	94.11	94.42	94.08
Crude protein	22.06	22.02	22.08	22.09	20.00	20.03	20.02	20.02	17.97	18.18	18.03	18.01
Crude fibre	3.35	3.42	3.15	3.48	3.50	3.01	3.64	3.68	3.27	3.76	3.07	3.17
Ether extract	7.18	7.60	7.95	8.15	7.34	8.03	7.90	8.38	7.03	7.77	7.80	8.59
Crude ash	5.29	5.24	5.53	5.17	5.59	5.38	5.73	5.35	4.98	5.30	5.20	4.96
N-free extract	53.22	53.34	52.62	52.49	54.19	54.06	52.89	52.86	61.14	59.10	60.32	59.35
Metabolisable Energy kcal/kg[b]	3169.9	3141	3109.4	3081.4	3185.4	3154.7	3124.9	3095.1	3230.4	3200.6	3169.9	3140.1

[a] KAVİMİX VM 214: Vit A: 12.000.000 IU; Vit D_3: 1.500.000 IU; Vit E: 30.000 mg; Vit K_3: 5.000 mg; Vit B_1: 3.000 mg; Vit B_2: 6.000 mg; Vit B_{12}: 30 mg; Folic Acid: 750 mg; Cal. D.Panth: 10.000 mg; D Biotine: 75 mg; Choline Chloride: 375.000 mg; Nicotine Amid: 40.000 mg; Mangan: 80.000 mg; Fe: 40.000 mg; Zn: 60.000 mg; Cu: 5.000 mg; Co: 100 mg; I: 400 mg; Se: 150 mg; Antioxidant: 10.000 mg (Per 2.5 kg.), [b] Calculated (TSE 1991)

Broiler Management

Three hundred and fifty-two one day old male and female Ross- 308 chicks were obtained local hatchery where they had been vaccinated in ovo for Marek's disease and had received vaccinations for New Castle Disease and Infectious Bronchitis post hatch via a coarse spray. The treatment was set up in a completely randomized design where 22 chicks (11 male and 11 female) randomly assigned to each of four treatments with four replicates. The experiment lasted for 42 days and the chicks were fed the experimental diets throughout the experimental period. Chicks had free access to feed and water. The lighting regime was 23 h/d. The temperature was maintained at 32°C for the first week and then reduced until a temperature of 22°C was achieved by the fourth week, gradually.

Measurements

The DDGS sample and experimental rations were analyzed by the methods of AOAC [14]. The ME levels of rations were calculated according to TSE [15]. The BWG of the chicks were determined at the beginning (0) and 7, 14, 21, 28, 35 and 42th d of study. At the same time all the replicates feed residues were weekly weighed to define the feed intake (FI) levels and FCR. At the end of the study thirty-two chicks (16 male and 16 female) per treatment were randomly chosen and slaughtered for determining the carcass yield. During slaughter, individual blood samples were taken *vena subcutena ulnaris* within each treatment and collected into dry clean centrifuge tubes containing drops of heparin and centrifuged for 15 min (3.500 rpm) to obtain plasma. Then, total protein, total cholesterol and total triglycerides were determined by DDS commercial kits.

Statistical Analysis

The importance's of the difference between the mean values of groups were evaluated by analysis of variance technique. Duncan multiple range test was used determine difference between treatment groups. The statistical analyses were performed of SPSS 16.0.

RESULTS

At the beginning of the study, there was no statistically significant difference between the groups in terms of body weight (P>0.05). The average BW obtained at the end of the trial in the control and trial groups were 1763.0, 1991.1, 2102.1 and 2060.1 g respectively, and this difference between the groups was found to be statistically significant (P<0.05). The highest BWG value was found in the trial group with a supplement of 10% DDGS. The highest FI was also found to occur in the same trial group. The lowest FCR in the 14-28 day period was found in the trial group that included 5% DDGS while in the other periods it was in the trial group supplemented with 15% DDGS. Broiler performance is provided in *Table 2* and *3*.

Carcass weights were higher in the trial groups and in the control group. Warm and cold carcass weights were highest in the trial group with DDGS added at a rate of 15%. It was also determined that the addition of DDGS in different amounts had no effect on carcass yield. Broiler carcass weight performance and carcass yields are provided in *Table 4*.

Supplementing broiler rations with different amounts of DDGS had no effect (P>0.05) on the weight of the liver or heart, but the groups did vary in terms of the weight of the gizzard (P<0.05). The addition of DDGS in differing amounts created a statistically significant difference (P<0.05) between the total protein and triglyceride values in blood serum, but it did not result in a difference (P>0.05) in total cholesterol values. Broiler performance regarding internal organ weight and blood parameters is provided in *Table 5*.

DISCUSSION

The BW values obtained on day 14 of this study were lower than the values of the control group in Oryschak et al.[16] but higher than the values they reported in groups supplemented with 5% and 10% DDGS. However, it was found to be lower than values obtained in other similar studies [10,11,17,18]. Body weight values on d 28 of the trial were lower than the values obtained in the control group and groups supplemented with 5 and 10% DDGS in the study by Oryschak et al.[16]. Results for BW were lower than those reported for the control group in the study by Loar et al.[19] and higher than the BW values found in the group supplemented with 15% DDGS. The numbers obtained in the study by Min et al.[17] with a supplement of 0 and 15% DDGS were lower than the values

Table 2. *Effects of dried distillers grains with solubles on the body weight of broilers. (g)*
Tablo 2. *Kurutulmuş damıtma çözünürlü tanelerinin broylerlerin canlı ağırlığı üzerine etkisi (g)*

Days	Control (X±Sx)	Group I (X±Sx)	Group II (X±Sx)	Group III (X±Sx)	P
1	47.78±0.37 (n=88)	47.16±0.36 (n=88)	47.60±0.35 (n=88)	46.50±0.32 (n=88)	-
14	305.82±4.98^{c} (n=88)	346.39±4.97^{b} (n=88)	351.68±4.53^{b} (n=86)	372.30±4.37^{a}	*
28	922.1±1.20^{c} (n=86)	1063.5±13.20^{b} (n=88)	1099.2±11.9^{a} (n=86)	1095.4±11.4^{ab} (n=86)	*
42	1763.0±13.9^{c} (n=83)	1991.1±15.9^{b} (n=86)	2102.1±16.4^{a} (n=83)	2060.1±18.1^{a} (n=86)	*

a,b,c: *Means on the same row followed by different letters differ significantly (P<0.05),* ***-:*** *Differences among the groups were not statistically significant (P>0.05),* ***n:*** *Deneme gruplarındaki hayvan sayıları*

Table 3. *Effects of dried distillers grains with solubles on the growth performance of broilers*
Tablo 3. *Kurutulmuş damıtma çözünürlü tanelerinin broylerlerin performansı üzerine etkisi*

Days	Parameters	Control (X±Sx)	Group I (X±Sx)	Group II (X±Sx)	Group III (X±Sx)	P
1-14	FI	415.55±1.93^{c}	483.51±10.03^{a}	478.21±10.11^{a}	449.26±9.53^{b}	*
	BWG	258.04±0.36^{c}	299.23±3.83^{b}	304.31±7.61^{b}	325.79±1.92^{a}	*
	FCR	1.61±0.008^{a}	1.61±0.04^{a}	1.57±0.02^{a}	1.38±0.02^{b}	*
14-28	FI	1223.9±8.15^{d}	1302±14.77^{c}	1392.3±11.96^{a}	1345.5±8.83^{b}	*
	BWG	616.37±4.08^{c}	717.12±9.11^{b}	747.56±7.89^{b}	723.01±2.90^{a}	*
	FCR	1.98±0.01^{a}	1.81±0.006^{c}	1.86±0.005^{b}	1.86±0.006^{b}	*
28-42	FI	1758±22.47^{c}	1890.1±27.75^{b}	2020±34.9^{a}	1928±25.93^{b}	*
	BWG	841.26±5.62^{d}	927.45±7.06^{c}	1003.1±9.42^{a}	964.47±10.56^{b}	*
	FCR	2.09±0.02^{a}	2.03±0.02^{b}	2.01±0.02^{b}	2.00±0.009^{b}	*
0-42	FI	3397.4±24.9^{c}	3675.8±41.8^{b}	3890.60±31.3^{a}	3722.7±32.7^{b}	*
	BWG	1715.7±8.89^{c}	1943.7±16.3^{b}	2055±15.8^{a}	2013.3±14.9^{a}	*
	FCR	1.98±0.007^{a}	1.89±0.009^{b}	1.89±0.009^{b}	1.84±0.004^{c}	*

a,b,c,d: *Means on the same row followed by different letters differ significantly. (P<0.05),* ***FI:*** *Feed Intake (g/chick),* ***BWG:*** *Body Weight Gain (g),* ***FCR:*** *Feed Conversion Ratio*

Table 4. *Effects of dried distillers grains with solubles on the slaughter weight (g), carcass weight (g) and yield (%) of broilers (n=32)*
Tablo 4. *Kurutulmuş damıtma çözünürlü tanelerinin broylerlerde kesim ağırlığı (g), karkas ağırlığı (g) ve verimi (%) üzerine etkisi (n=32)*

Parameters	Control (X±Sx)	Group I (X±Sx)	Group II (X±Sx)	Group III (X±Sx)	P
Slaughter weight	1757.90±31.5^{c}	2052.60±39.7^{b}	2114.50±32.0^{a}	2122.40±31.5^{a}	*
Warm carcass	1272.60±25.40^{c}	1480.0±30.10^{b}	1524.90±25.10^{ab}	1534.0±25.90^{a}	*
Cold carcass	1258.50±24.90^{a}	1463.40±28.90^{b}	1501.10±23.40^{ab}	1511.10±26.10^{a}	*
Warm carcass yield	72.37±0.23	72.90±0.17	72.09±0.15	72.35±0.20	-
Cold carcass yield	71.58±0.21	71.29±0.13	70.98±0.12	71.27±0.19	-

a,b,c: *Means on the same row followed by different letters differ significantly. (P<0.05),* ***-:*** *Differences among the groups were not statistically significant (P>0.05)*

Table 5. *Effects of dried distillers grains with solubles on the internal organ weights (g/ 100 BW) and some blood parameters (g/dl) of broilers*
Tablo 5. *Kurutulmuş damıtma çözünürlü tanelerinin broylerlerde iç organ ağırlıkları (g/ 100 CA) ve bazı kan parametreleri (g/ dl) üzerine etkisi*

Parameters	Control (X±Sx)	Group I (X±Sx)	Group II (X±Sx)	Group III (X±Sx)	P
Heart	13.26±0.45	12.53±0.49	13.14±0.57	13.62±0.49	-
Liver	39.87±1.21	40.76±1.37	41.48±1.22	39.78±1.15	-
Gizzard	28.78±1.22^{b}	31.46±1.38^{ab}	33.73±1.11^{a}	29.89±0.64^{b}	*
Total cholesterol	132.08±0.59	130.60±0.61	132.05±0.65	131.23±0.63	-
Total protein	2.95±0.02^{a}	3.22±0.01^{c}	3.39±0.01^{a}	3.32±0.01^{b}	*
Total triglyceride	86.77±0.67^{a}	84.66±0.36^{b}	82.32±0.31^{c}	81.23±0.20^{c}	*

a,b,c: *Means on the same row followed by different letters differ significantly (P<0.05),* ***-:*** *Differences among the groups were not statistically significant (P>0.05)*

found in this trial. The study conducted by Shalash et al.[20] found BW values in the control group and the group supplemented with 12% DDGS which were similar to those in this study. The BW values for day 35 were lower than the values reported by Wang et al.[10] for day 35. The values found in this study were higher than the values obtained as a result of supplementing with DDGS at the same rate in the study conducted by Wang et al.[18]. The BW findings obtained in the study were lower than the values found by Wang et al.[11] in the control group and the group supplemented with 10% DDGS. The BW values at the end of the trial were lower than those obtained by Min et al.[17] on day 42. These values were also lower than the BW values in the results obtained by Wang et al.[11] in the control group and the group supplemented with 10% DDGS and results in the control group as well as the group given 15% DDGS in Wang et al.[18]. The numbers in this study were lower than the BW values reported by Oryschak et al.[16] in the control group and the groups given 5% and 10% DDGS.

The BWG values obtained in days 14-28 as a result of this study were lower than the BWG values found by Loar et al.[19] for the same period in the control group and the trial groups given a DDGS supplement of 5%, 7.5% and 15%. The BWG values on days 28-42 of the trial were similar to those reported by Shalash et al.[20] during the same period in the control group and the group fed rations including with 12% DDGS. The body weight gains obtained in this study at the

conclusion of the trial were lower than the BWG values found during the same period by Lumpkins et al.[21] in a study supplementing 0, 6, 12 and 18% DDGS.

The FI values obtained in days 1-14 in this study were lower than the FI values found by Wang et al.[18] for days 1-14. Similarly, they were lower than the values found by Wang et al.[11] in the control group and the 10% DDGS group in their study. The values obtained in this study were similar to those found by Min et al.[17]. The FI values obtained in days 14-28 of the trial were lower than the values found by Loar et al.[19] in the control group and the trial groups given a 7.5% and 15% DDGS supplement. FI values from days 28-42 were found to be lower than the FI values found by Shalash et al.[20] during a similar period in the control group and that given 12% DDGS. At the conclusion of the trial, FI values in days 0-42 were found to be lower than the values obtained by Min et al.[17] in the control group and that given a supplement of 15% DDGS. Similarly, the results of this study were lower than the numbers obtained in the control groups and trial groups of Wang et al.[18] which received a 15% DDGS supplement. The results of this study were similar to those reported by Shalash et al.[20] in the control group and those obtained from adding 12% DDGS.

The FCR for d 1-14 of the study were 1.61, 1.61, 1.57 and 1.38 for the control group and trial groups supplemented with 5, 10 and 15% DDGS. The findings in this study were higher than those of the control group in the study by Min et al.[17] and similar to the findings in the study group receiving a 15% DDGS supplement. While the numbers in this study were higher than those Wang et al.[18] found in the control group at the conclusion of their study, they were similar to the group that received 15% DDGS. The FCR values obtained in this study were found to be higher than those in the control group and the groups given 10% DDGS in Wang et al.[11]. The FCR values for days 28-42 in the study that was conducted were similar to the FCR values found by Shalash et al.[20] during the same time in both the control group and the groups given a 12% DDGS supplement. FCR results for days 0-42 were higher than those found in the control group and the trial groups given 10% DDGS in Wang et al.[11]. Similarly, the FCR values obtained by Wang et al.[18] in the control group and the trial group given 15% DDGS were higher than the results of this study. The FCR values for the study were higher than those found by Shalash et al.[20] in the control group, but similar to the FCR values they found as a result of supplementing with 12% DDGS.

The reason for the differences seen at different times as a result of adding varying amounts of DDGS to broiler rations could be due to the composition of the ration, differences in the method used to obtain the DDGS added to the ration, or a difference in the composition of nutrients.

Slaughter weights in the study were higher than the live finishing weights reported by Lu and Chen [12] in their control group and the groups given 10% DDGS. The hot carcass values obtained in the trial were similar to those in the control group in the study by Lu and Chen [12] but higher than those obtained in the group supplemented with 15% DDGS. The numbers in this study were lower than those reported by Lumpkins et al.[21] in their control group and the trial groups given DDGS supplements of 6, 12 and 18%.

The hot carcass yields obtained in the study's control group and the trial groups that included 5, 10 and 15% DDGS were found to be lower than the yields that Min et al.[17] found in their control group and the trial group supplemented with 15% DDGS. The hot carcass yields reported by Wang et al.[11] in their control group and that which received 10% DDGS supplements were higher than the hot carcass yield results obtained in this study.

Liver weights from this study's control group and the trial groups supplemented with 5, 10 and 15 %DDGS were higher than those reported by Loar et al.[19] in their control group and trial groups receiving 7.5% and 15% DDGS. The liver weights reported by Shalash et al.[20] in the control group and trial group given a DDGS supplement of 12% were found to be lower than the results in this study. The values obtained in this study were higher than the liver weights reported by Lu and Chen [12] in their control group and the research groups given 10% DDGS. The heart weights obtained in the study's control and trial groups, on the other hand, were higher than those found by Shalash et al.[20] in their control group and the research group given 12% DDGS.

In this study, total blood cholesterol values in the control group and the trial groups receiving 5, 10 and 15% DDGS were 132.08, 130.6, 132.05 and 131.23 mg/dl respectively. The results of this experiment were higher than the values found by Shalash et al.[20] in their control group, but lower than the values reported for the research group supplemented with 12% DDGS. Total blood cholesterol values found in the trial were lower than the total blood cholesterol values reported by Awad et al.[22] as a result of supplementing with DDGS (0, 6, 12 and 18%) in ducks.

Total blood protein values in the study were lower than those found by Lu and Chen [12] in their control group and the research group given a 10% DDGS supplement. The total blood protein values found by Awad et al.[22] in trial groups where duck rations were supplemented with 0, 6, 12 and 18% DDGS were higher than the total protein values in this study.

The results for total triglycerides in samples of blood serum taken from broilers in the control group and those fed rations that included 5, 10 and 15% DDGS were higher than the total triglycerides in blood serum found by Lu and Chen [12] in their control group and trial groups given a 10% DDGS supplement. The values obtained in the study were lower than the total triglyceride values found in the blood serum of ducks fed a ration supplemented with 0, 6, 12 and 18% DDGS by Awad et al.[22].

It was concluded that supplementing broiler rations with up to 15% DDGS does not have a negative effect on performance, carcass yield or blood parameters and that it can be safely used in broiler rations.

REFERENCES

1. AAFCO: Official Publication of the Association of American Feed Control Officials, Inc. Oxford, IN, 2002.

2. Batal A, Dale, NM: Mineral composition of distillers dried grains with solubles. *J Appl Poult Res*, 12, 400- 403, 2003.

3. Noll SE, Parsons CM, Dozier III WA: Formulating poultry diets with DDGS- How far can go. *Proceeding 5th Mid Atlantic Nutrition Conference.* **In,** Zimmerman NG (Ed). Univ. Maryland, College Park, 2007.

4. Parsons CM, Baker DH, Harter JM: Distillers dried grains with solubles as a protein source fort he chick. *Poult Sci*, 62, 2445-2451, 1983.

5. Alenier JC, Combs GF: Effects of feed palatability of ingredients believed to contain unidentified growth factors of poultry. *Poult Sci,* 60, 215-224, 1981.

6. Runnels TD: The biological nutrient availability of corn distillers grains with solubles in broiler feeds. *Proc Dist Feed Res Counc,* 21, 11-15, 1966.

7. Noll SL, Abe C, Brannon J: Nutrient composition of corn distillers dried grains with solubles. *Poult Sci*, 82 (Suppl. 1): 71, 2003.

8. Waldroup PW, Owen JA, Ramsey BE, Whelchel DL: The use of high levels of distillers dried grains with solubles in broiler diets. *Poult Sci,* 60, 1479-1484, 1981.

9. Dale N, Batal A: Nutritional value of distillers dried grains and solubles for poultry. **In,** *19th Annual Carolina Nutrition Conference*, Research Triangle Park, NC, pp. 1-6, 2003.

10. Wang Z, Cerrate S, Coto C, Yan F, Waldroup PW: Use of constant or increasing levels of distillers dried grains with solubles (DDGS) in broiler diets. *Int J Poult Sci*, 6 (7): 501-507, 2007.

11. Wang Z, Cerrate S, Coto C, Yan F, Waldroup PW: Evaluation of high levels of distillers dried grains with solubles (DDGS) in broiler diets. *Int J Poult Sci,* 7 (10): 990-996, 2008.

12. Lu JJ, Chen YK: Effects of feeding diets containing U.S. corn distillers dried grains with solubles on growth performance and carcass quality of domestic colored broiler chickens in Taiwan. Department of Animal Science, National Chia- Yi University and AGAPE Nutrition Consultant, Taiwan, 2005.

13. NRC: Nutrient Requirements of Poultry. 9th Revised ed., National Academy Press, Washington, DC, 1994.

14. AOAC: Official Methods of Analysis of the Association of Official Analytical Chemist. 16 th ed., Arlington, VA. 1996.

15. TSE: Hayvan yemleri, metabolik (çevrilebilir) enerji tayini (Kimyasal metot). TSE No: 9610, 1991.

16. Oryschak M, Korver D, Zuidhof M, Meng X, Beltranena E: Comparative feeding value of extruded and nonextruded wheat and corn distillers dried grains with solubles for broilers. *Poult Sci,* 89, 2183- 2196, 2010.

17. Min YN, Liu FZ, Wang Z, Coto C, Cerrate S, Costa FP, Yan F, Waldroup PW: Evaluation of distillers dried grains with solubles in combination with glycerin in broiler diets. *Int J Poult Sci,* 7 (7): 646- 654, 2008.

18. Wang Z, Cerrate S, Coto C, Yan F, Waldroup PW: Effect of rapid and multiple changes in level of distillers dried grain with solubles (DDGS) in broiler diets on performance and carcass characteristics *Int J Poult Sci,* 6 (10): 725- 731. 2007.

19. Loar RE, Moritz S, Donaldson JR, Corzo A: Effect of feeding distillers dried grains with solubles to broilers from 0 to 28 days posthatch on broiler performance, feed manufacturing efficiency and selected interstinal characteristics. *Poult Sci,* 89, 2242- 2250, 2010.

20. Shalash SMM, Ali MN, Sayed MAM, El- Gabry HE, Shabaan M: Novel method for improving the utilization of corn dried distillers grains with solubles in broiler diets. *Int J Poult Sci,* 8 (6): 545-552, 2009.

21. Lumpkins BS, Batal AB, Dale NM: Evalution of distillers dried grains with solubles as a feed ingredient for broilers. *Poult Sci,* 83, 1891-1896, 2004.

22. Awad AL, Hussein MAA, Ghonim AIA, Kasim MG: Effect of dietary inclusion level of distillers dried grains with solubles on growth performance of omyati ducklings. Anim. Prod. Institue, Agric. Res. Center, Giza, 2011.

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RESEARCH ARTICLE

Türk Toplumunun Hayvan Hakları Kavramına Yaklaşımının Belirlenmesine Yönelik Bir Araştırma: I. Demografik Özelliklere Göre Tutum Analizi [1]

Türel ÖZKUL * Taner SARIBAŞ ** Ender UZABACI *** Erhan YÜKSEL ****

[1] *Bu çalışma TÜBİTAK tarafından (Proje No: SOBAG- 108 K 192) desteklenmiştir*

* Uludağ Üniversitesi, Veteriner Fakültesi, Veteriner Hekimliği Tarihi ve Deontoloji Anabilim Dalı, TR-16059 Görükle Kampüsü, Bursa - TÜRKİYE
** Ümitköy Pet Hospital, TR-06810 Çayyolu, Ankara - TÜRKİYE
*** Uludağ Üniversitesi Veteriner Fakültesi, Biyoistatistik Anabilim Dalı, TR-16059 Görükle Kampüsü, Bursa - TÜRKİYE
**** Erciyes Üniversitesi Veteriner Fakültesi , Veteriner Hekimliği Tarihi ve Deontoloji Anabilim Dalı, TR- 38039 Melikgazi, Kayseri - TÜRKİYE

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Özet

Bu çalışma, Türk toplumunun hayvan hakları konusunda bilinç düzeyi ve algısına yönelik saptamalar yapmak amacıyla gerçekleştirildi. Bu amaçla hazırlanan anket, Türkiye'nin coğrafi bölgelerini temsil eden yedi ilde değişik meslek, yaş ve cinsiyet gruplarından 2016 kişiye yüz yüze görüşme tekniği ile uygulandı. Anket verilerinin SPSS istatistik programı ile analizi sonucunda: büyükşehirlerde yaşayanların ($P<0.001$), kırk yaşın üstünde olanların ($P=0.002$), aylık gelirleri 1.000 TL'nin üzerinde olanların ($P=0.015$), bayanların ($P<0.001$) ve öğretmenlerin ($P<0.001$), diğer demografik gruplara kıyasla, hayvan hakları konusunda daha pozitif tutum sergiledikleri; hayvan hakları konusundaki tutum ile eğitim düzeyi arasında pozitif bir ilişki olduğu gözlendi ($P=0.005$).

Anahtar sözcükler: *Türk toplumu, Hayvan hakları, Tutum, Demografik özellikler*

A Survey to Identify the Turkish People's Approach on Animal Rights Concept: I. Attitude Analysis by Demographic Traits

Summary

The present study was undertaken to determine the Turkish people's cognitive level and perception on the concept of animal rights. For this aim, a sample of totally 2016 individuals was created from different groups of profession, age and gender. A questionnaire was developed for collecting data and was applied to participants in seven provinces representing geographical regions of Turkey. The survey was applied by interviewing with 2016 participants individually. The data obtained from questionnaire were analysed statistically and the results are given below: Those living in metropolitan ($P<0.001$), and over the age of forty ($P=0.002$), with incomes over 1.000 Turkish Liras per month ($P= 0.015$), women ($P<0.001$) and teachers ($P<0.001$) are showed more positive attitude towards animal rights than the other demographic groups. Positive attitude towards animals is also increased with the increase in educational level ($P=0.005$).

Keywords: *Turkish people, Animal rights, Attitude, Demographic traits*

GİRİŞ

Hayvan-bitki ve insan arasındaki simbiyotik ilişki çerçevesinde, canlılara-hayvanlara haklar tanınması ve onların korunmasına yönelik hareketlerin kökleri Antik Çağ'a kadar uzanmaktadır. Hayvan haklarının pozitif hukuka yansıtılma çabalarının en önemli göstergesi, hayvanları koruma yasaları olmuştur. Osmanlı Devletinde ve Batı Ülkelerinde hayvanları korumaya yönelik gerçekleştirilen bir takım öncü yasal düzenlemeler yanında ilk müstakil Hayvanları Koruma Yasası 1822 yılında İngiltere'de çıkartılmıştır. Hayvanları koruma hareketinin sosyal bir hareket olarak karşımıza çıkması ise

İletişim (Correspondence)
+90 224 2941371
turelozkul@uludag.edu.tr

yine İngiltere'de, 19. yüzyılın başlarında gerçekleşmiştir. İlk hayvan hakları hareketi "antiviviseksiyonist" hareket olarak İngiltere'de olgunlaşmış ve 1960'larda geniş kitlelerin desteğini almaya başlamıştır. Richard Ryder, 1970'li yıllarda "*türcülük*" terimini bulmuş, 1972 yılından itibaren hayvan özgürleşmesi hareketi faaliyete geçmiştir. 1980'lerin ilk yıllarında *PETA* "*People for the Ethical Treatment of Animals* (Hayvanlara Etik Muamele İçin Mücadele Edenler)", FARM "*Farm Animal Reform Movement*" (Çiftlik Hayvanları Reform Hareketi) gibi hayvanları korumaya yönelik önemli organizasyonlar ortaya çıkmaya başlamış ve günümüze gelene kadar tüm dünyada bu tür organizasyonların sayıları ve etkinlikleri giderek artmıştır [1-4]. UNESCO Evinde, 15 Ekim 1978 tarihinde "*Hayvan Hakları Evrensel Bildirgesi*" resmen ilan edilerek; haklarını savunamayan hayvanlara bir takım haklar tanınmıştır. İdeal olmamakla ve yeterince globalize olmamakla birlikte, olumlanabilecek yönleri de bulunan bu haklar; gelişmiş ülkelerde yasal statüye kavuşturulmuş ve hayvan haklarına yönelik kanunlar çıkartılmıştır [5,6].

Osmanlıda, hayvanları koruma hareketine ilişkin düzenlemeleri; İkinci Beyazıd döneminde hazırlanan Bursa, İstanbul ve Edirne İhtisab Kanunlarında ve daha sonraki dönemlerde hazırlanan fermannameler ile Belediye Kanunlarında görmek mümkünse de sahipsiz sokak hayvanlarını korumaya yönelik geniş kapsamlı ilk koruma yasası ancak Cumhuriyet Döneminin yakın zamanlarında, 2004 yılında çıkartılabilmiştir [7]. Avrupa Birliği uyum süreci kapsamında, hayvan hakları ve hayvan refahına yönelik yasal altyapı oluşturmaya başlanarak uygulamaya dönük gelişmeler kaydedilmiş; çiftlik ve deney hayvanlarını ilgilendiren yönetmelikler çıkartılmış, sivil toplumu bilinçlendirmek amacıyla dernekler kurulmuştur [8]. Ancak tüm bu gelişmelere rağmen, çıkartılan yasaların esas uygulayıcıları olması beklenen toplumun, konuya bakışına ve bilinçlilik düzeyine yönelik kapsamlı bir çalışma saptanamamış, bazı anket çalışmalarında [9-11] konuyla dolaylı olarak bağlantılı kısımlar belirlenebilmiştir. Bu durum, hayvan hakları konusunda Türk toplumuna yönelik ve özellikle uygulamaya dönük bir çalışma yapılması gerekliliğini ortaya koymaktadır. Bu çalışma ile küresel güncel olan bu konuda Türk toplumunun tutum ve yaklaşımları ortaya konularak uluslararası verilerle karşılaştırma yapmak amaçlanmaktadır.

MATERYAL VE METOT

Bu çalışmanın evrenini, son (2007) nüfus sayımı verilerine [12] göre, 20 yaş üstü 45.786.832 Türkiye Cumhuriyeti vatandaşı oluşturdu. Örneklem büyüklüğü, %95 güven düzeyini [13] sağlaması hedeflenen 2016 katılımcıdan oluşturuldu. Araştırmada veri toplama gereci olarak kullanılan anket formu, Wuensch ve ark.[14], Halis Yerlikaya ve ark.[11] ile Sharp ve ark.[15] tarafından yürütülen çalışmalardan yararlanarak geliştirildi. Anket, katılımcıların demografik özelliklerini belirlemek amacı ile toplam 6 adet kapalı uçlu soru ve katılımcıların hayvan hakları kavramına yönelik düşüncelerini belirlemeye yönelik 7'li Likert ölçeğiyle hazırlanmış 59 yargı içeren bir tutum ölçeğinden oluştu. Anketin güvenilirliği Cronbach Alfa katsayısı ile hesaplandı ve 0.928 değerine ulaşıldı.

Anketlerin uygulanacağı iller, Türkiye'nin yedi coğrafi bölgesini temsil edecek şekilde ve temsil yetenekleri dikkate alınarak; Bursa (Marmara Bölgesi, 14.211.086 kişi), Samsun (Karadeniz Bölgesi, 4.945.865 kişi), Antalya (Akdeniz Bölgesi, 5.692.760 kişi), İzmir (Ege Bölgesi, 6.552.066 kişi), Elazığ (Doğu Anadolu Bölgesi, 3.318.621 kişi), Urfa (Güneydoğu Anadolu Bölgesi, 3.278.965 kişi) ve Ankara (İç Anadolu Bölgesi, 7.787.469 kişi) olarak belirlendi [12]. Örneklem grubu, coğrafi bölgelerin 20 yaş üstü nüfus büyüklüğünün evren içerisindeki oranı dikkate alınarak orantılı-tabakalı örnekleme tekniği ile belirlendi. Bu orana göre değişik meslek, yaş ve cinsiyet gruplarından olmak üzere, Ankara'dan 340, Bursa'dan 625, Samsun'dan 220, Antalya'dan 253, İzmir'den 288, Elazığ'dan 147 ve Urfa'dan 143 katılımcı örnekleme alındı ve örneklemin oluşturulmasında tesadüfi örnekleme yöntemi uygulandı.

Yüz yüze görüşme yöntemiyle yürütülen çalışma sonucunda toplam 2016 kişiyle anket yapılarak, elde edilen veriler SPSS 13.0 for Windows (Chicago, IL) istatistik paket programı ile analiz edildi. Bağımsız değişkenleri belirlemeye yönelik sorular ile katılımcıların hayvan haklarına ilişkin tutumlarına yönelik sorularda sıklık (frekans) değerleri bulundu. Anketin uygulama tekniğinden ya da bazı katılımcıların bazı soruları yanıtlama isteksizliğinden kaynaklanan kayıp verilerin dağılım oranları üzerindeki olası yanıltıcı etkisini ortadan kaldırmak için frekanslar ve yüzdeler yerine, geçerli frekanslar ve geçerli yüzdeler kullanıldı. Sözü edilen frekans ve yüzde değerleri metin içerisinde tablolar halinde sunuldu.

Hayvan haklarına yönelik sergilenen tutum düzeyleri için elde edilen puanların karşılaştırılmalarında, ikiden fazla alt gruplu değişkenler için Kruskal Wallis Testi uygulandı. İki alt grubu bulunan değişkenlerle ilgili karşılaştırılmalar için ise Mann Whitney U Testi yapıldı. Tüm analizlerde anlamlılık düzeyi $P<0.05$ olarak kabul edildi.

Anket uygulamaları, 2009 Eylül - 2010 Mart tarihleri arasında gerçekleştirildi.

BULGULAR

Yaş dağılımı açısından en büyük grubu 20-30 yaş (%47.5) aralığındaki katılımcılar, en küçük grubu ise 60 ve üstü yaş (%2.3) aralığındaki katılımcılar oluşturdu. Yaş gruplarına göre tutum ölçeğinden elde edilen puanların ortalamaları karşılaştırıldığında 41-50, 51-60 ve 61 ve üstü yaş grubundaki bireylerin diğer gruplara göre daha pozitif bir tutum ($P=0.002$) sergiledikleri belirlendi *(Tablo 1)*.

Katılımcıların cinsiyet dağılımına bakıldığında %58.8'inin erkeklerden oluştuğu görüldü. Cinsiyet değişkenine göre tutum değerlendirilmesinde bayanların erkeklere oranla daha pozitif tutum sergiledikleri ($P<0.001$) gözlendi *(Tablo 1)*.

Katılımcılar eğitim düzeyi açısından sınıflandırıldığında en büyük grubu üniversite mezunlarının (%50.7) oluştur-

Tablo 1. *Katılımcıların bazı demografik özelliklere (yaş, cinsiyet, eğitim düzeyi, en uzun süre yaşanılan yerin niteliği, meslek ve gelir düzeyi) göre dağılımı ve tutum değerlendirmesi*

Table 1. *Distribution of participants according to some demographic traits (age, gender, education level, feature of living place for longest time, occupation, income level) and assessment of attitudes*

Demografik Özellikler	Parametre	n	%	X±Sx	Medyan	P	Minimum Değer	Maksimum Değer
Yaş	20-30	912	47.5	4.18±0.77	4.18	0.002	0.08	6.46
	31-40	530	27.6	4.15±0.80	4.14		0.00	6.69
	41-50	304	15.8	4.33±0.82	4.35		1.37	6.90
	51-60	132	6.9	4.36±0.72	4.32		2.53	5.95
	61 ve üstü	44	2.3	4.34±0.68	4.35		2.15	6.90
Cinsiyet	Bayan	827	41.2	4.41±0.74	4.40	<0.001	1.59	6.90
	Erkek	1180	58.8	4.10±0.78	4.10		0.00	6.46
Eğitim düzeyi	Okuryazar değil	3	0.2	3.75±0.53	3.54	0.005	3.36	4.36
	Okuryazar	7	0.4	4.02±0.52	4.18		3.19	4.59
	İlkokul	141	7.1	4.07±0.63	4.10		2.37	5.76
	Ortaokul	164	8.3	4.00±0.93	4.20		0.08	6.66
	Lise	497	25.1	4.16±0.85	4.22		0.00	6.69
	Üniversite	1006	50.7	4.26±0.72	4.34		1.83	6.90
	Lisansüstü	166	8.4	4.37±0.82	4.22		1.10	6.46
En uzun süre yaşanılan yerin niteliği	Köy	135	6.8	3.97±0.71	3.96	<0.001	1.10	5.76
	Kasaba	280	14.0	4.16±0.70	4.11		2.34	6.46
	Küçük Bir İl	254	12.7	3.96±0.71	4.02		1.37	5.69
	Büyükşehir	1326	66.4	4.29±0.81	4.32		0.00	6.90
	Diğer*	2	0.1	3.69±0.83	3.69		3.10	4.29
Meslek	Sağlık personeli	202	10.2	4.30±0.81	4.35	<0.001	1.10	6.36
	Mühendis	154	7.8	4.09±0.72	4.04		2.14	6.24
	Öğretmen	189	9.6	4.38±0.73	4.33		2.15	6.41
	İşçi	270	13.7	4.00±0.97	4.13		0.00	6.41
	Çiftçi	114	5.8	4.08±0.57	4.01		2.81	5.76
	Hukukçu	80	4.1	4.23±0.76	4.20		2.39	6.90
	Memur	199	10.1	4.28±0.71	4.27		2.19	6.58
	Serbest Meslek	374	18.9	4.14±0.79	4.18		1.51	6.69
	Öğrenci	253	12.8	4.23±0.73	4.15		1.80	6.41
	Diğer**	140	7.1	4.47±0.60	4.49		2.80	6.66
Gelir Düzeyi	500 ve altı	27	1.5	4.00±0.46	3.93	0.015	2.95	5.08
	501-1000	321	17.4	4.08±0.82	4.16		0.00	6.66
	1001-2000	676	36.7	4.25±0.73	4.25		1.51	6.41
	2001-3000	414	22.5	4.21±0.77	4.18		1.80	6.69
	3001-4000	170	9.2	4.21±0.77	4.22		1.92	6.37
	4001 ve üstü	236	12.8	4.27±0.97	4.30		1.10	6.90

n: *Frekans;* ***X±Sx:*** *Ortalama ± Standart hata; * Ada; ** Arkeolog, Bankacı (15), Bilgi İşlemci, Bilişim Teknolojileri Uzmanı, Ekonometri, Ekonomist (2), Ev Hanımı (53), Futbolcu, Finasman Uzmanı (2), Haberleşme, Hostes, İktisatçı (3), İşletmeci (6), Kimyacı, Mimar (2), Muhasebeci (7), Müzisyen (3), Özel Güvenlik (2), Psikolog, Radyo-Tv, Satış Yöneticisi, Sekreter (5), Sosyolog, Sporcu, Tasarımcı (2), Tiyatro Yönetmeni (2), Turizm (22), Uluslararası İlişkiler Uzmanı (2)*

duğu tespit edildi. Eğitim düzeyine göre yapılan tutum değerlendirmesinde pozitif tutum sergileyenlerden ilk üç sırayı lisansüstü, üniversite ve lise eğitimi alanlar (P=0.005) oluştururken, bu grupları sırasıyla; ilkokul mezunları, okuryazarlar, ortaokul mezunları ve okuryazar olmayanlar izledi *(Tablo 1)*.

En uzun süre yaşanılan yerin niteliği parametresine göre katılımcıların %66.4'ünün "büyükşehir"de yaşadığı, pozitif tutum sergileyenlerin ise sırasıyla; büyükşehir, kasaba, köy ve küçük bir ilde yaşayanlar şeklinde olduğu belirlendi (P<0.001) *(Tablo 1)*.

Serbest meslek mensupları (%18.9), işçiler (%13.7) ile sağlık personelinin (%10.2) en yüksek katılımı gösterdiği çalışmada, katılımcıların mesleklerine göre yapılan tutum değerlendirilmesinde *"diğer"* şıkkını işaretleyenlerin, öğretmenlerin ve sağlık personelinin tutum yönünden en yüksek düzeyde pozitif tutum sergiledikleri; çiftçi, mühendis ve işçilerin ise negatif tutum sergiledikleri gözlendi (P<0.001). *"Diğer"* şıkkını işaretleyenler arasında sayıca en yüksek grubu ev hanımları oluşturdu *(Tablo 1)*.

Katılımcıların gelir düzeyleri ile sergiledikleri tutum ilişkisi değerlendirildiğinde, yüksek gelir grubuna sahip olanların hayvan haklarına yönelik daha pozitif tutum sergiledikleri belirlendi (P=0.015) *(Tablo 1)*.

TARTIŞMA ve SONUÇ

Araştırmanın örneklem dağılımına bakıldığında, katılımcıların %75'lik kısmının 20-40 yaşlar arasındaki gruplara dâhil olduğu; yüzde 84'ünün lise ve üstü eğitim düzeyine sahip olduğu; yüzde 78'inin ise kentlerde yaşadığı anlaşılmaktadır. Bu dağılım çalışmanın örnekleminin kent merkezlerinden alındığını ve anketin daha çok üniversite yerleşkelerine yakın yerlerde uygulandığını düşündürebilir. Bu durum, çalışma sonuçlarından eğitim ve il parametreleri ile ilgili değerlendirmeleri etkileme olasılığını ortaya çıkarmaktadır.

Araştırma verileri, yaş değişkeni açısından incelendiğinde, kırk yaşın üstündekilerin altındakilere göre daha pozitif tutum sergilediği görülmektedir *(Tablo 1)*. Yurt dışında konuyla ilişkili yapılan çalışmalarda [16-21] gençlerin hayvan haklarına karşı daha duyarlı olduğu ortaya konulmuş ancak bu çalışmanın verileri ile uyum yakalanamamıştır. Bu farklılığın, ileri yaşta olanların daha genç olanlardan daha yüksek bir gelire sahip olduğu ön kabulüyle ve gelir düzeyi artışı ile katılımcıların hayvan haklarına yönelik sergiledikleri pozitif tutum arasındaki doğru orantıyla açıklanabileceği düşünülebilir.

Bayan katılımcıların erkek katılımcılara oranla hayvan hakları konusunda daha pozitif tutum sergilemesi *(Tablo 1)*, bu konu çerçevesinde yürütülen benzer çalışmaların sonuçlarıyla [22-26] uyum göstermektedir.

Lisansüstü, üniversite ve lise eğitimi alanların; ilkokul mezunlarına, okur yazarlara, ortaokul mezunlarına ve okur yazar olmayanlara göre daha pozitif tutum sergilemesi *(Tablo 1)*, İtalya'da tüketicilerin hayvan refahı ve gıda seçimlerine yönelik yürütülen çalışmanın [27] sonuçlarıyla paralellik göstermektedir. Söz konusu çalışmada, resmi eğitim düzeyi arttıkça, hayvanların yaşam koşullarına, çektikleri acıya ve psikolojik gereksinimlerine yönelik ilginin de arttığı belirtilmiştir. Konuya yer verilen diğer bir çalışmada [16] da, üniversite mezunlarının, hayvan haklarını destekleyenler arasında toplumun geneline göre daha fazla yer aldığı, eğitim düzeyi arttıkça hayvan haklarına yönelik farkındalık seviyesinin ve desteğin arttığı vurgulanmaktadır. Eğitim düzeyinin artışıyla hayvanlara yönelik duyarlılığın arttığının ortaya konulduğu bu ve benzeri çalışmaların yanı sıra anket sorularının eğitim düzeyi düşük kişiler tarafından tam olarak anlaşılamamış olması olasılığı da değerlendirilmelidir.

Büyükşehirde yaşayanların, kasaba ve köyde yaşayanlara göre hayvan hakları konusunda daha pozitif bir tutum sergilemesi *(Tablo 1)*, hayvan hakları kavramının şehir kökenli yaygınlaştığını, şehirlerde yaşayanların kırsal alanlarda yaşayanlara göre konuya daha duyarlı yaklaştığını, bu duyarlılığın şehirlerde yaşayanların kırsal bölgelerde yaşayanlara göre içinde hayvanları da barındıran doğal çevreden yeterince faydalanamamasından kaynaklandığını ortaya koyan [16,28] çalışmalara ait bulguları desteklemektedir. Ayrıca, bu durum eğitim parametresine ilişkin sonuçlarla beraber ele alınarak ve büyükşehirlerdeki eğitimli nüfusun fazlalılığı göz önünde tutularak da açıklanabilir. Rollin [29], İkinci Dünya Savaşının ardından yaşanan sanayileşmeye ve kırsal alanlardan şehirlere doğru yaşanan göçe bağlı olarak büyük geniş ailelerin yerini çekirdek ailelere bıraktığını, bu ve benzeri nedenlerle sosyal olarak yalnızlaşan bireylerin arkadaş hayvanlara olan ilgisinin arttığını ifade etmektedir. Bu ifadeler de büyükşehirde yaşayanların daha pozitif tutum sergilemesini açıklar nitelik taşımaktadır.

Meslek gruplarına göre yapılan değerlendirmede *"diğer"* şıkkını işaretleyenler, öğretmenler ve sağlık personeli tutum yönünden en pozitif tutum sergileyen grubu oluştururken; çiftçi, mühendis ve işçiler negatif tutum sergileyen grubu oluşturmaktadır *(Tablo 1)*. Yurt dışında demografik değerlerin hayvanlara yönelik tutumlara etkisini belirlemek amacıyla yürütülen bir çalışmada [26]; sağlık sektöründe çalışanlar ile eğitim sektöründe çalışanların pozitif tutum sergileyenler arasında ilk üç sırayı oluşturan gruplar arasında yer alması bu araştırmanın sonuçları ile uyum göstermektedir. Gelir düzeyi bakımından daha pozitif tutum göstermesi beklenen mühendislerin tutumu anlaşılamamaktadır.

Katılımcıların gelir düzeyleri ile sergiledikleri tutum ilişkisi değerlendirildiğinde, yüksek gelir grubuna sahip olanların hayvan haklarına yönelik daha pozitif tutum sergiledikleri belirlendi *(Tablo 1)*. Gelir düzeyi ile hayvan haklarını destekleme eğilimi arasında pozitif bir bağ olduğunu ifade eden çalışmalarda [16,30], bu durumun temel yaşam gereksinimlerini karşılayan insanların hayatlarındaki estetik unsurlara yönelik daha istekli ve olumlu tutum sergilemelerinden kaynaklandığının altı çizilmektedir. Bu bulguların yanı sıra gelir düzeyi en yüksek kategoride olanların daha olumsuz, en düşük kategoride olanların daha olumlu bir yaklaşım sergilediği bir çalışma [26] da bulunmaktadır.

Sonuç olarak, araştırma verileriyle; eğitim düzeyi arttıkça hayvanlara ve haklarına yönelik gösterilen pozitif tutumun arttığı, meslek gruplarından öğretmenlerin ve diğer meslekler grubunda yer alanların en yüksek pozitif tutumu sergilediği ortaya konulmuştur. Diğer meslek grupları içerisinde ev hanımlarının sayıca en büyük grubu oluşturduğu noktasından hareketle, ev hanımları tarafından okul öncesi ve öğretmenler tarafından okul döneminde hayvan hakları

konusunun bilinçli bir şekilde işlenmesi ile Türkiye'de hayvan hakları kavramının doğru temeller üzerine yapılandırılacağı söylenebilir.

KAYNAKLAR

1. Anonim: Animal Rights Movement. **In,** Bekoff M, Meaney CA (Eds): Encyclopedia of Animal Rights and Animal Welfare. 2nd ed., pp. 50-53, Greenwood Press, 1998.

2. Ertan B: Canlı Hakları: Çevrebilim Açısından. *Doktora Tezi,* Ankara Üniv. Sosyal Bil. Enst., 1998.

3. Singer P: Hayvan Özgürleşmesi. (Çev.: Hayrullah Doğan). Ayrıntı Yayınları, İstanbul, 2002.

4. Sungurbey İ: Hayvan Hakları: Bir İnsanlık Kitabı. İstanbul Üniversitesi Hukuk Fakültesi Yayınları, İstanbul, 1993.

5. Başağaç RT, **Öztürk R, Koluman A:** Hayvan-İnsan Sözleşmesi Üzerine. *II. Ulusal Tıbbi Etik Kongresi*, s. 97-104, Kapadokya, 18-20 Ekim 2001.

6. Regenstein L: Animal Rights, Endangered Species and Human Survival. **In,** Sınger P (Eds): In Defens of Animals. pp. 118-132, Harper&Row, Publisher, New York, 1985.

7. Menteş Gürler A, Osmanağaoğlu Ş: Türkiye'de hayvanları koruma kanununun tarihsel gelişimi. *Kafkas Univ Vet Fak Derg*, 15 (3): 325-330, 2009.

8. Özkul T: AB'ye Uyum Sürecinde Türk Veteriner Hekimliğine Bir Bakış. *I. Ulusal Veteriner Hekimliği Tarihi ve Mesleki Etik Sempozyumu Bildiri Kitabı,* s. 283-304, Elazığ, 30 Mart-1 Nisan 2006.

9. Anonim: Veteriner Sağlık Sektörü Algılama, İhtiyaç ve Beklentiler Araştırması. Avrupa Birliği Uyum Sürecinde Veteriner Hekimlik ve Hayvan Sağlığı Ürünleri, Ankara, 2005.

10. Ozen A, Ozturk R, Yasar A, Armutak A, Basagac RT, Ozgur A, Seker I, Yerlikaya H: An attitude of veterinary practitioners towards animal rights in Turkey. *Veterinární Medicína*, 49 (8): 298-304, 2004.

11. Yerlikaya H, Ozen A, Yasar A, Armutak A, Ozturk R, Bayrak S, Gezman A, Şeker I: A survey of attitudes of Turkish veterinary students and educators about animal use in research. *Veterinární Medicína,* 49 (11): 413-420, 2004.

12. Anonim: Türkiye İstatistik Kurumu, Adrese Dayalı Nüfus Kayıt Sistemi, 2007 Yerleşim Yeri Nüfusları. http://tuikapp.tuik.gov.tr/adnksdagitapp/adnks.zul, *Erişim tarihi*: 14.03.2008.

13. Lake C, Harper PC: Kamuoyu Araştırmaları. (Çev.: Nurettin Güz) Altın Küre Yayınları, Ankara, 2002.

14. Wuensch KL, Jenkins KW, Poteat GM: Misanthropy, idealism and attitudes towards animals. *Anthrozoös*, 15, 139-149, 2002.

15. Sharp HW, Wuensch KL, Eppler MA, Harju BL: Narcissism, empathy and attitudes towards animals. Presented at the Spring Conference of the North Carolina Psychological Association and North Carolina Psychological Foundation, April, 2006.

16. Allen MD: Teasing out the linkage between public opinion on environmentalism and animal rights. *Annual Meeting of the Midwest Political Science Association,* Chicago, IL, 7-10 April, 2005.

17. Anonim: On the Sacrifices of the Natural Order For Human Life. http://www.trinity.edu/mkearl/critter.html, *Accessed*: 25.07.2010.

18. Fielding WJ, Samuels D, Mather J: Attitudes and actions of West Indian dog owners towards neutering their animals: A gender issue? *Anthrozoos*, 15 (3): 206-226, 2002.

19. Jamison WV, William ML: Rights of animals, perceptions of science, and political activism: Profile of American animal rights activists. *Science, Technology, and Human Values,* 17, 438-458, 1992.

20. Jerolmack C: Tracing the profile of animal rights supporters: A Preliminary investigation. *Society and Animals*, 11 (3): 245-263, 2003.

21. Lowe BM, Caryn FG: Animal rights as a post-citizenship movement. *Society and Animals*, 10 (2): 203-215, 2002.

22. Eldridge JJ, Gluck JP: Gender differences in attitudes toward animal research. *Ethics Behav*, 6, 239-256, 1996.

23. Herzog JR, Harold A, Betchart NS, Pittman RB: Gender, sex role orientation, and attitudes toward animals. *Anthrozoös*, 4, 184-191, 1991.

24. Pifer L, Shimizu K, Pifer R: Public attitudes toward animal research: Some international comparisons. *Society and Animals*, 4 (1): 37-52, 1994.

25. Pifer L: Exploring the gender gap in young adults' attitudes about animal research. *Society and Animals*, 2 (2): 95-113, 1996.

26. Signal TD, Taylor N: Attitudes to animals: Demographics within a community sample. *Society and Animals*, 14 (2): 147-157, 2006.

27. Mara M: Consumer Concerns about animal welfare and the ımpact on food choice Italian report on laddering interview, Published by University of Pisa, 2000.

28. Garner R: Political Animals. New York: St. Martin's Press, 1998.

29. Rollin BE: An Introduction to Veterinary Medical Ethics: Theory and Cases. 2nd ed., Blackwell Publishing, 2006.

30. Van Leire KD, Riley ED: The social bases of environmental concern: A review of hypotheses, explanations and empirical evidence. *Public Opinion Quarterly*, 44, 181-197, 1980.

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RESEARCH ARTICLE

Use of ELISA for Preliminary Screening of 19 Nortestosterone Anabolic Steroid in Cattle Meat in Republic of Macedonia

Risto UZUNOV * Zehra HAJRULAI-MUSLIU * Elizabeta DIMITRIEVSKA-STOJKOVIC * Biljana STOJANOVSKA-DIMZOSKA * Pavle SEKULOVSKI * Velimir STOJKOVSKI *

* Faculty of Veterinary Medicine, Sts "Cyril and Methodius" University, Lazar Pop-Trajkov 5-7, 1000, Skopje, MACEDONIA

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Summary

In recent years, hormones and hormone like substances have been recently used in livestock production to obtain a high yield performance in a shorter period of time. These anabolic agents are used to increase the weight gain, to improve the food efficiency, storing protein and to decrease fatness. However, depending on the use of anabolic agent in animal feed, anabolic residues that may occur in meat and meat products present risks to human health. The aim of this study was to detect the levels of 19 nortestosterone residues in the market cattle meat in R. Macedonia. In this study, a total of 86 samples were obtained from different markets and used as a test material. 19 nortestosterone residues were analyzed with ELISA method. The average experimental level of 19 nortestosterone in cattle meat was 375.20 ppt. The recovery was between 79.54% and 114.39%, a working range between 50 to 3.000 ppt. The regression equation of the final inhibition curve was: $y = - 0.1453 x + 1.4057$, $R^2 = 0.9972$. The levels of 19 nortestosterone residues were below the international allowable levels set by the Macedonian Residue Control Plan and the European Union. According to the results of our study on this anabolic steroid, the obtained 86 cattle meat samples are safe for human consumption. However, it is still necessary to monitor this chemical as a food quality control measure.

Keywords: *19 nortestosterone, Cattle meat, ELISA, Residues, Public health*

Makedonya Cumhuriyeti'nde Etlerde 19 Nortestosteron Anabolik Steroidinin Ön Taramasının ELISA Kullanılarak Yapılması

Özet

Son yıllarda, hormonlar ve hormon benzeri maddeler kısa bir sürede yüksek verim performansı elde etmek için hayvancılık üretiminde kullanılmaktadır. Bu anabolik ajanlar, ağırlık kazancı artırmak, gıda verimliliğini ve protein depolamayı geliştirmek ve şişmanlığı azaltmak için kullanılmaktadır. Ancak, hayvan yemi olarak anabolik ajan kullanımına bağlı olarak, et ve et ürünlerinde oluşabilecek anabolik artıkları insan sağlığı için risk teşkil etmektedir. Bu çalışmanın amacı, Makedonya Cumhuriyeti'nde piyasadaki sığır etlerinde 19 nortestosteron kalıntı seviyelerini tespit etmektir. Bu çalışmada, farklı marketlerden toplam 86 numune elde edilmiş ve test materyali olarak kullanılmıştır. 19 nortestosteron artıkları ELISA yöntemi ile analiz edildi. 19 nortestosteronun ortalama deneysel seviyesi 375.20 ppt olarak bulundu. Düzelme 50 - 3.000 ppt çalışma aralığında %79.54 ve %114.39 arasında bulundu. Nihai inhibisyonun regrasyon eşitliği: $y = - 0.1453 x + 1.4057$, $R^2 = 0.9972$ olarak belirlendi. 19 nortestosteron kalıntı düzeyleri Makedonya Kalıntı Kontrol Planı ve Avrupa Birliği tarafından belirlenen uluslararası kabul edilebilir seviyelerin altında idi. Bu anabolik steroid üzerine olan bu çalışmanın sonuçlarına göre, elde edilen 86 sığır eti örneği insan tüketimi için güvenlidir. Ancak, gıda kalite kontrol önlemi olarak bu kimyasalın izlemesi hala gereklidir.

Anahtar sözcükler: *19 nortestosteron, Sığır eti, ELISA, Kalıntılar, Halk sağlığı*

GİRİŞ

Meat is one of the most important constituents of human diet as it provides proteins, energy, vitamins and minerals [1]. However, meat could also become a source of health hazards if it contains harmful material such as toxins, residues or chemical agents. Residues in meat may result from many sources such as animal drugs used to prevent or treat diseases

İletişim (Correspondence)
+389 23 240745
risteuzunov@fvm.ukim.edu.mk, zhajrulai@fvm.ukim.edu.mk

or to promote growth, pesticides, feed and agricultural or industrial chemicals [2]. 19 Nortestosterone, also called nandrolone (NT), (17β-hydroxyestra-4-en-3-one or $C_{18}H_{26}O_2$ (*Fig. 1* - structural formulas), MW = 274.40 Da), one of the most powerful anabolic steroids, has been widely used in veterinary medicine as well as human medicine for treatment of protein deficiency diseases, osteoporosis and male contraception. 19 nortestosterone has also been employed as a growth-promoting agent to accelerate weight gain, to improve feeding efficiency in meat producing animals and as a doping agent to boost muscular strength and performance in sports and horse racing [3]. The side effects of these substances, which include increased risk of coronary heart disease and hepatic carcinogenicity, are related to their androgenic and/or anabolic properties [4]. 19 nortestosterone and its metabolites in meat also produce some other important adverse effects, such as cardiomyopathy, coronary artery disease, peliosis hepatitis, hepatic carcinogenicity, cholestasis, hypoproteinemia, adrenal atrophy, cerebral dysfunction, and emotional instability (mood swings, aggressiveness depression, psychosis addiction etc.), testicular shrinkage, sperm count and sperm motility, alterations in sperm morphology, decreased semen production, infertility, prostatic hypertrophy, prostatic carcinoma, gynecomastia, menstrual cycle disorders, masculinization, deepening of the voice, shrinkage of the breasts, male-pattern baldness and an increase in sex drive, acne, body hair and clitoris size, uterine atrophy, breast atrophy [5-8]. As their possible harmful effects result from the intake of hormone residues and their metabolites, usage of growth-promoting drugs for fattening livestock have been banned in many countries. However, illegal use of 19-nortestosterone as a growth promoter has been widely reported in many countries. Thus, it is necessary to control 19-nortestosterone's abuse [3]. As in most countries and in EU use of 19 nortestosterone is banned and no residues of these substances are allowed in meat products. Therefore, any cattle kept for export to the EU must be shown to be free of these substances [9-11]. In addition monitoring hormonal residues of growth-promoters in animal materials is essential to enforce this ban and to protect public health against the harmful effects of these substances in food products.

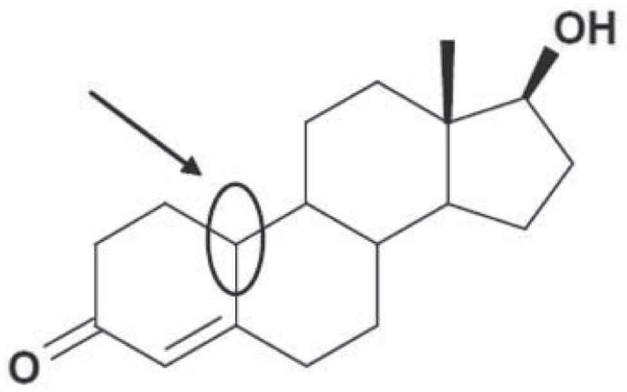

Fig 1. Structural formulas for 19 nortestosterone (nandrolone)
Şekil 1. 19 nortestosteron'un yapısal formülü

For determination of 19 nortestosterone is used immunoassay screening methods, gas chromatography coupled to mass spectrometric (GC/MS), liquid chromatography coupled to mass spectrometric (LC/MS) and other confirmatory methods. Confirmation methods are costly, time-consuming, require extensive sample preparation and highly trained personnel to operate sophisticated instruments and interpret complicated chromatograms [12,13]. The aim of this study was to monitor the use of 19 nortestosterone as anabolic substance and determination of 19 nortestosterone in cattle meat in Republic of Macedonia with screening ELISA method. ELISA method is simple, rapid, and cost-effective alternative to those traditional methods in cases where high-throughput and/or on-site screening is needed and permits analysis with little or no sample pre-treatment, thanks to the very high specificity of the bio specific reagents used (antibodies) [12-14].

MATERIAL and METHODS

Samples

A total of 86 meat samples were obtained randomly from 22 supermarkets, from 11 cities in Macedonia, from July 2010 to July 2011. The samples were kept frozen until use and the examinations were carried out according to the requirements of the European Community in five specialized veterinary diagnostic laboratories belonging to the Faculty of Veterinary Medicine, Food Institute.

Reagents

RIDASCREEN 19 nortestosteron test kit (R-Biopharm AG, Darmstadt, Germany) and its reagents were used to determine the presence and the 19 nortestosteron levels in the cattle meat. Methanol (Merck, 1060352500) and tertiary butyl methyl ether (Merck, 1018492500) used were of analytical grade. 20mM PBS buffer, pH 7.2, was prepared by mixing 0.55 g sodium dihydrogen phosphate hydrate (NaH_2PO_4 x H_2O) with 2.85 g disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O) and 9 g sodium chloride (NaCl) and filling up to 1.000 ml distilled water. 19 nortestosterone external standard (Fluca Chemica 74640) we used for recovery investigations. From this standard we prepared standard solution of 19 nortestosterone in 1 ml of methanol-water (80:20, v/v) corresponding to 1.000, 1.500 and 2.000 ppt, respectively for spiking of the blank cattle meat sample on three levels.

Extraction Procedure

Fat was removed from muscle and the muscle was ground. One gram from ground muscle was transferred in the test tube (graduated conical tube 50 ml, BSM477) and then muscle was homogenized in 1 ml of 20 mM PBS buffer by mixer (Ikalabortechnik, t 25 basic) for 10 min. The homogenate was mixed with 10 ml tertiary butyl methyl ether in a centrifugal screw vial and shaken carefully for 30 min and then

samples were centrifuged for 10 min at 4.000 rpm on 15ºC. The supernatant (ether layer) was transferred to another centrifugal vial, then the samples were evaporated to dryness and dissolved in 1 ml methanol/water (80:20; v:v). The methanolic solution was diluted with 2 ml of 20 mM PBS buffer and applied to a RIDA C18 column (RIDA® R-Biopharm AG, Darmstadt, Germany, R2002) in the following manner: column was rinsed by flowing of 3 ml methanol (100%); then the column was equilibrated by injection of 2 ml of 20mM PBS buffer; a sample (3 ml) was applied on column; column was rinsed by injection of 2 ml methanol/water (40:60; v:v); column was dried by pressing N_2 trough it for 3 min; the sample was eluted slowly by injection of 1 ml methanol/water (80:20; v:v) (flow rate: 15 drops/min). Next step was evaporation of the eluted sample to dryness, at 60ºC under a weak nitrogen flow. Dried residue was dissolved in 2 ml methanol/water (10:90; v:v). In the test 20 µl of standard/sample for per well was used [15].

Validation

The limit of detection (LOD) of the assay was defined as the concentration corresponding to the mean signal of 20 blank cattle meat samples plus 3 times of standard deviation of the mean. Blank cattle meat samples were obtained from untreated cattle. The accuracy was evaluated by determining the recovery of spiked blank cattle meat samples with three concentration of 19 nortestosterone standards (1.000, 1.500 and 2.000 ppt). Precision was expressed as the CV (Coefficient of variation) (%) of the calculated standards and sample concentrations. Detection capabilities (CCβ) is required to be at or lower than the MRPL [16]. CCβ was evaluated by analyzing 20 spiked blank cattle meat samples at 0.5 times MRPL (1.000 ppt for 19 nortestosterone in muscle) [17] level for 19 nortestosterone and calculated in accordance with European Commission Decision 2002/657/EC.

Test Procedure

RIDASCREEN® 19 Nortestosterone ELISA kits (R- Biopharm AG, Darmstad, Germany) were used in order to determine the presence and levels of 19 Nortestosterone in the cattle meat samples investigated. All reagents in the kit had to be brought to room temperature (20-25°C) before use. Standard used for 19 nortestosterone contain 0, 50, 150, 500, 1.000 and 3.000 ppt 19 nortestosterone in 10% of methanol.

100 µl of diluted antibody was added to each well, mixed gently by shaking the plate manually and incubated for 30 min at room temperature (20-25°C). Liquid was poured out of the wells and after complete removal of the liquid; all wells were filled with washing buffer. Washing was repeated two more times. Then 20 µl of each standard solution or prepared sample were added and after that 100 µl of the diluted enzyme conjugate was added. The solution in the microplate was carefully mixed by shaking the plate manually. The plate was then incubated at room temperature (20-25°C) for 30 min. The liquid poured out of the wells and after the complete removal of liquid, all wells were filled with washing buffer. After rinsing, the water was also discarded; the washing was repeated two more times. Then, 100 µl of substrate/chromogen (tetramethyl-benzidine) were added, and after mixing thoroughly and incubating for 15 min at room temperature in the dark, 100 µl of stop solution (1 N sulphuric acid) was added. After mixing, the absorbance was read at 450 nm by using a spectrophotometer (BIO RAD model 680) [15].

The assay of Cross Reactivity

The standards used for 19 nortestosterone contained 0, 50, 150, 500, 1.000 and 3.000 ppt 19 nortestosterone in 10% methanol, whereas the antibody used had cross reactions with other related compounds, as indicated by the manufacturer's literature and shown in *Table 1*.

RESULTS

Calculation of the gained results was made by RIDAWIN Software. For construction of the calibration curve the mean of absorbance values obtained for six standards was divided by the absorbance value of the first standard (zero standards) and multiplied by 100. The absorption is inversely proportional to the concentration of 19 nortestosterone. As can be seen in *Fig. 2*, the 19 nortestosteron calibration curve was found to be virtually linear between 50 and 3.000 ppt.

In *Fig. 3* the correlation between the absorbance ratio and 19 nortestosterone concentration was evaluated over the range 0-3.000 ppt. Linear regression analysis showed good correlation, with r^2 values 0.9972, ($y = - 0.1453 x + 1.4057$), where y was relative absorbance (%) and x was 19 nortestosterone concentration in ppt.

Table 1. *Cross reactivity of 19 nortestosterone antibody with various compounds*

Tablo 1. *19 nortestosterone antikorunun çeşitli bileşikler ile çapraz reaksiyonları*

Compound	Cross Reactivity
19-Nortestosterone 17β	100%
19-Nortestosterone 17α	approx. 80%
19-Norethisterone	approx. 74%
19-Norandrostendione	approx. 100%
18-Methyl-19 NT-17β	approx. 59%
17α-Ethyl-19 NT-17β	approx. 40%
15α, 16α-Methyl.-19 NT-17β-acetate	approx. 71%
Trenbolone	approx. 10%
17β-Estradiol	approx. 0.1%
Zeranol	< 0.1%
DES	< 0.1%
MPA	< 0.1%

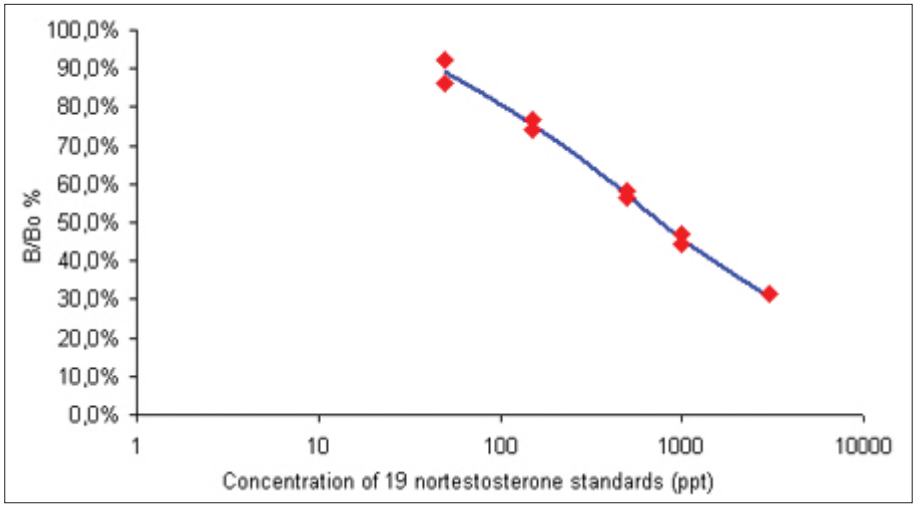

Fig 2. Linearity of calibration curve for 19 nortestosterones standards (50-3.000 ppt)

Şekil 2. 19 nortestosterone standartları için kalibrasyon eğrisinin doğrusallığı (50-3.000 ppt)

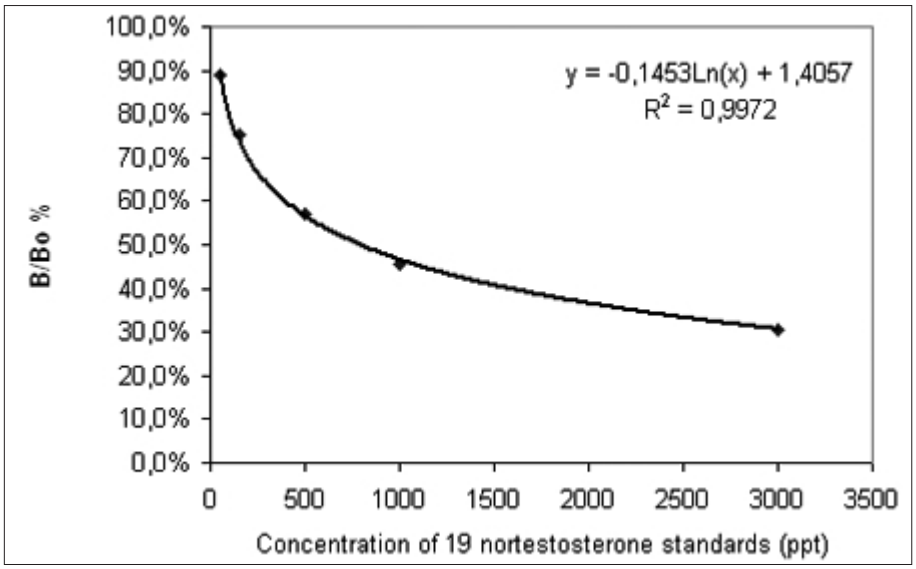

Fig 3. Calibration curve for 19 nortestosterone standards (0-3.000 ppt)

Şekil 3. 19 nortestosterone standartları için kalibrasyon eğrisi (0-3.000 ppt)

Table 2. *Precision of the method*
Tablo 2. *Metodun hassasiyeti*

Item	Concentration (ppt)	CV (%)
19 nortestosterone standards	0.0	1.6
	50.0	1.2
	150.0	1.4
	500.0	2.1
	1000.0	9.2
	3000.0	2.0
CV % for spiked sample	500.0	4.0
	1500.0	4.6
	2000.0	1.9

Results for the precision of the method are presented in *Table 2*. The precision (Coefficient of variation (CV) %) in 19 nortestosterone standards ranged from 1.2% to 9.2%. The precision (CV %) in spiked cattle meat sample ranged from 1.9% to 4.6%.

The accuracy was expressed as the recovery (%) of the estimated concentration. For the three target concentration

Table 3. *Accuracy of the method (recovery %)*
Tablo 3. *Metodun doğruluğu (geri kazanım %)*

Cattle Meat Sample n (Number of Replicates)	19 Nortestosterone		Recovery %
	Added (ppt)	Found (ppt)	
n = 6	1000	795.35	79.54
n = 6	1500	1715.84	114.39
n = 6	2000	2165.79	108.29

(1.000, 1.500, 2.000 ppt) the recoveries in cattle meat sample was 79.54%, 114.39% and 108.29% respectively and they are presented in *Table 3*.

These results are in agreement with the internationally accepted ranges for these parameters, and the standard deviations indicate that the method is sufficiently precise. Detection limit for 19 nortestosterone was found to be 124 ppt. The detection capability (CCβ) for 19 nortestosterone was 730 ppt, less than MRPL level of 1000 ppt. The analyzes of the cattle meat samples showed a values from 125.33 ppt to 625.07 ppt. In our case the calculated values of the analyzed samples are less than CCβ value.

DISCUSSION

Raw meat and meat products, which play an important role in human nutrition, should be safe and should not contain any factors or substances harmful for human health. However, the anabolic agents used for various purposes in animal husbandry tend to leave residues and this causes some problems in consumer health [18,19]. The European Economic Community (EEC) banned the use of anabolic compounds as growth accelerators in food animals [16]. In the present work, the ELISA method was used to achieve the unambiguous identification of 19 nortestosterone in cattle meat samples. This method was validated in accordance in the criteria of Commission Decision 2002/657/EC and is used in routine analyses in our laboratory. Because of the simple, rapid, and cost-effective of the method and its good recovery and precision it is applicable in official control laboratories as a screening method. In our opinion all ELISA kits aren't suitable for this purpose. For example P4 kits by Sorin and Tecna, and the T kit by Serono tended to underestimate and to give false negative results, while the P4 kit by Ovucheck and the T kit by Ridgeway Science tended to overestimate and to give false positive results [20].

In the case when the target analyte is clearly identified above CCβ the sample is considered as non compliant and we must confirm the results with confirmation method on GC/MS, LC/MS or another confirmatory method [16]. The methodologies and full procedure for confirmatory analysis require trained personnel with high expertise and they are costly in time, chemicals and equipments [21]. Identification is easier for a limited number of target analytes which are obtained with screening methods. In this study the calculated values of the analyzed samples are less than CCβ value, obtained with validation of the kit, so it seems that the present status of 19 nortestosterone in cattle meat is not at risk. But the number of samples included in this study is relatively lower compared to the total cattle meat sold in the market. These results do not exclude the possibility of misuse of 19 nortestosterone in the future. Due to the fact, it is still necessary to monitor this chemical as a food quality control measure.

REFERENCES

1. Gracey JF, Collins DS, Huey RJ: Meat Hygiene. 10th ed., pp. 261-287, WB Saunders Company, New York, USA, 1999.

2. Pullen M: Residues. **In,** Pearson AM, Dutson TR (Eds): Meat and Health Advances in Meat Research, Vol. 6, Elsevier Applied Science, New York, USA, 1990.

3. Tian Zh, Yu DH, Zhang YY, Guo JH, Peng Ch F, Chen Zh X and Xu Ch L: Development and optimization of an indirect enzyme-linked immunosorbent assay for 19-nortestosterone. *J Anim Feed Sci*, 17, 253-267, 2008.

4. Van Peteghem C, Maghuin-Rogister G, Cornelis M: Standardization of Hormone and Veterinary Drug Residue Analysis in Animal*, FFW, Ghent Univ, Belgium,* 7-13, 2001, http://cemu10.fmv.ulg.ac.be/OSTC, *Accessed:* May 2002.

5. Bahrke MS, Yesalis CE: Abuse of anabolic androgenic steroids and related substances in sport and exercise. *Curr Opin Pharmacol,* 4, 614-620, 2004.

6. Baume N, Avois L, Schweizer C, Cardis C, Dvorac J, Cauderay M, Mangin P, Saugy M: Nandrolone excretion in trained athletes: Interindividual variability in metabolism. *Clin Chem,* 50 (2): 355-364, 2004.

7. Committee on Sports Medicine and Fitness: Adolescents and Anabolic Steroids: A Subject Review. pp. 904-908, American Academy of Pediatrics, 1997.

8. Brännvall K, Bogdanovic N, Korhonen L, Lindholdm D: 19 Nortestosterone influences neural stem cell proliferation and neurogenesis in the rat brain. *Eur J Neurosci*, 21 (4): 871-878, 2005.

9. Council Directive: 96/22/EC of 29 April 1996 concerning the prohibition on the use in stock farming of certain substances having a hormonal or thyrostatic action and of β-agonists, and repealing, Directives 81/602/EEC, 88/146/EEC and 88/299/EEC, OJ L125 23/5/1996, *Off J Eur Comm,* L125, 3-8, 1996.

10. Council Directive: 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187EEC and 91/664/EEC, *Off J Eur Comm,* L125, 10-32, 1996.

11. Council Regulation: (EC) No. 882/2004, *Off J Eur Commun*, L165, 1-141, 2004.

12. Liu J, Jiang J, An Z, Zhang H, Fan G: Preparation and identification of artificial antigen against 19 nortestosteronerone. *Advances in Biomedical Engineering*, 9, 6-13, 2012.

13. Bineid M: Applicability of a yeast bioassay in the detection of steroid esters in hair. Gent University, 2009-2010.

14. Peng CF, Chen YW, Chen HQ, Xu Ch L, Jin1 ZY: A rapid and sensitive enzyme-linked immunosorbent assay (ELISA) method and validation for progestogen multi-residues in feed. *J Anim Feed Sci,* 17, 434-441, 2008.

15. R-Biopharm AG, Darmstadt, Germany: RIDASCREEN® 19-NORTESTOSTERONE, Enzyme immunoassay for the quantitative analysis of 19-nortestosterone, R2801.

16. Commission of the European Communities: European Commission Decision of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results (2002/657/EC), *Off J Eur Comm,* L 221, 8-36, 2002.

17. CRL Guidance Paper: CRLs view on state of the art analytical methods for National residue control plans, p. 3, 7 December, 2007.

18. Hoffman B: Problems of residue and health risks of anabolic agents with hormone like activites. *Proceedings of the Scientific Conference on Growth Promotion in Meat Production, European Comm. Dir. Gen. VI. Agric., Official Pub. European Comm., Brussels*, pp. 271-296, 1996.

19. Nazli B, Olak H, Aydin A, Hampukyan H: The presence of some anabolic residues in meat and meat products sold in Istanbul. *Turk J Vet Anim Sci,* 29, 691-699, 2005.

20. Simontacchi C, Marinelli L, Gabai G, Bono G, Angeletti R: Accuracy in naturally occurring anabolic steroid assays incattle and first approach to quality control in Italy. *Analys,* 124, 307-312, 1999.

21. Shankar B.P, Prabhu M, Chandan S, Ranjith D, Shivakumar V: rapid methods for detection of veterinary drug residues in meat. *Veterinary World,* 3, 241-246, 2010.

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SHORT COMMUNICATION

Nitric Oxide Levels, Total Antioxidant and Oxidant Capacity in Cattle with Foot-and-Mouth-Disease

Kadir BOZUKLUHAN * Emine ATAKISI ** Onur ATAKISI ***

* Kafkas University, Faculty of Veterinary Medicine, Department of Internal Medicine TR-36000 Kars - TURKEY
** Kafkas University, Faculty of Veterinary Medicine, Department of Biochemistry TR-36000 Kars - TURKEY
*** Kafkas University, Faculty of Art and Scince, Department of Chemistry TR-36000 Kars - TURKEY

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Summary

The aim of this study was to investigate total antioxidant (TAC), oxidant capacity (TOC) and nitric oxide levels (NO) in cattle with foot-and-mouth-diseases (FMD). Twenty Swiss Brown cattle aged between 24 and 48 months were used. Animals were divided into two groups as control (n=10) and FMD (n=10). Blood samples were collected from jugular vein and centrifuged. TOC and NO levels were found to be significantly higher in FMD group compared to those of control group. However, no significant differences were present in TAC levels between FMD and control groups. It was concluded that FMD increases serum NO levels and TOC, but do not affect TAC in cattle.

Keywords: *Cattle, Foot-and -Mouth Disease, Oxidative stress, Nitric oxide*

Şap Hastalıklı Sığırlarda Nitrik Oksit Düzeyi, Total Antioksidan ve Oksidan Kapasite

Özet

Çalışmanın amacı şap hastalığına (FMD) yakalanmış sığırlarda total antioksidan (TAC), oksidan kapasite (TOC) ve nitrik oksit (NO) seviyelerini araştırmaktır. Araştırmada, yaşları 24 ve 48 ay arasında olan 20 Montofon ırkı sığır kullanıldı. Hayvanlar kontrol (n=10) ve şap hastalıklı (n=10) olmak üzere iki gruba ayrıldı. Kan örnekleri Vena jugularis'ten alınarak santrifuj edildi. Şaplı hayvanlarda TOC ve NO seviyeleri kontrol grubundaki hayvanlara göre önemli düzeyde yüksekti. TAC seviyesinde ise şap hastalıklı ve kontrol grubu arasında önemli bir değişiklik saptanmadı. Sonuç olarak şap hastalığının sığırlarda serum NO ve TOC seviyesini artırırken, TAC seviyesini etkilemediği belirlendi.

Anahtar sözcükler: *Sığır, Şap, Oksidatif stres, Nitrik oksit*

INTRODUCTION

Foot-and-mouth-disease (FMD) is a highly contagious viral disease of all cloven-footed domestic and wild animals. The disease is distributed worldwide and is a major problem for cattle and sheep farmers. The virus, picarnovirus of genus aphthovirus, has a zoonotic potential and causes mild infection in farmers, veterinary surgeons, farm workers [1-3].

FMD has been shown to alter both haematological and biochemical parameters in cattle. In infected animals, erytrocytopeni, lymphositosis, monositosis and reduction in the serum concentrations of total protein, albumin, glucose, cholesterol, triglyceride and calcium have been reported elsewhere [3-7].

Nitric oxide is a cytotoxic factor released by a variety of cells. It is generated from the terminal guanidine nitrogen atom of L-arginine by NO synthase [8-11]. Despite its role in the primary defence against bacteria, viruses and parasites [12,13], it has also been reported to be immunosuppressive on immune system [14,15]. Therefore, NO may be protective or hazardous for mammalian tissues depending on concentration [16]. Oxidative stress is commonly observed in different pathological events of farm animals [17]. When the cellular oxidant state is overwhelmed by excessive production of reactive oxygen species and the condition may end up with cellular damage due to oxidative stress and lipid peroxidation [18,19].

İletişim (Correspondence)
+90 474 2426800/5226
kbozukluhan@hotmail.com

These reactive oxygen species are eliminated through enzymatic and non-enzymatic antioxidative mechanisms [18,20,21]. It has been known that picarnovirus induces NO synthesis in naturally infected cattle with FMD5. However, the relationship between NO and its antioxidant capacity on free radicals is not well-known in FMD. The aim of the study was to investigate total antioxidant, oxidant capacity and nitric oxide levels in cattle with foot-and-mouth-diseases.

MATERIAL and METHODS

In this study, 20 Swiss-Brown cattle aged between 24-48 months old were used. Initially, animals suspected of FMD were tested serologically by the Etlik Central Veterinary Control and Research Institute, Ankara for FMD. Ten cattle were diagnosed with FMD, serotype Asia 1. Then, the blood samples were collected from the animals which were confirmed to have FMD. The remaining cattle were clinically healthy and used as control in the study. The blood samples were collected from all the animals and used to prepare serum samples. These samples were then used to determine NO levels, TAC and TOC.

Serum nitric oxide concentrations were determined using a spectrophotometer (PowerWave XS, BioTek, Instruments, USA) as described by Miranda and others [22]. Initially, serum samples were deproteinized with 10% zinc sulphate and serum nitrate was reduced to nitrite by vanadium (III) chloride. Total nitrite, an indicator of nitric oxide, were then determined calorimetrically using acidic Griess reaction [22].

Serum total antioxidant and oxidant capacities were measured colorimetrically (PowerWave XS, BioTek, Instruments, USA) using a commercial kit (Rel Assay Diagnostic, Turkey). Trolox and hydrogen peroxide were used as standards to calculate for total antioxidant and total oxidant capacities, respectively [18].

All values were expressed as mean±SEM. ANOVA and then Tukey test were used to analyze the significance of differences between the groups using SPSS Windows 10.0. The differences between the groups were considered significant if *P* value was less than 0.05 ($P<0.05$).

RESULTS

Clinical examination revealed that the animals with FMD had characteristic clinical symptoms including fever, blisters or vesicles, erosions and ulcers in the mucosa of mouth, tongue, lips, gums and palate. Lesions were also observed in foot and teats. Furthermore, picarnovirus serotype Asia 1 was also isolated and identified from all the animals having lesions of FMD. In the present study, TOC and NO levels were found to be significantly higher in FMD group compared to those of control group. However, no significant differences were obtained in TAC levels between FMD and control groups *(Table 1)*.

Table 1. *Serum nitric oxide levels, total antioxidant and oxidant capacity in cattle with Foot-and-Mouth-Disease and control groups*

Tablo 1. *Şap hastalıklı sığırlarda ve kontrol grubunda serum nitrik oksit seviyesi, total antioksidan ve oksidan kapasite*

Parameters	Control (n=10)	FMD (n=10)	P
TAC (mmolTrolox Equiv./L)	0.79±0.047	0.66±0.051	Non significant
TOC ($mmolH_2O_2$ Equv./L)	7.15±0.66	11.76±0.84	<0.001
NO (mmol/L)	4.23±0.75[b]	15.80±1.53[a]	<0.05

** Different letters in the same line indicate significant differences between the groups*

DISCUSSION

Nitrate and nitrite in serum are formed by the decomposition of NO [23,24]. Their concentrations in serum are used as a direct measure of NO production [22]. In the present study, a significantly high level of nitrate was determined in serum samples obtained from FMD group. The results of the present study indicate that picornavirus induce the production of NO *in vivo*. It is well-known that NO plays an important role in the primary defence mechanism against several bacteria [25,26], viruses and parasites [27,28]. The production of NO is known to be induced by various viruses which inhibit virus replication *in vivo* and *in vitro* [29,30]. On the other hand, the protective or harmful effect of NO is suggested to be associated with the NO concentration [16].

Oxidative stress is generally defined as imbalance between the oxidant and antioxidant molecules [31]. Peroxynitrite radical, a reactive molecule, is formed due to reaction of NO and super oxide anion during inflammation [32]. Peroxynitrite radicals are formed from the lipid peroxides and free radicals via oxidizing long chained lipid acids located on the cell membranes [33-35]. In the present study, serum TOC in FMD group was shown to be increased significantly, whereas TAC in FMD group was lower than in control group. However, the difference was not statistically significant. It can be speculated that increased levels of NO found in this study might be due to the production of oxidant molecules. Establishment of TAC values in a sample allow us to determine all of the exogenous and endogenous antioxidants on a large spectrum [20]. However, there was no correlation between TOC and TAC values obtained from FMD group.

In conclusion, presence of FMD in cattle increase serum NO and TOC values, but does not affect TAC values in these animals.

REFERENCES

1. Lubroth J: Foot and mouth disease a review for the practitioner. *Vet Clin Food Anim*, 18, 475-499, 2002.

2. Tunca R, Sozmen M, Erdogan HM, Citil M, Uzlu E, Özen H, Gökce E: Determination of cardiac troponin I in the blood and heart of calves with foot mouth disease. *J Vet Diagn Inves,* 20, 598-605, 2008.

3. Yarım GF, Nisbet C, Cenesiz S, Coşkuner A: Şap hastalıklı koyunlarda

serum nitrik oksit düzeyi ve adenozin deaminaz aktivitesinin araştırılması. *Ankara Univ Vet Fak Derg,* 53, 161-164, 2006.

4. Elitok B, Balıkçı E, Kececi H, Yılmaz K: Şaplı sığırlarda serum kreatinin fosfokinaz (CPK), laktat dehidrogenaz (LDH), aspartat aminotransferaz (AST) aktiviteleri, glukoz düzeyleri ve EKG bulguları. *Kafkas Univ Vet Fak Derg, 5* (2): 161-166, 1999.

5. Gokce G, Gokce HI, Gunes V, Erdoğan HM, Citil M: Alterations in some haematological and biochemical parameters in cattle suffering from foot-and-mouth disease. *Turk J Vet Anim Sci,* 28 (4): 723-727, 2004.

6. Or ZS, Fidancı UR: Şap virusu ile enfekte ve aşılı danalarda serum proteinlerinin elektroforetik dağılımı. *Ankara Univ Vet Fak Derg,* 56, 13-18, 2009.

7. Şahal M, Imren HY, Ozlem MB, Tanyel B: Süt ineklerinde şap hastalığı ve diabetes mellitus arasındaki ilişki. *Ankara Univ Vet Fak Derg,* 41 (2): 169-181, 1994.

8. Stuer JD: Mammalian nitric oxide synthases. *Biochim Biophys Acta,* 41, 217-230, 1999.

9. Griffith OW: Nitric oxide synthase inhibitors: Mechanisms of action and *in vivo* studies. **In,** Weisman BA, Allon N, Shapira S (Eds): Biochemical, Pharmacological and Clinical Aspects of Nitric Oxide. pp. 21-36, Plenum Press, New York, 1995.

10. Groves JT, Wang C: Nitric oxide synthase: Models and mechanisms. *Curr Opin Struct Biol,* 4, 687-695, 2000.

11. Feldman PL, Stuehr DJ, Griffith OW: Mechanisms of mammalian nitric oxide biosynthesis. **In,** Weisman BA, Allon N, Shapira S (Eds): Biochemical, Pharmacological and Clinical Aspects of Nitric Oxide. pp. 14-20, Plenum Press, New York, 1995.

12. Akaike T, Noguchi Y, Ijiri S, Setoguchi K, Suga M, Zheng YM, Dietzschold B, Maeda H: Pathogenesis of influenza virus-induced pneumonia: Involvement of both nitric oxide and oxygen radicals. *Proc Natl Acad Sci USA,* 93, 2448-2453, 1996.

13. James SL: Role of nitric oxide in parasitic infection. *Microb Rev,* 59, 533-547, 1995.

14. Albina JE, Abate JA, Henry WL: Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation: Role of IFN-γ in the induction of the nitric oxide-synthesizing pathway. *J Immunol,* 147, 144-148, 1991.

15. Rockett KA, Awburn MM, **Rockett EJ, Cowden WB, Clark IA:** Possible role of nitric oxide in malarial immunosuppression. *Parasit Immunol,* 16 (5): 243-249, 2007.

16. Zelnickova P, **Matiasovic J, Pavlova B**, **Kudlackova H**, **Kovaru F**, **Faldyna M:** Quantitative nitric oxide production by rat, bovine and porcine macrophages. *Nitric Oxide* 19 (1): 36-41, 2008.

17. Lykkesfeldt J, Svendsen O: Oxidants and antioxidants in disease: Oxidative stress in farm animals. *Vet J,* 173, 502-511, 2007.

18. Erel O: A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem* 37, 277-285, 2005.

19. Halliwell B, Gutteridge JMC: Free Radicals in Biology and Medicine. 3rd ed., Oxford University Press, New York, 1999.

20. Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V: Method for the measurement of antioxidant activity in human fluids. *J Clin Pathol,* 54, 356-361, 2001.

21. Somogyi A, Rotsa K, Pusztai P, Tulassay Z, Nagy G: Antioxidant measurements. *Physiol Meas,* 28 (4): 41-55, 2007.

22. Miranda KM, Espey MG, Wink DA: A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide,* 5 (1): 62-71, 2001.

23. Marletta MA: Mammalian synthesis of nitrite, nitrate, nitric oxide, and nitrosating agents. *Chem Res Toxicol,* 1, 249-257, 1988.

24. Tayeh MA, Marletta MA: Macrophage oxidation of L-arginine to nitric oxide, nitrate, and nitrite: Tetrahydrobiopterin is required as a cofactor. *J Biol Chem,* 264, 19654-19658, 1989.

25. Fang FC: Perspectives series, host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest,* 99, 2818-2825, 1997.

26. Mancinelli RL, Mckay CP: Effects of nitric oxide and nitrogen dioxide on bacterial growth. *Appl Environ Microbiol*, 46 (1): 198-202, 1983.

27. Moncada S, Palmer RM, Higgs E: Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev,* 43 (2): 109-134, 1991.

28. De Groote MA, Fang FC: NO inhibitions: Antimicrobial properties of nitric oxide. *Clin Infect Dis,* 21 (2): 162-165, 1995.

29. Croen KD: Evidence for an antiviral effect of nitric oxide. *J Clin Invest,* 91, 2446-2452, 1993.

30. Schoedon G, Schneemann M, Walter R, Blau N, Hofer S, Schaffner A: Nitric oxide and infection: Another view. *Clin Infect Dis*, 2, 152-157, 1995.

31. Gulbahar MY, Davis WC, Guvenc T, Yarım M, Parlak U, Kabak YB: Myocarditis associated with foot-and-mouth disease virus type O in lambs. *Vet Pathol,* 44 (5): 589-599, 2007.

32. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA: Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci, USA,* 87, 1620-1624, 1990.

33. Al-Sadoni H, Ferro A: S-nitrosohiols: A class of nitric oxide- donor drugs. *Clin Sci,* 98, 507-520, 2000.

34. Pryor WA, Squadrito GL: The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am J Physiol Lung Cell Mol Physiol,* 268, 699-722, 1995.

35. Wang PG, Xian M, Tang X, Wu X, Wen Z, Cai T, Janczuk AJ: Nitric oxide donors: Chemicals activites and biological applications. *Chem Rev,* 102, 1091-1134, 2002.

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