Journal of Advances in VetBio Science and Techniques

VETBIO

E-ISSN: 2548 - 1150 - Period: Tri-annual

J Adv VetBio Sci Tech - Volume 7(3) - December 2022

JOURNAL OF ADVANCES IN VETBIO SCIENCE AND TECHNIQUES

e-ISSN 2548-1150 | **Period** Tri-annual | **Founded:** 2016 | *J adv **VetBio** Sci Tech*

Year 2022 | **Volume 7** | **Issue 3**

EDITORIAL ARCHIVE

December 31, 2022 / Copyright© VetBio

Journal of Advances in **VetBio** Science and Techniques is aimed to serve as scientific research journal.

VetBio is a **triannual** (April, August, and December), open access, and fully refereed **international** journal.

VetBio is to publish high-quality scientific research articles on animal-related fields including science branches such as veterinary medicine, fisheries, biological sciences, food science, wildlife and zoology. In addition, short communications and reports, case reports, letter to the editor and reviews are also accepted. Publishing languages are Turkish and English. The editorial policy of the journal is based on independent, unbiased, and **double-blind** peer-review. The VetBio **does not charge** submission, processing or publication fee.

VetBio has been **indexed** by **TRDizin**, **CAB International (CABI)** index, **Index Copernicus International (ICI)** World of Journals, **Google Scholar,** Academic Research Index (**Research Bib**), Root Society for Indexing and Impact Factor Service (**Rootindexing**), Eurasian Scientific Journal Index (**ESJI**), **Cosmos** Impact Factor, Scientific Indexing Services (**SIS**), **OpenAIRE,** Directory of Open Access Scholarly Resources (**ROAD**), and **CiteFactor** databases.

e-mail: vetbiojournal@gmail.com
Web Page: http://dergipark.gov.tr/vetbio
Phone: 05536203468

JOURNAL OF ADVANCES IN VETBIO SCIENCE AND TECHNIQUES

e-ISSN 2548-1150 | **Period** Tri-annual | **Founded:** 2016 | *J adv **VetBio** Sci Tech*

VetBio 2022, 7(3)

CONTENTS

Pages

Research Articles

Some reproductive and gynecological characteristics of Morkaraman ewes **274-282**

Bekir YILMAZ, Buket BOĞA KURU, Mushap KURU

Turunç ekstraktı uygulamasının saha koşullarında ishalli ve ishali olmayan buzağılarda dışkı kıvamı ile gizli temizlik üzerine etkinliğinin araştırılması **283-288**

Deniz ALIÇ URAL, Songül ERDOĞAN, Hasan ERDOĞAN, Gökhan ZARARYOK, Barış DOĞAN, Kerem URAL

An examination of blind mole-rat (Nannospalax xanthodon) brain, cerebellum, and spinal cord tissues: A histological and stereological study **289-295**

Ayşe İKİNCİ KELEŞ, Burcu BİTERGE SÜT, Teoman KANKILIÇ

Histochemical and immunohistochemical investigation of the effects of Sambucus nigra on mast cells and VEGF in diabetic rat spleen **296-304**

Elif Tuğba ERGEL, Tuğrul ERTUĞRUL

Investigation of Escherichia coli septicemia and antibiotic susceptibility in neonatal lamb deaths in the Aegean region. **305-312**

Volkan ÖZAVCI, Çağatay NUHAY, Mehmet ÖZDEN

Evaluation of the effect of ozone therapy on the treatment of cutaneous wounds with tissue-loss in dogs and cats **313-320**

Osman AYDIN, Ali KUMANDAŞ

Afyonkarahisar ilinde meme tümörü saptanan köpek ve kedilerde ırk ve yaş dağılımı **321-326**

Duygu BAKİ ACAR, Ece TUNÇ

JOURNAL OF ADVANCES IN VETBIO SCIENCE AND TECHNIQUES

e-ISSN 2548-1150 | **Period** Tri-annual | **Founded:** 2016 | *J adv **VetBio** Sci Tech*

The inhibitory effects of tyrosol on clinical Candida glabrata planktonic and biofilm cells **327-337**

Zarifeh ADAMPOUR, Betül YILMAZ ÖZTÜRK, İlknur DAĞ

Çeşitli gıda örnekleri ve kesimhanelerden izole edilen bazı patojen bakterilerin biyofilm oluşturma yeteneğinin araştırılması **338-345**

Tolga UYANIK, Ayşegül BÖLÜKBAŞ, Ali GÜCÜKOĞLU, Özgür ÇADIRCI

Pathological and biochemical investigation of the effects of l-carnitine and gemfibrozil on peroxisome proliferator activated Receptors (PPARS) and lipidosis in rabbits on a high-fat diet **346-360**

Mehmet ÇİTİL, Recai TUNCA, Erdoğan UZLU, Mahmut KARAPEHLİVAN, Yasemen ADALI, Kürşat YAPAR, Hüseyin Avni EROĞLU, Ekin Emre ERKILIÇ, Mustafa MAKAV, Hidayet Metin ERDOĞAN

Determination of reticular fibers in tissues fixed with sugarcane molasses **361-365**

Hatice Yaren KULOĞLU

Experimental intravaginal and intrauterine endometritis model: which model is more useful? **366-375**

Mustafa MAKAV, Mushap KURU, Hatice BEŞEREN, Yasemen ADALI, Mustafa Reha COŞKUN, Hüseyin Avni EROĞLU

The Influence of blood, seminal plasma testosterone, growth hormone and cortisol levels on the sperm quality in merino rams **376-384**

Hale ERGİN EĞRİTAĞ, Nagehan ÖZGÖKÇEN, Halil HARMAN, Şükrü GÜNGÖR, Caner OZTURK, Behiç SERPEK

Investigation of antifungal activity mechanisms of alpha-pinene, eugenol, and limonene **385-390**

Hatice Büşra KONUK, Bengü ERGÜDEN

Case Report

Changes in cardiac markers in a calf died of foot and mouth disease **391-394**

Cemalettin AYVAZOĞLU, Erhan GÖKÇE, Hidayet Metin ERDOĞAN

Some reproductive and gynecological characteristics of Morkaraman ewes

Research Article

Bekir Yılmaz[1a]
Buket Boğa Kuru[2b]
Mushap Kuru[3c]

[1]Ministry of Agriculture and Forestry, Iğdır, Türkiye
[2]Department of Animal Breeding and Husbandry, Faculty of Veterinary Medicine, Kafkas University, Kars, Türkiye
[3]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Kafkas University, Kars, Türkiye

ORCİD-
[a]0000-0002-5477-0380
[b]0000-0002-7170-270X
[c]0000-0003-4409-251X

Correspondence
Mushap KURU
mushapkuru@hotmail.com

Article info
Submission: 26-01-2022
Accepted: 11-05-2022
Online First: 15-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

ABSTRACT

The aim of this study is to determine some reproductive parameters and gynecological and lamb yield characteristics in Morkaraman ewes in Iğdır province, Türkiye. Reproductive parameters and fertility characteristics of Morkaraman ewes were recorded in two breeding seasons. Clinically healthy 600 Morkaraman ewes were used. Estrus duration was statistically different in the first and second breeding seasons (P=0.034). The estrus rates were 95% and 97% in the seasons, respectively (P=0.211). According to the breeding seasons (first and second year), lambing rate was 90.7% and 92%, infertility rate was 5% and 3%, placental retention rate was 2.6% and 1.5%, uterine infection rate was 8.2% and 6.6%, vaginal and uterine prolapse rate was 1.8% and 2.2%, follicular cyst rate was 1.3% and 0.7%, mastitis rate was 3.7% and 2.2%, insufficient milk production rate was 2.6% and 3.6%, abortion rate was 3.2% and 4.5%, dystocia rate was 4.4% and 5.4%, congenital anomaly rate was 1.1% and 1.5%, twinning rate was 10.7% and 12%, birth weight in singleton was 3.8 kg and 3.6 kg, birth weight in twins 3.2 kg and 3.3 kg, survival rate was 94.4% and 96.5%, respectively (P>0.05). In conclusion, Morkaraman ewes show high reproductive performance, do not have many gynecological problems, and are more likely to have single births.

Keywords: Birth, ewes, gynecological, lamb, Morkaraman, reproductive

INTRODUCTION

Sheep breeding is carried out extensively or semi-extensively in small family-type farms in rural areas, depending on geographic and climatic conditions. Small ruminants are raised intensively when snowfall begins in the Eastern provinces, Türkiye. Morkaraman ewes have adapted to harsh winter condition, poor quality pasture, high altitude plateau and is a great breed for small family-type farms (Akçapınar, 1994; Kaymakçı, 2016).

Morkaraman (also known as Red Karaman), is the second most abundant of sheep breeds in Türkiye (15.8% of ewes, 17.1% of local breed ewes). The body color is reddish-brown. The nose and mouth area can be light in color, and the head and feet can be darker. Morkaraman is a fat-tailed native sheep breed and the tail end-piece form is "S" shaped (Kaymakçı, 2016; Yalçin, 1986). Live weight is 50-60 kg in ewes, live weight in rams is 60-70 kg, greasy fleece weight is 1.5-2.5 kg, lactation period is 150-160 days, twin birth rate is 8-30% in Morkaraman ewes. Lambs can give 20-25 kg of carcass in 3-month fattening after weaning (Akçapınar, 1994; Yalçin, 1986; Yilmaz et al., 2013).

How to cite this article
Yılmaz, B., Boğa Kuru, B., Kuru M. (2022). Some reproductive and gynecological characteristics of Morkaraman ewes. *Journal of Advances in VetBio Science and Techniques, 7*(3), 274-282.
https://doi.org/10.31797/vetbio.1063461

In Morkaraman ewes, gestational length is 148.9-150.1 days (Akçapınar & Kadak, 1982; Odabaşıoğlu et al., 1996), estrus rate 100%, pregnancy rate 92%, birth rate 88%, twinning rate 26.7-30%, litter size 1.1-1.35 (Akçapınar et al., 1984; Odabaşıoğlu et al., 1996; Özbey & Akcan, 2000), lamb birth weight is 4.0 kg and survival rate in lambs is 93% (Odabaşıoğlu et al., 1996). In Morkaraman ewes, lactation milk yield is 40-77.6 kg (11.6-148 L), milk fat ratio 6.6-7.3% (3.9-8.4%) and lactation period 117-143.8 (68-173) days (Akçapınar, 1994; Yilmaz et al., 2013). In a study, the duration of lactation, milk yield and daily milk yield in Morkaraman ewes were determined as 137 days, 88.3 L, 645 mL, respectively (Kırmızıbayrak et al., 2005).

Strong body condition, rapid adaptation to the environment and resistance to cold climate are the most important characteristics of Morkaraman ewes. There are studies on some yield characteristics of Morkaraman ewes. However, in our literature reviews, we did not find detailed studies on the reproductive and gynecological characteristics of Morkaraman ewes. The aim of this study is to determine some reproductive parameters and gynecological and lamb yield characteristics in two breeding seasons in Morkaraman ewes.

MATERIAL and METHOD

Location, animals and feeds

The ewes and rams in the study were obtained from a Morkaraman sheep farm at an altitude of 860 m in Iğdır province, Türkiye.

A total of 600 (300 in first breeding seasons + 300 in second breeding seasons) Morkaraman ewes, 2-5 years old and 50-65 kg, without any clinical problem, were used. Body condition score of ewe varied between 2.5-3.5 (1 = Extremely thin, 5 = Obese) (Kenyon et al., 2014). Fifteen Morkaraman rams with no clinical health problems and weighing 70-80 kg were used for estrus detection and mating. The ewes were grazed on the pasture in the summer and were housed in the barns when the weather was getting colder or when the snow started.

The ewes were not fed any extra feed while they were in the pasture, but in winter, the ewes were fed with alfalfa, wheat straw, corn silage, bran and barley when they were brought to the barns. In the pregnancy period, additionally, 0.4 kg ewe/day barley-wheat meal was given. Water was given ad libitum.

Two-year herd data of the study were obtained from the farm logbook. Two-year examinations of reproductive parameters and determination were made by the veterinarian. Different sheep from each other in the first and second breeding seasons were included in the study.

The estrus is the time between ewes accepting to mate and refusing to mate (Kuru et al., 2017b). The time between mating and parturition was the gestation period (Kuru et al., 2017a). Sheep were exposed to rams during the breeding season (August-December) and outside of this period rams were removed from the herd (Kuru et al., 2017b). Lambing rates were determined by recording the ewes that gave birth. The time of birth was recorded as daytime (6.00-18.00) or night (18.01-05.59) (Kuru et al., 2017a). Sheep that were not in heat or not pregnant during the breeding season were considered infertile (Bekyürek, 2017). Retained placenta was clinically diagnosed when the placenta could not separate spontaneously in the third stage of labor (12-24 hours after birth) (Fthenakis, 2004). Purulent or mucopurulent vaginal discharges in the postpartum period were diagnosed as uterine infection. In such cases, examination with vaginal speculum was also performed (Scott, 2015). If the uterus passed through the cervix and protruded from the vulva, it was diagnosed as prolapse uteri (Oral & Kuru, 2016). Sheep that continued estrus after mating or showed estrus again 2-3 days after mating were diagnosed as follicular

cysts. These sheep were examined by ultrasonography and when a Graff follicle 1.2-1.5 cm in diameter or larger was detected in the ovary, it was recorded as a follicular cyst (Khodakaram-Tafti & Davari, 2013). Swelling and pain in the breast and deterioration of milk composition (such as pus, watery, smelly, clotted) were evaluated as clinical mastitis (Menzies & Ramanoon, 2001). Insufficient milk yield was defined as less than 50-100 mL of milk during the lactation period of sheep (Kuru et al., 2017a). Births requiring all kinds of intervention were diagnosed as dystocia (Kuru et al., 2016). Lambs born between 130-140 days of gestation were diagnosed as premature. These lambs had no incisors, the claws were soft, and the belly was hairless (Şahal et al., 1994).

Some reproductive and gynecological parameters

In the study, some reproductive and gynecological parameters and lamb yield characteristics were calculated according to the formulas (Kuru et al., 2017a; 2017b; Kuru et al., 2020).

Estrus duration = The time between accepting and rejecting mating.

Gestational length = Time between accepting to mate and parturition day.

Estrus rate (%) = Sheep in heat / All sheep x 100.

Lambing rate (%) = Sheep giving birth / All sheep x 100.

Infertility rate (%) = Non-pregnant sheep at the end of the breeding season / All sheep x 100.

Placental retention rate (%) = Number of sheep detected / Number of sheep giving birth x 100.

Uterine infection rate (%) = Sheep detected / All sheep x 100.

Vaginal – uterine prolapse rate (%) = Sheep detected / Sheep giving birth x 100.

Follicular cyst rate (%) = Sheep with follicular cyst / All sheep x 100.

Clinical mastitis rate (%) = Sheep with clinical mastitis / Sheep giving birth x 100.

Insufficient milk yield rate (%) = Detected sheep / Number of whole sheep x 100.

Abortion rate (%) = Abortions / Sheep giving birth x 100.

Dystocia rate (%) = Dystocia / All births x 100.

Premature birth rate (%) = Premature birth / All births x 100.

Congenital anomaly rate (%) = Congenital anomalies / All births x 100.

Twinning rate (%) = Sheep giving birth twins / Sheep giving birth x 100.

Litter size = Total number of lambs / Sheep giving birth x 100.

Lack of maternal instincts rate – rejected of lamb = Sheep with lack of maternal instincts / All sheep x 100.

Survival rate (%) = Number of lambs alive / Number of lambs born x 100.

Time periods were recorded according to whether the sheep gave birth during the daylight (06:00-18:00) or at night (18:01-05:59).

Statistical analysis

Estrus duration, gestational length and birth weight were given as mean ± standard error (SEM). These parameters, which showed normal distribution in the Shapiro-Wilk test, were compared with the independent samples t-test according to two seasons. Other reproductive and gynecological characteristics in the two seasons were compared with the Chi-square test. SPSS® (SPSS Version 18.0, Chicago, IL, USA) program was used for statistical analysis. $P<0.05$ was considered statistically significant.

RESULTS

Estrus duration (Fig. 1a) was 33.1 ± 0.8 and 30.8 ± 0.7 hours in the first and second breeding season, respectively ($P=0.034$). The effect of years on gestational length (Fig. 1b) was not statistically significant ($P=0.219$).

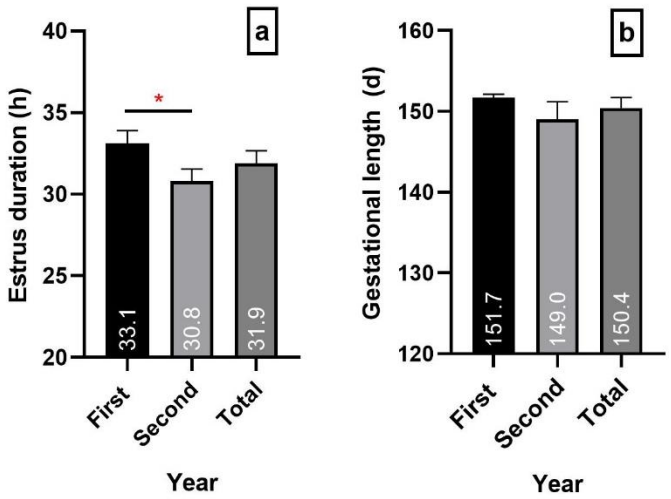


Figure 1. a: Estrus duration (h) in two breeding seasons and total value, b: Gestation length (d) in two breeding seasons and total value. *: The difference between estrus duration in the first and second breeding season was statistically significant (P=0.034).

The differences in estrus, lambing infertility, placental retention, uterine infection, vaginal-uterine prolapse, follicular cyst, mastitis, insufficient milk yield, abortion, dystocia, premature birth, congenital anomaly, lack of maternal instincts, twinning, gender, litter size were not statistically significant (P>0.05) in the two breeding seasons (Table 1).

Table 1. Parameters in two breeding seasons and their total values

Parameters	First Year % (n / Total n)	Second Year % (n / Total n)	Total % (n / Total n)
Estrus	95 (285 / 300)	97 (291 / 300)	96 (576 / 600)
Lambing	90.7 (272 / 300)	92 (276 / 300)	91.3 (548 / 600)
Infertility	5 (15 / 300)	3 (9 / 300)	4 (24 /600)
Placental retention	2.6 (7 / 272)	1.5 (4 / 276)	2 (11 / 548)
Uterine infection	8.2 (23 / 281)	6.6 (19 / 289)	7.4 (42 / 570)
Vaginal – uterine prolapse	1.8 (5 / 272)	2.2 (6 / 276)	2 (11 / 548)
Follicular cyst	1.3 (4 / 300)	0.7 (2 / 300)	1 (6 / 600)
Mastitis	3.7 (10 / 272)	2.2 (6 / 276)	2.9 (16 / 548)
Insufficient milk yield	2.6 (7 / 272)	3.6 (10 / 276)	3.1 (17 / 548)
Abortion	3.2 (9 / 281)	4.5 (13 / 289)	3.9 (22 / 570)
Dystocia	4.4 (12 / 272)	5.4 (15 / 276)	4.9 (27 / 548)
Premature birth	1.1 (3 / 272)	0 (0 / 276)	0.6 (3 / 548)
Congenital anomaly	1.1 (3 / 272)	1.5 (4 / 276)	1.3 (7 / 548)
Lack of maternal instincts	3.7 (10 / 272)	5.4 (15 / 276)	4.6 (25 / 548)
Twinning	10.7 (29 / 272)	12 (33 / 276)	11.3 (62 /548)
Gender ♂	56.8 (171/301)	58.3 (180/309)	57.5 (351 / 610)
Gender ♀	43.2 (130/301)	41.8 (129/309)	42.5 (259 / 610)
Litter size	110.7 (301 / 272)	112 (309 / 276)	111.3 (610 / 548)

The effect of year on birth weight in single lambs was statistically significant (P=0.01). Year had no significant effect on birth weight in twin lambs (P=0.229). However, birth weights

of twin lambs were statistically lower than single lambs in both the first (P<0.001) and the second (P=0.035) year (Fig. 2).

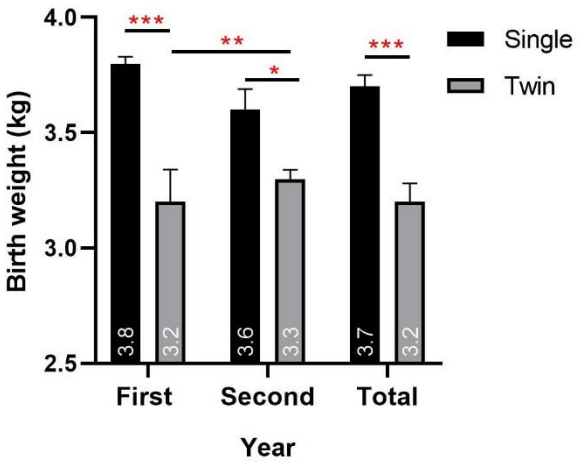


Figure 2. Birth weight (kg) in single and twin lambs in two breeding seasons and total values. *: In the second breeding season, the statistically significant difference between single and twin lamb birth weights was significant (P=0.035). **: The statistical difference between birth weight of single lambs in the first and second breeding season was significant (P=0.01). ***: The statistical difference was significant between the birth weight of the single and twin lambs in the first breeding season and total data (P<0.001).

The survival rate of Morkaraman lambs born in the first breeding season was 94% in the first month and 97.5% in the second month. The survival rate of Morkaraman lambs born in the second breeding season was 94.8% in the first month and 95.6% in the second month. In general, the survival rate was high in the Morkaraman lamb (Table 2).

Table 2. Survival rate of Morkaraman lambs born in two breeding seasons and total values.

Parameter	Season	First month after birth % (n / Total n)	Second month after birth % (n / Total n)	Total % (n / Total n)
Survival rate (%)	First	94 (283/301)	97,5 (276/283)	95.7 (559 / 584)
	Second	94,8 (293/309)	95,6 (280/293)	95.2 (573 / 602)
	Total	94.4 (576 / 610)	96.5 (556 / 576)	95.5 (1132 / 1186)

The Morkaraman ewes gave birth more frequently at night during the two breeding seasons. In the total of two seasons, ewes birth at a rate of 55.85% at night and 46.16% in the daytime (Fig. 3).

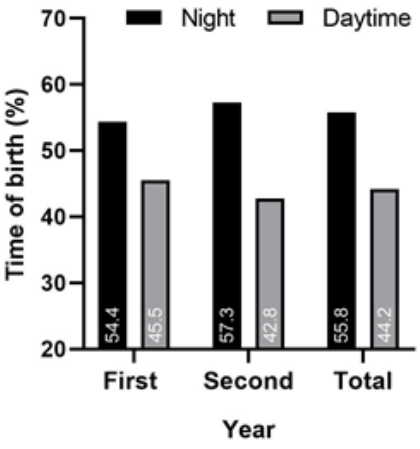


Figure 3. Night or daytime birth rates for two breeding seasons and total values (%). Night: Between 18:00 and 06:00 (h). Daytime: Between 05:59 and 18:01 (h).

DISCUSSION

Sheep breeding can provide an excellent economic gain for families living in rural areas. Many sheep breeds raised in Türkiye and genetic diversity is also high. There are many sheep breeds have been adapted to different feeding methods and different climate and environmental conditions in Türkiye. Morkaraman sheep is also adapted to the harsh climatic and environmental conditions of Eastern Anatolia and is the second most abundant sheep breed in the region. There are generally studies on fattening performance and some yield characteristics about Morkaraman sheep, but there is not enough information about the reproductive and gynecological characteristics. Therefore, in this study, some reproductive and gynecological parameters and lamb yield characteristics of Morkaraman ewes were determined in two breeding seasons.

Sheep are seasonally polyestrous. Behavioral signs of estrus in ewes last 1-2 d and average 35-36 h (Gordon, 1997). The duration of estrus in British ewe breeds is 30 h and may be 10 h shorter in young ewes. The duration of estrus in Merino ewes is 48 h (Robinson & Noakes, 2019). Estrus duration is 37 (Kutluca et al., 2006) or 41-45 h (Emsen & Yaprak, 2006) in Morkaraman ewes. In our study, the estrus duration was between 30-33 h according to two breeding seasons in Morkaraman ewes. Our result was consistent with the duration of estrus in sheep but was shorter than the reported in Morkaraman ewes (Emsen & Yaprak, 2006; Kutluca et al., 2006). This may be due to the use of external hormone injection for estrus synchronization in studies.

The gestation length is generally 143-150 days in ewes (Pugh & Baird, 2012). The gestation length is between 149-151 days in Merinos and Rambouillet ewes (Gordon, 1997), 147-153 days in Menz ewes (Mukasa-Mugerwa & Lahlou-Kassi, 1995), 148.9-154.2 days in Morkaraman ewes (Akçapınar & Kadak, 1982; Gimenezdiaz et al., 2005; Odabaşıoğlu et al., 1996). The gestation lenght in our study was between 149-151 days and it was compatible with the studies. The estrus rate was 89.6% in Ramlıç ewes (Ceyhan et al., 2010), 100% in Kıvırcık ewes (Koyuncu & Akgün, 2018), 92.5% (Emsen & Yaprak, 2006) and 100% (Özbey & Akcan, 2000) in Morkaraman ewes. The lambing rate was 69.4% in Ramlıç ewes (Ceyhan et al., 2010), 76.1% and 81.3% in Horro and Menz ewes, respectively (Berhan & Van Arendonk, 2006), 91.8% in Dorset ewes (Brash et al., 1994), 100% in Kivircik ewes (Koyuncu & Akgün, 2018), and 80%-95% in Morkaraman ewes (Emsen & Yaprak, 2006; Esenbuğa & Dayıoğlu, 2002; Özbey & Akcan, 2000). In our study, the estrus rate was between 95% and 97%, the birth rate was between 90.7% and 92%, and according to the results, it can be said that the reproductive ability of Morkaraman ewes was high.

More than 90% of ewes have a high fertility rate during the breeding season (Scott, 2015). Infertility rate was determined as 6.46% in Morkaraman ewes (Gimenezdiaz et al., 2005), 7.7% in Norduz ewes, 8.9% in Karakaş ewes (Ülker et al., 2004) and 9% in Karagül ewes (Erol & Akçadağ, 2009). In our study, the rate of infertility in Morkaraman sheep was between 3% and 5%, and this rate was lower than in some studies. Infertility is affected by many factors such as season, feeding, lameness, infectious diseases, abortions, and dystocia. Morkaraman ewes do not have much infertility problems and many of them become pregnant during the breeding season.

Placental retention can be caused by selenium or vitamin A deficiency, infectious abortion (e.g., toxoplasmosis, chlamydiosis, listeriosis), obesity, hypocalcemia, dystocia, and is between 1.25% and 1.6% in ewes (Fthenakis, 2004; Fthenakis et al., 2000). We determined an average of 2% placental retention rate in two breeding seasons in Morkaraman ewes. This situation may be affected by factors such as dystocia and abortions. Uterine infections between 4.04% and 5.06% (Khodakaram-Tafti & Davari, 2013; Saberivand & Haghighi, 2006) were detected in ewes in the slaughterhouse. In our study, uterine infection was an average of 7.4%. Uterine infections, which are very low in sheep and goats, may increase after aseptic intervention of dystocia by breeders.

Preparturient vaginal prolapse can be seen in the last month of pregnancy and its incidence is around 1% in ewes. The uterine prolapse rate is 0.1% in ewes (Oral & Kuru, 2016; Scott, 2015). In our study, the rate of vaginal-uterine prolapse in Morkaraman ewes in two breeding seasons was between 1.87% and 2.22%. Cystic ovarian disease is more common in goats than ewes. The incidence in small ruminants can range from 0.01% to 2.4% (Palmieri et al., 2011; Pugh & Baird, 2012; Regassa et al., 2009). In our study, follicular cysts rate in two breeding

seasons was 0.7% to 1.3%, and the rates were consistent with the literature.

The prevalence of clinical mastitis is between 1% and 3% in ewes (Menzies & Ramanoon, 2001). Clinical mastitis should be below 5% in sheep herds (Bergonier et al., 2003; Pugh & Baird, 2012). In our study, the incidence of mastitis was between 2.17% and 3.68%. Colostrum may be insufficient for lambs in 30% of ewes that give birth to twins or triplets and in 10% of ewes that give birth to single (Nowak & Poindron, 2006). In small ruminants, the colostrum after parturition to be less than 50-100 mL is known as insufficient milk yield. Insufficient milk yield was determined as 5.6% in Gurcu goats (Kuru et al., 2017a). In our study, the insufficient milk yield in two breeding seasons was between 2.6% and 3.6%. Insufficient milk yield problem was observed more frequently in primiparous ewes.

Small ruminants have a higher abortion incidence compared to other farm animals, and the overall abortion rate is 5% (Pugh & Baird, 2012). Abort rate is 2.5% in Karakaş and Norduz ewes (Ülker et al., 2004) and 3.8% in Gıcık ewes (Çimen et al., 2003). In our study, the abortion rate was 3.9% in Morkaraman ewes. Dystocia rate in small ruminants is between 3-5%. Although dystocia is uncommon, mortality may be high in lambs in such cases (Kuru et al., 2016; Rook et al., 1990). It has been reported that 9% of lamb deaths are caused by dystocia (Refshauge et al., 2016). In our study, the average dystocia rate was 4.9% in Morkaraman ewes. Congenital anomaly rate in lambs can vary between 0.2% and 2.0%. In addition, the mortality rate can be 50% in this type of lamb (Dennis, 1993; Tuzcu, 2015). In our study, congenital anomaly rates were 1.1% and 1.5% in two breeding seasons.

The twinning rate is 8-28% (Akçapınar et al., 1982; Emsen & Yaprak, 2006; Turkyilmaz & Esenbuga, 2019) and litter size is 1.13-1.28 in Morkaraman ewes (Kayalık & Bingöl, 2015). In our study, twinning rate was 11.3% and litter size was 1.11 and our results were consistent with the literature. Birth weight of Morkaraman lambs was determined as 3.6-4.7 kg in singleton and 3.4-3.6 kg in twin (Emsen & Yaprak, 2006; Kopuzlu et al., 2014). In our study, the average birth weight in single and twin lambs was 3.7 and 3.2 kg, respectively. The birth weight of twin lambs was lower than the literature. This may be due to the younger and lower body weight of the ewes or feeding differences.

Mortality rate in lambs and kids should be below 15% until weaning and aggressive prevention are taken in mortality above this rate (Mukasa-Mugerwa et al., 2002). The survival rate is 83.6% in Horro ewes (Berhan & Van Arendonk, 2006), 85% in Dorset ewes (Brash et al., 1994), 94.9% in Ramlıç ewes (Ceyhan et al., 2010) and 93.4% in Morkaraman ewes (Odabaşıoğlu et al., 1996). The survival rate of Morkaraman lambs 30 and 60 days after birth was determined as 100% and 93%, respectively (Özbey & Akcan, 2001). In our study, the survival rate of Morkaraman lambs 30 and 60 days after birth was 94.43% and 96.52%, respectively. According to our results, we can say that survival rate is high and mortality rate is low in Morkaraman lambs.

CONCLUSION

In conclusion, Morkaraman ewes, which is one of the local gene resources in the Eastern Anatolia Region and adapted to the harsh climate-geographical conditions, may be superior to most of the native sheep breeds in terms of reproductive performance. In addition, Morkaraman ewes has a high reproductive performance, does not have many reproductive / gynecological and udder problems, mostly gives birth to singleton and lambs have a high survival rate.

ACKNOWLEDGMENT

Ethical approval:

This study was carried out after the approval obtained from the Kafkas University Animal Experiments Ethics Committee, (HADYEK 2018/071) and the permission obtained from the Ministry of Agriculture and Forestry.

Conflict of interest: There is no conflict of interest between the authors

REFERENCES

Akçapınar, H. (1994). *Koyun yetiştiriciliği*. Ankara: Medisan Kitabevi.

Akçapınar, H., Aydın, İ., & Kadak, R. (1984). Morkaraman koyunlarının Erzurumda özel bir işletmede kuzu ve süt verimleri. *Ankara Üniversitesi Veteriner Fakültesi Dergisi*, *31*(1), 114–127.

Akçapınar, H., & Kadak, R. (1982). Bazı faktörlerin Akkaraman ve Morkaramanlarda gebelik süresi ve doğum ağırlığı üzerine etkileri. *Ankara Üniversitesi Veteriner Fakültesi Dergisi*, *29*, 392–400.

Akçapınar, H., Kadak, R., & Odabaşıoğlu, F. (1982). Morkaraman ve Kangal-Akkaraman koyunlarının döl verimi ve süt verimi üzerinde karşılaştırmalı araştırmalar. *Ankara Üniversitesi Veteriner Fakültesi Dergisi*, *29*, 379–391.

Bekyürek, T. (2017). Koyunlarda infertiliteyle seyreden hastalıkların tanı ve tedavi yöntemleri. *Türkiye Klinikleri Veteriner Bilimleri-İç Hastalıkları - Özel Konular*, *3*: 61–74.

Bergonier, D., de Crémoux, R., Rupp, R., Lagriffoul, G., & Berthelot, X. (2003). Mastitis of dairy small ruminants. *Veterinary Research*, *34*(5), 689–716. doi: 10.1051/vetres:2003030

Berhan, A., & Van Arendonk, J. (2006). Reproductive performance and mortality rate in Menz and Horro sheep following controlled breeding in Ethiopia. *Small Ruminant Research*, *63*(3), 297–303. doi: 10.1016/j.smallrumres.2005.03.003

Brash, L. D., Fogarty, N. M., & Gilmour, A. R. (1994). Reproductive performance and genetic parameters for Australian Dorset sheep. *Australian Journal of Agricultural Research*, *45*(2), 427–441. doi: 10.1071/AR9940427

Ceyhan, A., Sezenler, T., Yildirir, M., & Erdogan, I. (2010). Reproductive performance and lamb growth characteristics of Ramlıç sheep. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, *16*(2), 213–216. doi: 10.9775/kvfd.2009.529

Çimen, M., Soylu, D., Soylu, İ., & Özsoy, M.K. (2003). Gıcık koyunlarında vücut ölçüleri, döl verimi ve kuzularda büyüme özellikleri. *Lalahan Hayvancılık Araştırma Enstitüsü Dergisi*, *43*(1), 29–34.

Dennis, S. M. (1993). Congenital defects of sheep. *The Veterinary Clinics of North America: Food Animal Practice*, *9*(1), 203–217. doi: 10.1016/S0749-0720(15)30681-2

Emsen, E., & Yaprak, M. (2006). Effect of controlled breeding on the fertility of Awassi and Red Karaman ewes and the performance of the offspring. *Small Ruminant Research*, *66*(1–3), 230–235. doi: 10.1016/j.smallrumres.2005.09.022

Erol, H., & Akçadağ, H. İ. (2009). Halk elinde yetiştirilen Karagül koyun sürülerinde bazı verim özellikleri. *Lalahan Hayvancılık Araştırma Enstitüsü Dergisi*, *49*(2), 91–104.

Esenbuğa, N., & Dayıoğlu, H. (2002). İvesi ve Morkaraman koyunlarının döl verim özelliklerine kimi çevre faktörlerinin etkileri. *Turkish Journal of Veterinary and Animal Sciences*, *26*(1), 145–150.

Fthenakis, G. C. (2004). Effects of retention of fetal membranes on subsequent reproductive performance of dairy ewes. *Theriogenology*, *61*(1), 129–135. doi: 10.1016/S0093-691X(03)00188-2

Fthenakis, G. C., Leontides, L. S., Amiridis, G. S., & Saratsis, P. (2000). Incidence risk and clinical features of retention of foetal membranes in ewes in 28 flocks in southern Greece. *Preventive Veterinary Medicine*, *43*(2), 85–90. doi: 10.1016/S0167-5877(99)00088-4

Gimenezdiaz, C. A., Emsen, E., Koycegiz, F., Emsen, B., Yaprak, M., & Kutluca, M. (2005). Synchronization of estrus in fat tailed sheep using melengestrol acetate (MGA) in the breeding season. *Journal of Applied Animal Research*, *28*(1), 25–27. doi: 10.1080/09712119.2005.9706782

Gordon, I. (1997). *Controlled Reproduction in Sheep & Goats. Controlled Reproduction in Farm Animal Series - Volume 2*. USA: CAB International.

Kayalık, M. Ş., & Bingöl, M. (2015). Tüm yönleriyle Morkaraman koyunları. *Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi*, *5*(2), 89–97.

Kaymakçı, M. (2016). *İleri Koyun Yetiştiriciliği*. İzmir: Meta Basım.

Kenyon, P., Maloney, S., & Blache, D. (2014). Review of sheep body condition score in relation to production characteristics. *New Zealand Journal of Agricultural Research*, *57*(1), 38–64. doi: 10.1080/00288233.2013.857698

Khodakaram-Tafti, A., & Davari, A. (2013). Congenital and acquired abnormalities of reproductive tract of non-pregnant ewes slaughtered in Fars province, Iran. *Iranian Journal of Veterinary Research*, *14*(2), 140–144. doi: 10.22099/IJVR.2013.1588

Kırmızıbayrak, T., Aksoy, A. R., Saatcı, M., & Tilki, M. (2005). Tuj ve Morkaraman koyunların süt verimi ve meme özellikleri ile bu özellikler arasındaki ilişkiler. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*, *11*(1), 11–15.

Kopuzlu, S., Sezgin, E., Yuksel, S., Ozluturk, A., Biberoglu, O., Esenbuga, N., Bilgin, O. C., Bayram, M., & Keskin, M. (2014). Phenotypic and genetic parameters for growth characteristics of Morkaraman sheep. *Journal of Applied Animal Research*, *42*(1), 97–102. doi: 10.1080/09712119.2013.822808

Koyuncu, M., & Akgün, H. (2018). Ekstansif koşullarda yetiştirilen Akkaraman melezi koyunlarda süt verimi

ve sütteki bazı bileşenlerin belirlenmesi. *Hayvansal Üretim, 59*(1), 33–40.

Kuru, M., Boğa Kuru, B., Kulaksız, R., Çağın Arı, U., & Oral, H. (2017a). Gürcü keçilerinin bazı reprodüktif özellikleri. *Harran Üniversitesi Veteriner Fakültesi Dergisi*, *6*(2), 119–125.

Kuru, M., Boga Kuru, B., Sogukpinar, O., Cebi Sen, C., Oral, H., & Kirmizibayrak, T. (2020). Oestrus synchronisation with progesterone-containing sponge and equine chorionic gonadotropin in Pirlak ewes during the non-breeding season: Can Toryum improve fertility parameters? *Journal of Veterinary Research*, *64*(4), 573–579. doi: 10.2478/jvetres-2020-0074

Kuru, M., Mülazımoğlu, S. B., & Kaya, D. (2016). Koyun ve keçilerde güç doğum. *Turkiye Klinikleri Veterinary Sciences-Obstetrics and Gynecology - Special Topics*, *2*(1), 74–77.

Kuru, M., Sogukpinar, O., Makav, M., & Cetin, N. (2017b). Effect of barium selenate injections on fertility of Pirlak ewes subjected to estrus synchronization during non-breeding season. *Medycyna Weterynaryjna*, *73*(8), 479–482. doi: 10.21521/mw.5758

Kutluca, M., Emsen, E., Emsen, H., & Gimenez Diaz, C. A. (2006). Postpartum reproductive activity and body weight changes in fat tailed ewes. *Journal of Animal and Veterinary Advances*, *5*(12), 1220–1222.

Menzies, P. I., & Ramanoon, S. Z. (2001). Mastitis of sheep and goats. *The Veterinary Clinics of North America. Food Animal Practice*, *17*(2), 333–358. doi: 10.1016/S0749-0720(15)30032-3

Mukasa-Mugerwa, E., Anindo, D., Sovani, S., Lahlou-Kassi, A., Tembely, S., Rege, J. E. O., & Baker, R. L. (2002). Reproductive performance and productivity of Menz and Horro sheep lambing in the wet and dry seasons in the highlands of Ethiopia. *Small Ruminant Research*, *45*(3), 261–271. doi: 10.1016/S0921-4488(02)00155-4

Mukasa-Mugerwa, E., & Lahlou-Kassi, A. (1995). Reproductive performance and productivity of Menz sheep in the Ethiopian highlands. *Small Ruminant Research*, *17*(2), 167–177. doi: 10.1016/0921-4488(95)00663-6

Nowak, R., & Poindron, P. (2006). From birth to colostrum: Early steps leading to lamb survival. *Reproduction Nutrition Development*, *46*(4), 431–446. doi: 10.1051/rnd:2006023

Odabaşıoğlu, F., Arslan, M., & Yertürk, M. (1996). Morkaraman ve Corriedale x Morkaraman (F1) kuzularda doğum ağırlığı ve yaşama gücüne, Morkaraman koyunlarda gebelik süresine bazı faktörlerin etkisi. *YYU Veteriner Fakultesi Dergisi*, *7*(1–2), 1–7.

Oral, H., & Kuru, M. (2016). Koyun ve keçilerde klinik ve deneysel reprodüktif cerrahi. *Turkiye Klinikleri Veterinary Sciences-Obstetrics and Gynecology - Special Topics*, *2*(1), 83–88.

Özbey, O., & Akcan, A. (2000). Akkaraman, Morkaraman ve İvesi koyunlarının yarı-entansif şartlardaki verim performansı I. Döl ve süt verimi özellikleri. *Veteriner Bilimleri Dergisi*, *16*(1), 109–120.

Özbey, O., & Akcan, A. (2001). Morkaraman, Akkaraman ve İvesi koyunlarının yarı entansif şartlardaki verim performansı II. Kuzularda büyüme ve yaşama gücü özellikleri. *Veteriner Bilimleri Dergisi*, *17*(1), 57–66.

Palmieri, C., Schiavi, E., & Salda, L. Della. (2011). Congenital and acquired pathology of ovary and tubular genital organs in ewes: A review. *Theriogenology*, *75*(3), 393–410. doi: 10.1016/j.theriogenology.2010.09.020

Pugh, D. G., & Baird, A. N. (2012). *Sheep & goat medicine.* Missouri: Saunders-Elsevier.

Refshauge, G., Brien, F. D., Hinch, G. N., & Van De Ven, R. (2016). Neonatal lamb mortality: Factors associated with the death of Australian lambs. *Animal Production Science*, *56*(4), 726–735. doi: 10.1071/AN15121

Regassa, F., Mengesha, D., Dargie, M., & Tolosa, T. (2009). Abattoir evidence on association between uterine and ovarian abnormalities in Ethiopian highland ewes. *Animal Reproduction Science*, *111*(2–4), 384–390. doi: 10.1016/j.anireprosci.2008.03.020

Robinson, B., & Noakes, D. E. (2019). Reproductive physiology of the female. In D. E. Noakes, T.J. Parkinson, & G. C. W. England (Eds.), *Veterinary reproduction and obstetrics* (pp.2–34).W.B. Saunders.

Rook, J. S., Scholman, G., Wing-Proctor, S., & Shea, M. (1990). Diagnosis and control of neonatal losses in sheep. *The Veterinary Clinics of North America: Food Animal Practice*, *6*(3), 531–562. doi: 10.1016/S0749-0720(15)30831-8

Saberivand, A., & Haghighi, M. (2006). Acquired reproductive tract abnormalities of ewes in northwest of Iran: An abattoir survey. *Iranian Journal of Veterinary Research*, *7*(1), 44–48. doi: 10.22099/IJVR.2006.2680

Scott, P. R. (2015). *Sheep medicine*. London: CRC Press.

Şahal, M., Kurtdede, A., İmren, H.Y. (1994). Prematüre asfeksili bir kuzuda asidozis'in sodyum bikarbonat ve glukoz ile sağaltımı. *Ankara Üniversitesi Veteriner Fakültesi Dergisi*, *41*,439–450.

Turkyilmaz, D., & Esenbuga, N. (2019). Increasing the productivity of Morkaraman sheep through crossbreeding with prolific Romanov sheep under semi-intensive production systems. *South African Journal of Animal Sciences*, *49*(1), 185–191. doi: 10.4314/sajas.v49i1.21

Tuzcu, M. (2015). Koyunların konjenital anomalileri. *Turkiye Klinikleri Veterinary Sciences- Internal Medicine - Special Topics*, *1*(3), 18–23.

Ülker, H., Gökdal, Ö., Aygün, T., & Karakuş, F. (2004). Karakaş ve Norduz koyunlarının temel üreme özellikleri bakımından karşılaştırılması. *Yüzüncü Yıl Üniversitesi Tarım Bilimleri Dergisi*, *14*(1), 59–63.

Yalçin, B. C. (1986). *Sheep and Goats in Turkey*. Food and Agriculture Organization of the United Nations.

Yilmaz, O., Cengiz, F., Ertugrul, M., & Wilson, R. T. (2013). The domestic livestock resources of Turkey: Sheep breeds and cross-breeds and their conservation status. *Animal Genetic Resources*, *52*, 147–163. doi: 10.1017/s2078633613000015

Turunç ekstraktı uygulamasının saha koşullarında ishalli ve ishali olmayan buzağılarda dışkı kıvamı ile gizli temizlik üzerine etkinliğinin araştırılması

Investigation the effectiveness of citrus extract application on stool consistency and hide cleanliless in diarrhoeic and non-diarrhoeic calves at field conditions

ÖZET

Fitoterapötik ve doğal ürünlerin kullanımı ve/veya immunonutrisyon yükselen değer taşımaktadır. Doğal ürünlere dönüş ve normalde besinsel öğe gibi görünen fitoterapötik unsurların kullanımı ile veteriner hekimliği alanında sayısız hastalığın sağaltılabildiği görülmektedir. Yüksek ekonomik değere sahip turunçgillerin (Sitrus ailesi) geviş getiren hayvanlarda rasyona katkısı bilinse de, terapötik amaçla kullanımına dair bilinmeyenler mevcuttur. Bu çalışmanın çıkış amacı ülkemizdeki en yeni doğal müstahzarlardan birisi olan turunç çekirdeği ekstraktının ishalli ve ishali bulunmayan 2 farklı grup buzağıda gizli temizlik skoru ile dışkı kıvamı skorları üzerine etkinliğinin belirlenmesi amaçlandı. Aydın İli' nde ticari faaliyette bulunan bir işletmede 11 ishalli, 7 sağlıklı olmak üzere toplamda 18 buzağıda dışkı kıvamı ve gizli temizlik skorları 0 ila 3'lük skalada değerlendirildikten sonra her 2 grup buzağıya da turunç çekirdeği ekstraktı rektal enema şeklinde 1 hafta süre ile 20'şer ml uygulandı. Uygulama sonrası dışkı kıvamı ve gizli temizlik skorlarında azalma (0-1 arası) görüldü. Her iki skorda da belirgin azalma olması turunç çekirdeği ekstraktının etkinliği ile önerilen dozda sahada pratik kullanımına dair ipuçları verebilir.

Anahtar Kelimeler: Buzağı, fitoterapi, ishal, rektal enema, turunçgiller.

ABSTRACT

Phytotherapeutic and natural compounds and/or immunonutrition has gained increased use. It is seen that various diseases in veterinary medicine can be treated with returning to natural products and using of phytotherapeutic compounds that as normally appear to be nutritional elements. Even if it is known that citrus by-products being high economic value can be added to ruminant rations, there are unknowns about therapeutic using. The aim of this study was to state the effect of citrus seed extract, which is one of the novel natural preparations in our country, on hide cleanliness and stool consistency scores in diarrhoeic and non-diarrhoeic 2 different calves group. After evaluating the stool consistency and hide cleanliness which scored on a scale of 0 to 3 in totally 18 calves of 11 with diarrhea and 7 healthy, citrus seed extract was applied to both group by rectal enema with 20 ml for one week in a commercial enterprise farm in Aydın Province. After application, a decrease of stool consistency and hide cleanliness scores (between 0-1) was observed. Significant reduction in both scores after application may give clues about the effectiveness of citrus seed extract and its practical use in the field at the recommended dose.

Keywords: Calf, phytotherapy, diarrhea, rectal enema citrus by-products.

How to cite this article

Alıç Ural, D., Erdoğan, S., Erdoğan, H., Zararyok, G., Doğan, B., Ural K. (2022). Investigation the effectiveness of citrus extract application on stool consistency and hide cleanliless in diarrhoeic and non-diarrhoeic calves at field conditions. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 283-288. https://doi.org/10.31797/vetbio.1014827

Research Article

Deniz Alıç Ural[1a]
Songül Erdoğan[2b]
Hasan Erdoğan[2c]
Gökhan Zararyok[2d]
Barış Doğan[2e]
Kerem Ural[2f]

[1]Faculty Farm, Veterinary Faculty,Aydın Adnan Menderes University, Aydın, Türkiye
[2]Department of Internal Medicine, Veterinary Faculty Aydın Adnan Menderes University, Aydın, Türkiye

ORCİD-
[a]0000-0002-2659-3495
[b]0000-0002-7833-5519
[c]0000-0001-5141-5108
[d]0000-0002-3014-4609
[e]0000-0001-7111-1005
[f]0000-0003-1867-7143

Correspondence
Songül Erdoğan
songultp.09@gmail.com

Article info
Submission: 27-10-2021
Accepted: 04-08-2022
Online First: 18-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

GİRİŞ

Standardize edilmiş aköz-alkolik *Citrus aurantium* (olgunlaşmamış meyvesinden) ekstraktı iştahın düzenlenmesi, kilo kontrolü, performansın iyileştirilmesi ile enerji sağlanmasına yönelik olarak yem katkı unsuru şeklinde tüketime haiz durumdadır. Turunç ürünleri aynı zamanda meyve suyu ve marmelat olarakta tüketilmektedir (Karoui vd., 2010; Stohs ve Shara, 2013). Sitrus grubuna ait yan ürünlerin ruminant beslenmesinde dahi rolü bulunmaktadır. Agro-endüstriyel yan ürünlerin ekonomik olarak önemli olsa da, çiftlik hayvanlarında konvansiyonel beslenme materyali olarak kullanımı pahalı karşılanmaktadır. Geviş getiren hayvanlarda beslenme sistemleri göz önünde bulundurulduğunda lokal olarak elde edilen hayvan yemi yan ürünleri (Hyyü) pratikte karşılığını alternatif olarak bulduğu; rumen mikrobiyel ekosisteminin kendi nutrisyonel ihtiyaçlarını karşılayabilmek adına yüksek oranda strüktürel lif içeren Hyyü'den yaralanabildiği ve bu sayede büyüme, üreme ve üretim sağlanabildiği bilinmektedir (Mirzaei-Aghsaghali ve Maheri-Sis,2008). Ülkemizde yeni müstahzarlardan birisi olarak yem katkı maddesi ya da tamamlayıcı yem sınıfında yer alan turunç çekirdeği ekstraktının (tçe) bu çalışma kapsamında ishalli ve ishali bulunmayan 2 farklı grup buzağıda gizli temizlik skoru (gTs) ile dışkı kıvamı (dK) skorları üzerine etkinliğinin belirlenmesi amaçlandı.

MATERYAL VE METHOD

Saha çalışması

Çalışma Aydın ilinde 128 başlık bir işletmede gerçekleştirildi. Deneysel bir çalışma olmayıp, multidisipliner katılım ile (ziraat zootekni ve veteriner iç hastalıkları alanından çalışma grubu) 11 ishalli, 7 sağlıklı olmak üzere toplamda 18 buzağıda gerçekleştirildi. İshalin etiyolojisine yönelik hızlı test kitleri ile gerçekleştirilen ön çalışmada herhangi bir enfeksiyöz etken belirlenemedi. Buzağıların bireysel kulübelerde barındırıldığı, süt ikame yemine geçildiği öğrenildi.

Dışkı kıvamının belirlenmesi

Dışkı kıvamı 0 ila 3'lük skalada [dışkı skoru 0 = normal kıvam, 1 = yarı oluşmuş veya pasta kıvamında, 2 = gevşek dışkı ve 3 =sulu dışkı] değerlendirildi (Tablo 1). Dışkı skoru≥2-3 olan buzağılarda diyare pozitif kabul edildi (McGuirk, 2008). Bu çalışmada skorlama gerçekleştirilirken elde edilen görsel arşivden bir kısım örneklemeler Resim 1'de gösterilmiştir.

Şekil 1. Dışkı skorları sırası ile a) 2, b) 1, c) 3, d) 2 ve e) 1.

Tablo 1. Dışkı skorlama (McGuirk, 2008)

Dışkı Skoru	Dışkı kıvamı
0	normal kıvamda
1	yarı-şekillenmiş ve pasta kıvamında
2	gevşek dışkı
3	Sulu kıvamda dışkı

Gizli temizlik skorlaması

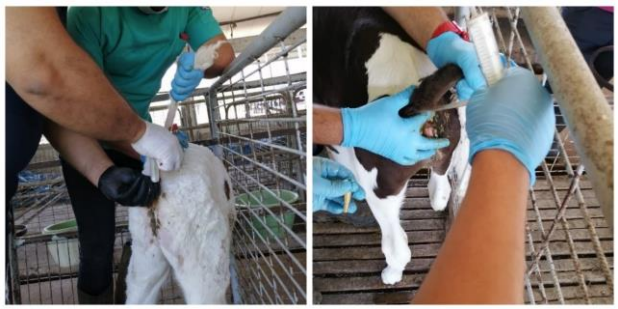
Önemli bir hijyen parametresi olarak değerlendirdiğimiz gTs Tablo 2 dahilinde ilgili literatürler (Panivivat vd., 2004; Sutherland vd., 2014) eşliğinde gerçekleştirildi.

Tablo 2. Gizli temizlik skorlaması (Panivivat vd., 2004; Sutherland vd., 2014)

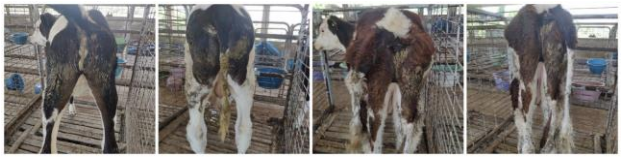
0	Baldır ve tüm vücut temiz, üzerilerinde alt bacaklarda herhangi bir dışkı ya yok ya da çok az
1	Buzağının kuyruk başı ve arka son bölümü dışkı ile bulaşık/kirli
2	Kuyruk başı, arka son bölümü ve baldır ya da bacaklar dışkı ile bulaşık/kirli
3	Kuyruk başı, arka son bölümü ve baldır ile bacaklar dışkı ile bulaşık/kirli

Turunç çekirdeği ekstraktının uygulaması

Çiftlik sahibinden alınan bilgi onam formu ile yem katkı maddesi olarak görünen turunç meyvesi ekstraktı uygulandı. Piyasada mevcut AuraCalf L (Auranta, İrlanda; Türkiye tarafı distribütörü İnterhas Hayvan Sağlığı A.Ş., Ankara) ticari hazır preparatı içerisinde yer alan tçe (sıvı formda, 1.15-1.25 g/cm3 yoğunlukta, kahverengi) rektal enema (Resim 2) şeklinde 20'şer ml 7 gün süre ile günde 1 defa uygulandı.



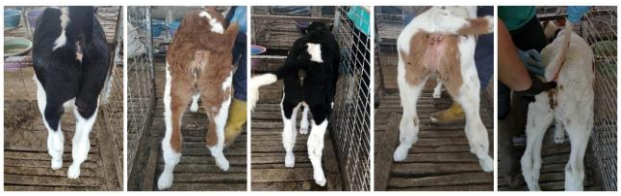
Şekil 2. Araştırmacılar rektal yolla tçe'yi saha koşullarında uygularken. Rektal enema tercih sebebimiz.



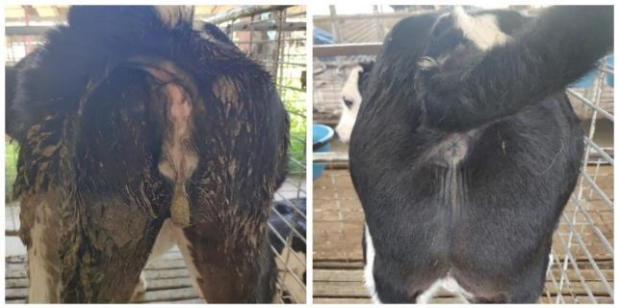
Şekil 3. Gizli temizlik skorları sırası ile a)1, b) 2, c) 3 ve d) 3.

BULGULAR

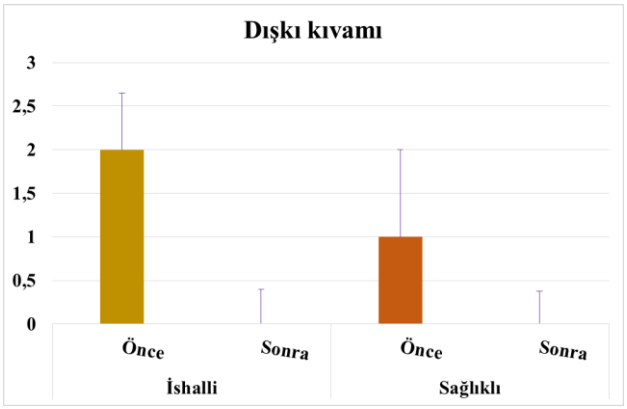
Gereç ve yöntem bölümünde belirtildiği üzere dK ile gTs 0 ile 3 puantaj arası skorlandı. İshalli buzağılarda 1 ile 3 arası değişen dK'ya ait skorlar tçe uygulaması sonrası 2 olguda 1 seviyesine gerilerken diğerlerinde 0 olarak saptandı. Sağlıklı olgularda yine başlangıçta herhangi bir uygulamada bulunulmadan önce 0 ile 2 arasında değişen dK skorları 1 olguda 1 iken diğerlerinde 0 olarak saptandı. Diğer yandan gTs' ye bakıldığında ishalli buzağılarda 2-3 arası değişirken, tçe uygulaması sonrası 0-1 arası saptandı (Resim 3-5). Yine sağlıklı buzağılarda uygulama öncesi ve sonrası sırası ile 0-3 ile 0-1 arası değiştiği görüldü. Şekil 6 ile 7'de skorlara ait bar grafik görünümlere yer verildi.



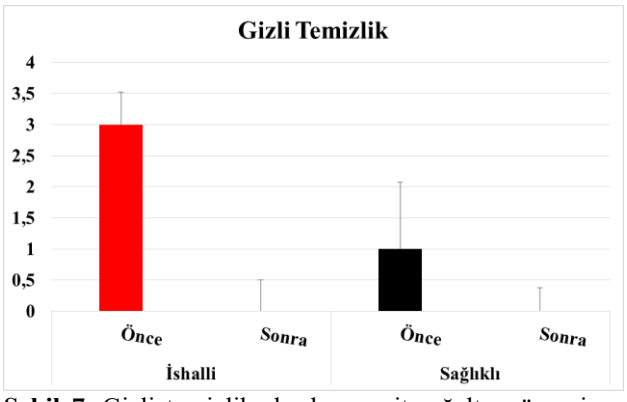
Şekil 4. Gizli temizlik skorları sırası ile a) 0, b)1, c)1, d) 2 ve e) 2.



Şekil 5. Auroracalf uygulaması öncesi ve sonrası gTs ile dK skoru arasında belirgin farklılık mevcut. Uygulama öncesi sırası ile 3 ve 2 olan gTs ile dK, uygulama sonrası 7. günde sırası 0 ve 1.



Şekil 6. Dışkı kıvamına ait skorlara ilişkin sağaltım öncesi ve sonrası bar grafik görünüm.



Şekil 7. Gizli temizlik skorlarına ait sağaltım öncesi ve sonrası bar grafik görünüm.

TARTIŞMA

Sitrus yan ürünlerinin düşük maliyet nedeni ile yem katkı maddesi olarak kullanılabilirliği mevcut olup sığır rasyonlarında yer verilebilmektedir. Böylelikle sitrus yan ürünlerinin ruminal mikroorganizma havuzunda karışarak gerek *Escherichia coli*, gerekse Salmonella türlerinin gelişimin durdurduğu tespit edilmiştir (Duoss-Jenning vd., 2013). Sitrus cinsi meyve ağacı türlerinin içine alan bitki topluluğu; narenciye ya da turunçgiller aslında ekonomik değeri yüksek özelliktedir. Bu yönü ile taze sitrus küspesi, sitrus silajı, kurutulmuş sitrus küspesi, sitrus yaprakları ve gövdesi, sitrus melası, narenciye kabuğu likörü, aktif narenciye silajı olarak geviş getiren hayvanların beslenmesinde kullanılabilmektedir. Sitrus Hyyü yüksek enerji kaynağı olarak büyümeyi destekleyici amaçla kullanılırken, nişastaca zengin yemlere oranla, rumen fermentasyonu üzerine çok az olumsuz etkisi mevcuttur (Bampidis ve Robinson, 2006). Bizim bu çalışmamızda rafta ticari ve ithal hazır bir ürün olan AuraCalf L (Auranta, İrlanda, Türk tarafı distribütörü İnterhas A.Ş., Ankara, Türkiye) isimli preparat içerisinde yer alan tçe kullanıldı.

Turunç, Latince *Citrus aurantium L.* ya da İngilizce tabirle Bitter orange, nutrisyonel destek ya da gıda ürünleri ile meyve sularında kullanılabilen önemli bir üründür. İçerisinde yer alan primer aktif bileşen unsur p-sinefrin, toplam protoalkaloidlerin %90'nını teşkil etmektedir. Ratlarda %50'lik standardize edilmiş p-sinerfin sırası ile 100, 300 ve 1000 mg/kg/gün dozda 3 ay süre ile uygulanarak subkronik güvenliği ya da toksisitesi tayin edilmiştir. İncelenen parametrelerle nörolojik, nefrolojik, oftalmolojik, hematolojik ya da serum biyokimyasal değişkenlerin etkilenmemesi ya da yan etki olmayışı dikkat çekicidir. En yüksek doz olan 1000 mg/kg uygulamada dahi yan etki olarak ilişkilendirilmeyen tamamen geri dönüşümlü bazı klinik bulgular saptanmış, geri dönüşümlü BUN ve üre artışları gözlemlenmiştir. İlgili çalışmada 300 mg/kg üzerinde etkinlik alt sınırı belirlenerek, 1000 mg/kg dozda herhangi bir yan etki şekillenmeksizin yüksek güvenilirlikle kullanım önerilmiştir (Deshmukh vd., 2017). Bu çalışmada rektal enema şeklinde (acımtırak tadı nedeni ile oral değil, rektal kullanım tercih edilmiştir) kullanılan müstahzara ilişkin önerilen dozda hiçbir yan etki ile karşılaşılmadı. Bu bulgumuzu destekler mahiyette insanlara yönelik önceki bir araştırmada sağlıklı yetişkinlerde günlük 50 mg p-sinefrin tek başına kullanımının herhangi bir yan etkiye ya da sağlık sorununa neden olmadığı tespit edilmiştir (Stohs ve Ratamess, 2017).

P-sinefrinin anti-inflamatuvar aktivitesinin belirlenmesine yönelik çokça çalışma literatürde yerini almıştır. Anılan etken maddenin NF-κB sinyalizasyon yolağını inhibe ederek farelerde lipopolisakkarid kaynaklı akut akciğer hasarını baskılamıştır (Wu vd., 2014). Lipopolisakkarid ile indüklenen farelerde p-sinefrinin akciğerde yangısal hücrelerin miktarını azalttığı, reaktif türlerin seviyesini düşürdüğü, superoksit dismutaz aktivitesini uyararak, tümör nekrozan faktör alfa ve interlöykin-6'yı azalttığı buna karşın interlöykin-10'u arttırdığı belirlenmiştir. İlgili çalışmada dozajlamanın farelerde 5 ila 15 mg/kg olduğu; bunun 80 kiloluk bir insanda sırası ile 32.4 ve 97.3 mg' karşılık denk geldiği saptanmıştır (Wu vd., 2014). Bizim çalışmamızda üretici firma tarafından buzağı başına günlük oral ya da süt ile 5 gr/gün önerilen tçe, rektal enema şeklinde 20'şer ml uygulandı.

Sitrus meyvelerinin yüksek miktarda polifenol içerdiğinden, insan fizyolojisi üzerinde koruyucu etkiye sahiptir (Zhang vd., 2018). *Citrus aurantium L.* vb. çok sayıda sitrus meyvesi tıbbi amaçlarla kullanılmakta; antioksidan, anti-inflamatuvar ve hepatoprotektif etkinlikleri öne çıkmaktadır (Liu vd., 2020). *Citrus aurantium L.* 'nin total

fenolik ekstraktı diyetle gelebilecek flavanoid (glikozil derivatları) flavonlar, flavonolller, polimeoksiflavonlar ve kumarinler içermektedirler (Liu vd., 2020). Sitrus sağaltımının ilk hasat kuersetin içeriğinin bağırsak mikrobiyotasında değişikliklere yönelik olumlu yönde etkileşime neden olduğu bildirilmektedir (Jin vd., 2010; Minamisawa vd., 2017). *Citrus aurantium L.* 'nin total fenolik ekstraktı doğal olarak insan sağlığı üzerinde yarar sağlamaktadır. Karaciğerde üretilen safra asitleri, sonrasında bağırsak mikrobiyotası vasıtasıyla bağırsakta metabolize edilmektedir. Faklı birçok çalışma safra asitleri kompozisyonudaki değişim ile antibiyotik kaynaklı disbiyozis arasındaki ilişkiyi irdelemiştir (Swann vd., 2011). Bağırsak mikrobiyotasındaki değişime neden olan faktörlerin anlaşılması ile buna yönelik sağaltım stratejilerinin bağırsak ekolojisinin restorasyonu ile safra asitleri metabolizmasına yönelik olduğu anlaşılmaktadır. Karaciğer-bağırsak ekseni karaciğeri bağırsaklara bağlarken, her iki organında sağaltımına yönelik yeni perspektif gelişimine salık vermektedir.

Antibiyotik kullanımının buzağı ishallerinde ne kadar yaygın olduğu ('malumun ilanı') bilindiğinden sitrus ile gerçekleştirilen önceki bir çalışmanın sonuçları dikkate alınmalıdır. Antibiyotik kullanılan farelerde serumda endotoksin seviyelerini bariz şekilde artırdığı, bunun da antibiyotik kullanımı sonrası kontrol grubuna göre bağırsak permeabilitesinin arttığına işaret ettiği belirlenmiştir. Aynı çalışmada *Citrus aurantium L.* 'nin total fenolik ekstraktı ve probiyotik uygulanan farelerde ZO-1 ile okludin seviyelerinin belirgin şekilde arttığı ve endotoksin seviyelerinini dramatik şekilde azaldığı belirlenmiştir (Liu vd., 2020). Benzer bir durum ilgili analizler gerçekleştirilemese de (kısıtlı ve self-bütçe nedeniyle) bu çalışmaya dahil edilen buzağılarda gereçli olmuş olabilir. Destekleyici olarak gT ve dK skorlarındaki belirgin değişim tçe'nin etkinliği ile bağırsak sağlığı üzerine olumlu etkilerine işaret eder niteliktedir. Nitekim farelerde *Citrus aurantium L.* 'nin total fenolik ekstraktının güçlü sağaltım etkinliği intestinal permabilitenin düzeltilmesi ile ilişkidedir (Liu vd., 2020). Son sözü edilen fenolik ekstrakt mikrobiyel biyoçeşitliliği düzenlemekte, bağırsak mikrobiyotası topluluğunun yapısını restore etmekte, bozulan bağırsak geçirgenliğini tamir ederek (bağırsak bariyer bütünülüğünün sağlanması) safra asidi metabolizmasını düzenlemektedir (Liu vd., 2020). Anılan değişimlerin bizim çalışmamızda klinik skorlandırma esasına dayalı parametrelere ilişkin destekleyici unsurları olarak bağırsak mikrobiyomu analizleri ile ilgili biyobelirteç tayini, bir sonraki çalışmamızın ana hedefi olacaktır. Yine çalışmamızın hedef ve çıktıları ile ilişkide olarak yüksek antioksidan içeriği ile tamamlayıcı yem unsuru olan tçe süt emen buzağı, kuzu ve oğlaklarda kullanıma haiz olup, doğal bitkisel ektrakt oluşu ile organik asit içeriği sayesinde bağırsak sağlığını destekleyebilmektedir.

SONUÇ

Sonuç olarak çalışmamızda büyükbaş hayvan yetiştiriciliğinde daha çok yem katkı maddesi olarak kullanılan tçe' nin ishalli buzağılarda rektal enema şeklinde uygulanabilirliği ortaya konulmuş olup non-enfeksiyöz ishalli buzağılardaki etkinliği dK ve gTs' deki uygulama sonrası azalma ile desteklenmiştir.

AÇIKLAMALAR

Etik beyan: 15.02.2014 tarihli 28914 sayılı gazetede yayınlanan Hayvan Deneyleri Etik Kurulu Çalışma Usul ve Esaslarına Dair Yönetmeliğin 2. Maddesinin b bendinde deneysel olmayan klinik veteriner hekimliği uygulamalarında etik kurul onayına gerek olmadığı beyan edilmiştir. İlaveten

aydınlatılmış hasta sahibi bilgi onam formu alınmıştır.

Çıkar çatışması: Yazarlar çıkar çatışması olmadığını beyan ederler.

KAYNAKLAR

Bampidis, V. A., & Robinson, P. H. (2006). Citrus by-products as ruminant feeds: A review. *Animal Feed Science and Technology*, *128*(3-4), 175-217. doi: 10.1016/j.anifeedsci.2005.12.002.

Deshmukh, N. S., Stohs, S. J., Magar, C. C., Kale, A., & Sowmya, B. (2017). Bitter orange (Citrus aurantium L.) extract subchronic 90-day safety study in rats. *Toxicology Reports*, *4*, 598-613. doi: 10.1016/j.toxrep.2017.11.002.

Duoss-Jennings, H. A., Schmidt, T. B., Callaway, T. R., Carroll, J. A., Martin, J. M., Shields-Menard, S. A., & Donaldson, J. R. (2013). Effect of citrus byproducts on survival of O157: H7 and non-O157 Escherichia coli serogroups within in vitro bovine ruminal microbial fermentations. *International Journal of Microbiology*, 2013. doi: 10.1155/2013/398320.

Jin, M. J., Kim, U., Kim, I. S., Kim, Y., Kim, D. H., Han, S. B., & Yoo, H. H. (2010). Effects of gut microflora on pharmacokinetics of hesperidin: a study on non-antibiotic and pseudo-germ-free rats. *Journal of Toxicology and Environmental Health, Part A*, *73*(21-22), 1441-1450. doi: 10.1080/15287394.2010.511549.

Karoui, I. J., Wannes, W. A., & Marzouk, B. (2010). Refined corn oil aromatization by Citrus aurantium peel essential oil. *Industrial Crops and Products*, *32*(3), 202-207. doi: 10.1016/j.indcrop.2010.04.020.

Liu, X., Wang, Y., Chen, Y., Xu, S., Gong, Q., Zhao, C., & Sun, C. (2020). Characterization of a flavonoid 3'/5'/7-O-methyltransferase from Citrus reticulata and evaluation of the in vitro cytotoxicity of its methylated products. *Molecules*, *25(*4), 858. doi: 10.3390/molecules25040858.

McGuirk, S. M. (2008). Disease management of dairy calves and heifers. *Veterinary Clinics of North America: Food Animal Practice*, *24*(1), 139-153. doi: 10.1016/j.cvfa.2007.10.003.

Minamisawa, M., Suzuki, K., Kawai, G., Yamaguchi, A., & Yamanaka, S. (2017). Functional evaluation of yuzu (Citrus junos) extracts containing limonoids and polyamine for life extension. *Journal of Functional Foods*, *38*, 591-600. doi: 10.1016/j.jff.2017.09.043.

Mirzaei-Aghsaghali, A., & Maheri-Sis, N. (2008). Nutritive value of some agro-industrial by-products for ruminants-A review. *World Journal of Zoology*, *3*(2), 40-46.

Panivivat, R., Kegley, E. B., Pennington, J. A., Kellogg, D. W., & Krumpelman, S. L. (2004). Growth performance and health of dairy calves bedded with different types of materials. *Journal of Dairy Science*, *87*(11), 3736-3745. doi: 10.3168/jds.S0022-0302(04)73512-2.

Stohs SJ, Shara M. (2013). Review of the safety and efficacy of Bitter Orange (Citrus aurantium) and its primary protoalkaloid, p-Synephrine, in weight management. In: D. Bagchi, & H. G. Preuss (Eds), *Obesity: Epidemiology, Pathophysiology, and Prevention*. (pp. 903-918). CRC Press.

Stohs, S. J., & Ratamess, N. A. (2017). Effects of p-synephrine in combination with caffeine: a review. Nutrition and Dietary Supplements, 9, 87-96. Doi: 10.2147/NDS.S144761.

Sutherland, M. A., Worth, G. M., & Stewart, M. (2014). The effect of rearing substrate and space allowance on the behavior and physiology of dairy calves. *Journal of dairy Science*, *97*(7), 4455-4463. doi: 10.3168/jds.2013-7822.

Swann, J. R., Want, E. J., Geier, F. M., Spagou, K., Wilson, I. D., Sidaway, J. E., & Holmes, E. (2011). Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. *Proceedings of the National Academy of Sciences*, *108*(1), 4523-4530. doi: 10.1073/pnas.1006734107.

Wu, Q., Li, R., Soromou, L. W., Chen, N., Yuan, X., Sun, G., & Feng, H. (2014). p-Synephrine suppresses lipopolysaccharide-induced acute lung injury by inhibition of the NF-κB signaling pathway. *Inflammation Research*, *63*(6), 429-439. doi: 10.1007/s00011-014-0715-7.

Zhang, H., yang, Y. F., & Zhou, Z. Q. (2018). Phenolic and flavonoid contents of mandarin (Citrus reticulata Blanco) fruit tissues and their antioxidant capacity as evaluated by DPPH and ABTS methods. *Journal of Integrative Agriculture*, *17*(1), 256-263. doi: 10.1016/S2095-3119(17)61664-2.

An examination of blind mole-rat *(Nannospalax xanthodon)* brain, cerebellum, and spinal cord tissues: A histological and stereological study

Research Article

Ayşe İkinci Keleş[1a]
Burcu Biterge Süt[2b]
Teoman Kankılıç[3c]

[1]Department of Histology and Embryology, Faculty of Medicine, Aksaray University, Aksaray, Türkiye
[2]Department of Medical Biology, Faculty of Medicine, Niğde Ömer Halisdemir University, Niğde, Türkiye
[3]Department of Biotechnology, Faculty of Science and Literature, Niğde Ömer Halisdemir University, Niğde, Türkiye

ORCİD-
[a]0000-0003-0716-5695
[b]0000-0001-5756-5756
[c]0000-0002-9576-5887

Correspondence
Ayşe İKİNCİ KELEŞ
ayse_ikinci@hotmail.com

Article info
Submission: 13-06-2022
Accepted: 07-09-2022
Online First: 18-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

ABSTRACT

The purpose of this study was to perform a histological examination of blind mole-rat (*Nannospalax xanthodon*) brain, cerebellum, and spinal cord tissues. Six blind mole-rats were caught in a natural environment, anesthetized with ether, and sacrificed. Brain, cerebellum, and spinal cord tissues were then removed. All tissues were kept in 10% formaldehyde for one week, at the end of which they were subjected to routine histological procedures and embedded in blocks. Five micron-thick sections were taken from the blocks (5 and 15 micron thick from spinal cord tissues). All sections were then stained with hematoxylin-eosin, Cresyl Violet, and DAPI. These sections were then evaluated under light and fluorescent microscopes. The blind mole-rats weighed 201.3±61 g, the brains and cerebella weighed 1.8±0.3 mg and 0.32±0.05 mg, respectively, and the brain, cerebellum, and spinal cord volumes were 1.49±0.46 ml, 0.33±0.08 ml, and 2.53±0.19 µm^3, respectively. No histological variation was observed in the brain or cerebellum tissues. However, examination of the spinal cord tissue revealed differences compared to other rodents. The spinal cord exhibited a segmented, lobulated appearance, each lobe itself exhibiting the characteristics of a small spinal cord. No butterfly appearance was observed, and white and gray matter transitions were irregular, with less white and more gray matter. The location of the anterior and posterior horns was unclear. The motor neuron cells were also small in size. No significant variations were observed at nuclear organization (DAPI signals) between any tissues. In conclusion, the blind mole-rats compared to rats were normal in weight, increased brain and cerebellum tissue weight and volumes were observed, while a decrease was determined in spinal cord tissue volumes. The brain and cerebellum were normal at histological examination, while structural differences were detected in the spinal cord.

Keywords: Brain, Cerebellum, Spinal Cord, Nanosplax xanthodon, Stereology, Blind mole-rat

INTRODUCTION

Blind mole-rats are organisms adapted to a subterranean life. They spend their entire lives in tunnels they build underground and rarely emerge above the surface. They feed on the roots, rhizomes, and bulbs of root plants they encounter when digging their tunnels (Sözen, 2005). Blind mole-rats are long-lived (>20 years) rodents with tolerance to hypoxia (O_2 as low as 3%) (Figure 1). They have therefore recently been used as a model organism in medical studies. These animals exhibit no aging or age-related disorders or symptoms. Studies of blind mole-rats over an extended period (50 years) have reported no spontaneous tumors among thousands of individuals (Hadid et al., 2012; Kardong, 1995; Keleş et al., 2020; Manov et al., 2013; Nevo et al., 1995; Tian et al., 2013).

How to cite this article
İkinci Keleş, A., Burcu Biterge Süt, B., Kankılıç T. (2022). An examination of blind mole-rat (nannospalax xanthodon) brain, cerebellum, and spinal cord tissues: a histological and stereological study. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 289-295. https://doi.org/10.31797/vetbio.1130201

Such studies have involved various branches of science, such as histology, anatomy, embryology, morphology, ecology, geography, behavior, biochemistry, cytogenetics, molecular biology, and geology. Although numerous studies have been carried out, research into these organisms is continuing due to the fact that they exhibit chromosomal changes, the difficulty of maintaining their habitats in the clinical environment, and that the reasons for their resistance to diseases have not been fully resolved. Some studies of *Nannospalax* species examining the central nervous system have described enlargement of the brain, cerebellum, and motor structures (Frahm et al., 1997). Another study reported that the spinal nerves forming the brachial plexus in the spinal cord and the interconnection of these spinal nerves differ from those of other rodents and mammals (Aydın and Karan, 2012). Although several studies involving the optic nerve have been performed, adequate information has not been obtained. Studies on this subject have reported that although the animal is blind, a structure has developed instead of the eye, and that this is connected to the brain through optic nerves and even a retina (Cernuda-Cernuda at al., 2002; Keleş at al., 2020). It is thought that the absence of visual organs may have led to greater development of the nervous system.

The above factors have all led to blind mole-rats being employed in scientific research. The starting point for the present study was to determine whether any difference exists in the central nervous system of these unique organisms. Our scan of the literature revealed some studies on the subject, although no clarity has been achieved. The purpose of this study was therefore to perform a histological examination of the brain, cerebellum, and spinal cord of the blind mole-rat *(Nannospalax xanthodon)*.

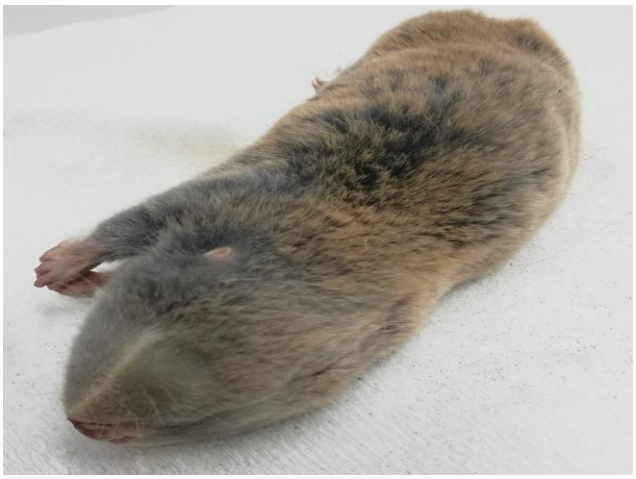


Figure 1: A photo of a blind mole-rat, Nannospalax xanthodon

MATERIAL and METHOD

Location, animals and feeds

Ethics statements and animal care

All animal procedures were approved by the institutional ethics committee at Niğde Ömer Halisdemir University (protocol number 2019/24 dated 10.09.2019) and were carried out in accordance with the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Wild-type *Nannospalax xanthodon* blind mole-rats were obtained from Niğde/Turkey. Six animals were weighed and sacrificed via ether inhalation without being exposed to any treatment. The spinal cord in the upper thoracic region, the brain, and the cerebellum were removed and also weighed.

Histological procedures

The harvested brains and cerebella were fixed in 10% formaldehyde for a week. Paraffin-embedded blocks were prepared through routine procedures. The spinal cord was kept in 10% formaldehyde overnight and subjected to decalcification [formic acid (5 ml), 10% formalin (5 ml), and distilled water (90 ml)] (Boncroft, 1996). At the end of the decalcification process, the tissues were kept in running water overnight. The spinal cord was

then subjected to routine histological procedures.

Five micron-thick transverse sections were cut from the brain, cerebellum, and spinal cord tissues using a Leica Biosystems RM 2245 Microtome (Germany). All sections were stained with Eosin Y (Product Code: HST-EOQ-0500, Histoplus) and hematoxylin Harris (Product Code: HEMH-OT-100, Biognost) for histological evaluation and with cresyl violet (Product Code: C5042). All sections were evaluated under a light microscope (Olympus, BX53, digital camera: DP 80, Olympus and cellSens standard software version 1.17, Japan).

DAPI procedures

Five micron-thick transverse sections were cut from the brain, cerebellum, and spinal cord tissues using a Leica Bio systems RM 2245 Microtome (Germany). Tissue sections were then deparaffinized, washed in phosphate buffered saline washed and kept in 0.4% Triton-X-100 (1x10 minutes). Sections were processed consecutıvely in Twin 20 (3x1 minutes), 2% Paraformaldehyde (1x40minutes), and Twin 20 (3x1 minutes). Next, the sections were blocked with 4% bovine serum albumin (1 hour) and Twin 20 (3x1 minutes). 4′,6-diamidino-2-phenylindole (DAPI) was kept in the dark for 15-20 minutes and then sealed and stored at 4°C in the dark. The resulting sections were evaluated histologically with the aid of a fluorescent microscope attached to a light microscope (Olympus, BX53, Japan) equipped with a digital camera (DP 80, Olympus, Japan) and cellSens standard software (version 1.17).

Brain and cerebellum volume measurement

Archimedes' principle (Keleş et al., 2020) was used to measure the volume of the brains and cerebella. Both organs were submerged separately into a graduated cylinder filled with water. The volume of each tissue was equal to that of the displaced water.

Spinal cord volume measurement

Five tissue sections per animal each 15 microns in thickness, were taken and stained with hematoxylin-eosin (H&E for stereological analyses. The section sampling fraction was 1/10. Volumetric evaluation of the spinal cord tissues was performed using the Cavalieri principle (Keleş and Biterge Süt, 2021) (Table 1) (Figure 2)

Table 1: Parameters used in stereological analysis

Stereological analysis parameters	Values
Section sampling fraction	1/10
Sampled sections from each spinal cord	10
Paraffin section thickness (µm)	15
Objective lens for thickness	x20

Spinal cord volume was calculated manually using a point grid. The following formula was employed when calculating the results -

Ai=Σ Pi x a / p

where **Ai** represents the total section area, **Σ Pi** the total number of points on the section image of interest, and the center of each “plus” (+) sign on the grid represents a specific unit area **(a/p)** (Keleş, 2019).

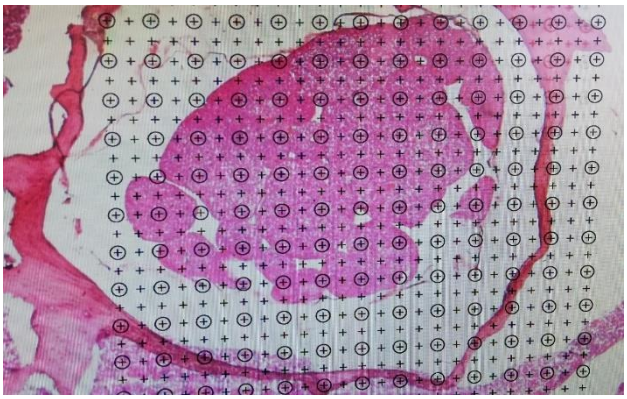


Figure 2: Placing the point-counting probe on the spinal cord tissue (x4, Hematoxylin & Eosin)

Statistical analysis

Statistical analysis was performed on Statistical Package for the Social Sciences (SPSS) version 22.0 software (BM Corporation, Armonk, NY, USA). Values were calculated as mean± standard deviation.

RESULTS

Brain, cerebellum, and spinal cord volume analysis results

The body, brain, and cerebellum volumes of the six blind mole-rats in the study are shown in Table 2**.**

Body, brain, and cerebellum weight

The brain, cerebellum, and spinal cord weights of the six blind mole-rats are shown in Table 2.

Table 2: Weight and volume measurements (n=6)

Measured parameters	
Blind mole-rats (n=6)	**Mean±standard deviation**
Body weight (g)	201.3 ± 61
Brain weight (mg)	1.8 ±0.30
Cerebellum weight (mg)	0.32 ±0.05
Cerebellum volume (ml)	0.33 ±0.08
Brain volume (ml)	1.49±0.46
Spinal cord volume (µm3)	2.53± 0.19

Histopathological evaluation of brain, cerebellum, and the spinal cord tissues

Analysis following H&E and cresyl violet staining of the brain, and cerebellum tissues of the blind mole-rat revealed no histological difference in the brain and cerebellum tissues of the other rodent (Figure 3-4).

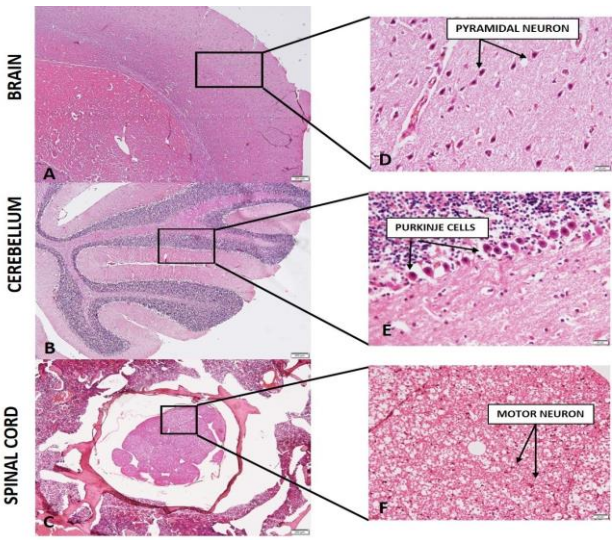


Figure 3: Representative micrographs of the brain (prefrontal cortex), cerebellum, and spinal cord (A, B, C x4; D, E, F x40, Hematoxylin & Eosin)

However, examination of spinal cord tissues revealed differences compared to both humans and other rodents. The pia mater surrounding the spinal cord was normal and easily visible. In general terms, it exhibited a segmented and lobulated appearance. However, the butterfly appearance that would normally be expected in the spinal cord was not observed. White and gray matter transitions were irregular, with less white and more gray matter. The site of the anterior and posterior horn was unclear. Examination of the cellular structures of the spinal cord revealed neuroglial cells and motor and sensory neurons, although their location (in the anterior and posterior horn) was unclear, and the motor neurons were smaller in size. Each lobe contained a cord resembling a small spinal cord, white matter, gray matter, neurons, and glial cells. Ependymal cells were observed around the medulla spinalis. The epithelium of the ependymal cells was single-layered and cuboidal (Figures 3-4).

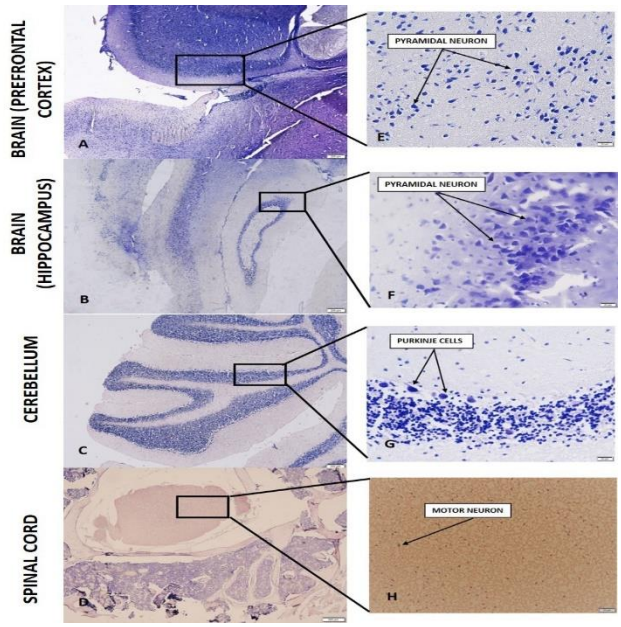


Figure 4: Representative micrographs of the brain (prefrontal cortex and hippocampus), cerebellum, and spinal cord (A, B, C, D x4; E, F, G, H x40, Cresyl viole

Immunofluorescence evaluation of the brain, cerebellum, and spinal cord

Cell signals were evaluated with DAPI staining in areas with dense cells in the brain (hippocampus and prefrontal region), cerebellum, and spinal cord tissues on immunofluorescence images. Cell signals were

localized in the nuclei of the cells in the prefrontal cortex, hippocampus [cornu ammonis 1 (CA1), cornu ammonis 2 (CA2) and cornu ammonis 3 (CA3), dentate gyrus and hilus], cerebellum, and spinal cord regions in the brain (Figures 5-6).

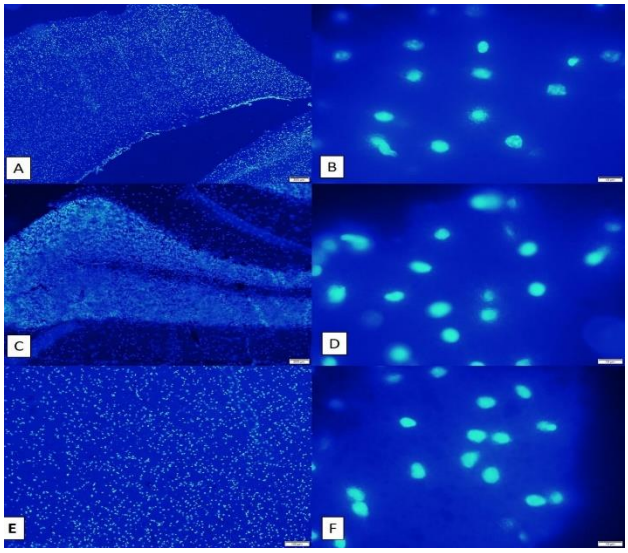


Figure 5: Representative micrographs of the brain (prefrontal cortex) (A, B), cerebellum (D, C), spinal cord (E, F). DNA stained with DAPI (blue) marks the nucleus) DAPI: 4′,6-diamidino-2-phenylindole. (A, C x4, E x10, B, D, F x100)

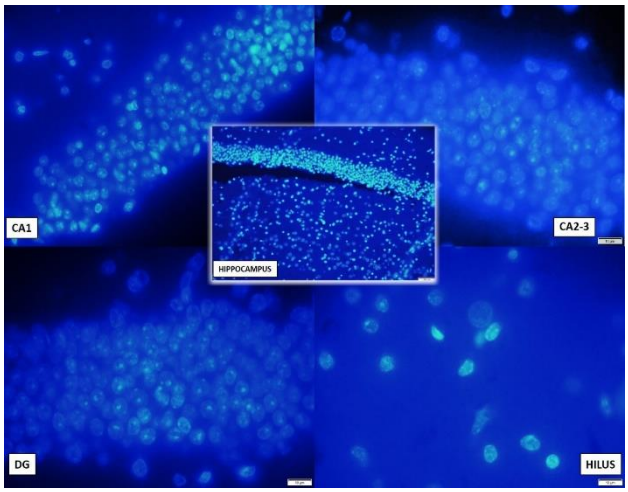


Figure 6: Representative micrographs of the brain. Hippocampus x20 and CA1, CA2-3, DG, Hilus x100. DNA stained with DAPI (blue) marks the nucleus. (C1, cornu ammonis 1; C2-3, cornu ammonis 2-3; DG, dentate gyrus) DAPI: 4′,6-diamidino-2-phenylindole

DISCUSSION

The histological structures of the brain, cerebellum, and spinal cord of the blind mole-rat were examined due to these animals' high tolerance to hypoxia (Nevo et al., 1994), behavior, emotion-perception, and sensitivity to different stresses (Avivi et al., 2005; Avivi et al., 1999), the fact they do not experience cancer (Altwasser et al., 2019), and are even resistant to induced carcinogenesis (Manov et al., 2013).

The bodies, brains, and cerebella of six randomly caught blind mole-rats were weighed. The mean body weight of these six blind mole-rats was 201.3 ± 61g. A previous study reported a mean body weight of 191.7 ± 26.5 g in 25-day-old Sprague Dawley rats (Keleş et al., 2019). Blind mole rats appear have a higher body weight. However, considering the biological, genetic, and environmental factors, we do not think it would be appropriate to directly compare the animals caught in this study with normal rats.

The mean weight of the brains of the blind mole-rats in this study was 1.8 ± 0.3 mg, and the mean weight of the cerebella was 0.32 ± 0.05 mg. A previous study of male Sprague Dawley rats weighing 191.7 ± 26.5 g determined a mean brain weight of 1.2 ± 0.05 mg (Keleş et al., 2019). Another study reported a mean brain weight of 1.43 ± 0.07 mg in female Sprague Dawley rats (mean weight 241.83 ± 20.10 g) (Aktürk et al., 2014). Another study reported a cerebellar weight of 0.19 g in 21-day-old female Wistar rats (Fang et al., 2020). Our findings indicate greater brain and cerebellum weights compared to those of normal rats.

The mean brain and cerebellum volumes of the blind mole-rats in this study were 1.49±0.46 ml and 0.33± 0.08 ml, respectively. Aktürk et al. (2014) determined rat weights of 241.83±20.10 g, with a mean brain volume of 4.61±0.16 mm^3 (0.004 ml). Another study reported a cerebellar volume of 1.4 mm^3 (0.001 ml) in 21-day-old female Wistar rats (Fang et al., 2020).

Frahm et al. (1997) compared the brain and cerebellum volumes of rats and blind mole-rats

and concluded that these were greater in the latter. The present study also suggests a volume increase. The findings show that the increases in the weights and volumes of the brain and cerebellum are directly proportional. Spinal cord volume was also evaluated in this study, and was calculated at 2.53± 0.19 μm^3. We encountered no previous studies investigating spinal cord volume in blind mole-rats. A rat study reported a volume of 2.88±0.26 μm^3 (Keleş and Biterge Süt, 2021). This was lower than the spinal cord volume of blind mole-rats. The volumetric decrease in spinal cord tissue appears not to be normal in the light of the rats' body weights. The volume would normally be expected to be greater than the value determined. In addition, the reasons for the decrease in spinal cord volume, despite greater brain and cerebellum weights and volumes, is unclear.

Histological examination of spinal cord tissue revealed differences compared to humans and other rodents. The pia mater surrounding the spinal cord was normal and clearly visible. It exhibited a segmented, lobulated appearance. However, the butterfly appearance that would normally be expected in the spinal cord was not present. The white and gray matter transitions were irregular, with less white matter being seen and more gray matter. The location of the anterior and posterior horn was unclear. Examination of the cellular structures of the spinal cords revealed neuroglial cells and motor and sensory neurons, although their locations (in the anterior and posterior horns) were unclear. Cells were also smaller than normal compared to other rodents. Each lobe contained a medulla spinalis, white matter, gray matter, neurons, and glial cells, resembling its own small spinal cord. Ependymal cells were observed around the medulla spinalis. The epithelium of the ependymal cells was single-layer and cubic in appearance. These findings suggest the presence of significant differences in the spinal cord structures. One previous study reported that the spinal nerves forming the brachial plexus and the interconnection of these spinal nerves in the spinal cord of blind mole-rats differ from those of other rodents and mammals (Aydın and Karan, 2012). Although this does not fully support the present study, we nevertheless think that it shows the presence of differences in spinal cord structures. Our examination of the literature in terms of the structures of the blind mole-rat spinal cord structures revealed no previous studies on the subject.

No difference was detected in the analysis of cell signals in brain, cerebellum or spinal cord tissues under fluorescent microscopy. No difference was found in terms of structural or genetic (DAPI signal) evaluation when nucleus organization was examined in these tissues.

CONCLUSION

In conclusion, based on a comparison with rata data in the literature, the weight of the blind mole-rats in this study was normal, an increase was observed in volume and weight in brain and cerebellum tissues, and a volumetric decrease in spinal cord tissue. Structural and volumetric differences were also observed in spinal cord tissue, and cells were smaller than other rodents.

ACKNOWLEDGMENT

This study was supported by the Niğde Ömer Halis Demir University Scientific Research Projects Coordination Unit as a Project No: SAT 2021/4-BAGEP

Ethical approval:

All animal procedures were approved by the institutional ethics committee at Niğde Ömer Halisdemir University (protocol number 2019/24 dated 10.09.2019) and were carried out in accordance with the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

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

REFERENCES

Altwasser, R., Paz, A., Korol, A., Manov, I., Avivi, A., Shams, I. (2019). The transcriptome landscape of the carcinogenic treatment response in the blind mole rat: insights into cancer resistance mechanisms. *BMC Genomics*, *8*, 20-17. https://doi.org/10.1186/s12864-018-5417-z.

Aktürk, Z., Odacı, E., İkinci, A., Baş, O., Canpolat, S., Çolakoğlu, S., Sönmez, O.F. (2014). Effect of Ginkgo biloba on brain volume after carotid artery occlusion in rats: a stereological and histopathological study. *Turk J Med Sci*, *44*, 546-53. https://doi.org/10.3906/sag-1305-40.

Aydın, A. ve Karan, M. (2012). The spinal nerves forming the brachial plexus in mole-rats (Spalax leucodon), *Veterinarni Medicina, 57*, 430–433.

Avivi, A., Resnick, M.B., Nevo, E., Joel, A., Levy, A.P. (1999). Adaptive hypoxic tolerance in the subterranean mole rat Spalax ehrenbergi: the role of vascular endothelial growth factor. *FEBS Lett*, *452*, 133–140. https://doi.org/10.1016/S0014-5793(99)00584-0.

Avivi, A., Shams, I., Joel, A., Lache, O., Levy, A.P., Nevo, E. (2005). Increased blood vessel density provides the mole rat physiological tolerance to its hypoxic subterranean habitat. *Federation of American Societies for Experimental Biology (FASEB J)*, *19*(10), 1314-6. https://doi.org/10.1096/fj.04-3414fje.

Cernuda-Cernuda, R., DeGrip, W.J., Cooper, H.M., Nevo, E., García-Fernández, J.M. (2005). The retina of Spalax ehrenbergi: novel histologic features supportive of a modified photosensory role. *Invest Ophthalmol Vis Sci*, *43*, 2374- 83.

Frahm, H.D., Rehkämper, G., Nevo, E. (1997). Brain structure volumes in the mole rat, Spalax ehrenbergi (Spalacidae, Rodentia) in comparison to the rat and subterrestrial insectivores. *J Hirnforsch*, *38*, 209-22.

Fang, Y., Che, X., You, M., Xu, Y., Wang, Y. (2020). Perinatal exposure to nonylphenol promotes proliferation of granule cell precursors in offspring cerebellum: Involvement of the activation of Notch2 signaling. *Neurochem Int*, *140*, 104843. https://doi.org/10.1016/j.neuint.2020.104843.

Hadid, Y., Németh, A., Snir, S., Pavlíček, T., Csorba, G., Kázmér, M., Major, A., Mezhzherin, S., Rusin, M., Coşkun, Y., Nevo, E. (2012). Is evolution of blind mole rats determined by climate oscillations? *PLoS One*, *7*(1):e30043.

Keleş, A.İ. (2019). Sağlık alanında kullanılan kantitatif yöntem, Stereoloji. *Dicle Tıp Dergisi / Dicle Med J, 46*, 615- 621. https://doi.org /10.5798/dicletip.536434.

Keleş, A.İ., Nyengaard, J.R., Odacı, E. (2019). Changes in pyramidal and granular neuron numbers in the rat hippocampus 7 days after exposure to a continuous 900-MHz electromagnetic field during early and mid-adolescence. *J Chem Neuroanat*, *26*,101-101681. https://doi.org/10.1016/j.jchemneu.2019.101681.

Keleş, A.İ., Süt, B.B., Kankiliç, T. (2020). Histopathological analysis of the eye and optic nerve structure in the blind mole rat. Dicle Med J *47* (3): 638-644. . https://doi.org /10.5798/dicletip.800025.

Keleş, A.İ., Süt, B.B. (2021). Histopathological and epigenetic alterations in the spinal cord due to prenatal electromagnetic field exposure: an H3K27me3-related mechanism. *Toxicology and Industrial Health*, *37*(4), 189-197. https://doi.org//10.1177/0748233721996947.

Kardong, K.V. (1995). "Vertebrates'', Comparative Anatomy, Function, Evolution, Dubuque, Melbourne, Oxford, Wm. C. Brown Publishers (Times Mirror International Publishers), *17*, 777.

Manov, I., Hirsh M., Lancu T.C., Malik A., Sotnichenko N., Band M., Avivi A., Shams I. (2013). Pronounced cancer resistance in a subterranean rodent, the blind mole-rat, Spalax: in vivo and in vitro evidence. *BMC biology*, *9*, 11-91.

Nordmann, A. (1840). Observations sur la faune pontique. A. *Demidoff Voyage dans la Russie Meridion*, *3*(35).

Nevo, E., Filippucci, M.G., Redi, C., Simson, S., Heth, G., Beiles, A. (1995). Karyotypeandgeneticevolution in speciation of subterraneanmolerats of thegenusSpalax in Turkey. *BiologicalJournal of theLinneanSociety*, *54*, 203-29. https://doi.org/10.1111/j.1095- 8312.1995.tb01034.x.

Sözen, M. (2005). A biological investigation on Turkish Spalax Guldenstaedt, 1770 (Mammalia: Rodentia). *G.Ü. Fen Bilimleri Dergisi*, *18*(2), 167-181.

Tian, X., Azpurua, J., Hine, C., Vaidya, A., Myakishev-Rempel, M.,Ablaeva, J., Mao, Z., Nevo, E.,Gorbunova, V., Seluanov, A. (2013). High-molecular-mass hyaluronan mediates the cancer resistance of the naked mole rat. *Nature*, *499*, 346-349. https://doi.org/10.1038/nature12234.

Histochemical and immunohistochemical investigation of the effects of Sambucus nigra on mast cells and VEGF in diabetic rat spleen

Research Article

Elif Tuğba Ergel[1a]
Tuğrul Ertuğrul[2b]

[1]Samsun Bafra State Hospital, Samsun, Türkiye
[2]Department of Histology and Embryology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Türkiye

ORCİD-
[a]0000-0001-6734-4834
[b]0000-0002-9310-1200

Correspondence
Tuğrul ERTUĞRUL
tugrulertugrul06@hotmail.com

Article info
Submission: 15-06-2022
Accepted: 10-11-2022
Online First: 19-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

ABSTRACT

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia due to disturbed insulin secretion or insulin function. The purpose of this study was to look into the effect of Sambucus nigra (S. nigra) extract on mast cell number and immunohistochemical expression of VEGF-immune positive cells in experimental diabetic rat spleen. Thirty-two male were used in this study. Control group, Diabetes group, S. nigra group and Diabetes + S. nigra group. When the groups were evaluated, the least number of mast cells (MC) was detected in the control group, whereas the highest number of MCs was observed in the diabetes group. The MC numbers in the S. nigra and diabetes + S. nigra groups were close. The application of S. nigra to diabetic rats resulted in a considerable reduction in the number of MCs, which was a notable finding in our investigation. The number of immune positive VEGF cells increased only in the diabetes group when the groups were examined individually. VEGF expression was similar in the control group, S. nigra group, and diabetes + S. nigra group. As a remarkable finding in our study, it was observed that the application of S. nigra caused a decrease in the number of immune-positive VEGF cells in diabetic rats. As a result, this study showed that S. nigra caused a decrease in the number of MC and immune-positive VEGF cells that increase with diabetes. These findings suggest that MCs may be both a source and a target of VEGFs.

Keywords: Diabetes, mast cell, Sambucus nigra, VEGF

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia due to disturbed insulin secretion or insulin function (Darenskaya et al., 2021). It has been reported that the development of chronic hyperglycemia in diabetes is accompanied by damage, dysfunction, and failure of various organs and tissues, and the development of micro and macrovascular complications (Harding et al., 2019).

Vascular endothelial growth factor (VEGF) is a multifunctional cytokine that plays important role in both normal physiological as well as pathological vasculogenesis and angiogenesis (Leung et al., 1989). It is known to help preserve tissue barrier functions, as well as neuroprotection, endothelial cell proliferation, and migration. Furthermore, VEGF receptors are located on various cells and govern cell survival and physiological function (Huang et al., 2012). Additionally, experimental evidence shows that VEGF plays a central role in mediating diabetes-induced disorders in many organs. It has also been reported that it may cause diabetic complications by affecting glucose levels simultaneously (De Vriese et al., 2001).

How to cite this article
Ergel, ET., Ertuğrul T. (2022). Histochemical and immunohistochemical investigation of the effects of Sambucus nigra on mast cells and VEGF in diabetic rat spleen. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 296-304. https://doi.org/10.31797/vetbio.1131079

Mast cells (MCs) are multifunctional immune cells that play a role in many different health and disease states. The local microenvironment has a direct impact on MC maturation and function. They are known to be able to perceive and respond to different stimuli by releasing a physiologically active sequence mediator (Galli et al., 2011). MCs might be activated by pattern-recognition receptors or tissue injury, and they can express the FcR1 and Fc receptors, allowing them to respond to adaptive immune system targets (Dwyer et al., 2016). Furthermore, MCs are a significant source of cytokines, chemokines, and growth factors. It has been known that VEGF is present in the cytoplasmic granules of MCs and that these cells can secrete VEGF both on their own and in response to activation (McHale et al., 2019). MCs are thought to be important cells that can function in pro- or anti-inflammatory roles in various immune processes such as pathogen clearance and autoimmune diseases (Kumar & Sharma, 2010). Also, MCs are known to play essential roles in preventing obesity and diabetes in experimental animal models (Sismanopoulos et al., 2012).

Natural products are known to provide a numerous supply of bioactive substances that can be employed in medicine. Many plants are used medicinally to prevent and delay illness development and progression, increase health span, and improve quality of life (Yang et al., 2020). Sambucus nigra (S. nigra) is used in traditional medicine because of its rich chemical composition, which contains many bioactive components, including vitamins, minerals, terpenes, sterols, and polyphenols (Ferreira et al., 2020). S. nigra has been also shown to contain water-soluble chemicals that promote intramuscular glucose absorption and utilization and activate insulin release (Gray et al., 2000).

Although there are studies describing the relationship between MCs and VEGF cytokine, there are few studies on the effect of S. nigra on MC distribution and VEGF expression in diabetic spleen tissue. This study aimed to investigate the effects of S. nigra on MC and VEGF immune-positive cell distribution in rat spleen in a model of STZ-induced diabetes.

MATERIAL and METHOD

Animal material

Thirty-two male rats weighing 250-300 g were used in this study. The rats were housed in conventional cages with 12 hours of light and 12 hours of darkness in a 21-23°C ambient temperature environment, and they were fed tap water and ad libitum food. The rats used in the study were randomly divided into four groups equal in number. Experimental groups: Group 1: Control group, Group 2: Diabetes group, Group 3: S. nigra group and Group 4: Diabetes + S. nigra group.

In our study, to form a diabetes model in animals in diabetes and diabetes + S. nigra group, animals received a single dose intraperitoneal (i.p.) injection of streptozotocin (STZ; 50 mg/kg). It was prepared by dissolving 450 mg of STZ (SO130; Sigma-Aldrich, USA) in 10 mL of distilled water (Çetin et al., 2013). After confirming the occurrence of diabetes in rats, S. nigra at a dose of 1.5 mg/kg (Bidian et al., 2021) was administered by oral gavage to all animals in the S. nigra group and diabetes + S. nigra group for 14 days. During the experiment, no application was made to the rats in the control group.

Following the experiment, the rats were sacrificed, and spleen tissue samples were taken. The spleen tissue samples were fixed in a 10% formaldehyde solution, then passed through standard histological tissue processing and blocked in paraffin.

Determination of blood glucose levels

A glucometer (PlusMED Accuro) was used to take blood from the hungry animals' tail vein 8 hours before the start of the trial to determine

their blood glucose level preprandial. Animals involved in the study with a glucose level of 300 mg/dL had their preprandial blood glucose level measured for 8 hours on the 3rd day of STZ practice.

Mast cell histochemistry

To count the MCs, ten serial cross-sections of 5 µm thickness were obtained from the prepared blocks at 30 µm intervals and stained with toluidine blue (92-31-9; Sigma-Aldrich, %0,5 ve pH=0.5) (Enerback, 1966). To determine the numerical distribution of MCs in the prepared serial crosssections, cell counts were performed with a 100 square ocular micrometer. At a magnification of 40x, the MCs in the ocular graticule were counted in per unit. For each piece of spleen tissue, the cells were counted in ten randomly selected regions. All these data were then converted to the number of MCs per 1 mm^2 unit area.

Immunohistochemical staining

To determine the expression of VEGF in 5 µm thick tissue sections taken from spleen tissue, one of the immunohistochemical methods, the "streptavidin-biotin-complex method," was used (True, 1990). The primary antibody utilized in immunohistochemistry was mouse monoclonal VEGF (1/800 dilution, Santa Cruz Biotechnology, sc-7269). Antibody diluent reagent solution was used for reconstitution (Zymed 00-3118). As a secondary antibody, the Histostain® Plus kit was employed (Zymed kit: 85-6743). To reveal the antigen in the tissues, after the deparaffinization process, the sections were taken into a citrate buffer solution and heated in a microwave oven at 700 watts. The same procedure was performed three times, each for 5 minutes. At the end of the process, the sections in the citrate buffer solution were left to cool at room temperature for 20 minutes. Sections washed with Phosphate Buffered Saline (PBS) were incubated in 3% hydrogen peroxide solution for 10 minutes to block endogenous peroxidase activity. After the tissues removed from the PBS solution were thoroughly dried, the serum in the kit was dripped onto them to prevent non-specific protein binding. The blocking solution of the Histostain® Plus kit was used as protein blocking solution. The primary antibody was then dripped over the sections, which were then stored at +4 °C overnight. Only PBS solution was used in negative control group tissues. After washing, the sections were dripped with biotinylated secondary antibody and then incubated in streptavidin-horseradish peroxidase complex. In the last step, 3,3'-diaminobenzidine (DAB) was used as the chromogen (Zymed, 31079800) and the preparations were counterstained with hematoxylin and sealed with enthallan.

Immunohistochemical examination

In the immunohistochemical examination, the distribution of VEGF-positive cells was analyzed semiquantitatively. The following criteria were used in the evaluation: no stained cells in the scanned area no positive cell (–), 1-2 cells (±), 3-4 cells (+), 5-6 cells (++), 7 and more cells (+++) (Ertuğrul et al., 2021).

Statistical analysis

The Shapiro-Wilk test was employed to determine normality. One-way analysis of variance (ANOVA) was performed to assess the data depending on the normality of the data, and Duncan's test was employed to identify any differences within groups. Obtained data are shown as mean ± standard error of the mean (mean ± SEM). A p-value of <0.05 was considered statistically significant

RESULTS

Histochemical findings

MCs were metachromatically stained with toluidine blue in the spleen tissue of all groups, and their granules could not be distinguished individually. Morphological differences were not observed among the MCs in the whole

group, and cells were determined to be round and oval with different sizes. When the location of MCs in the spleen tissue is examined, they are rarely observed in the subcapsular region. It was remarkable that MCs were localized in the red pulp sinusoids. MCs were rarely found in the lymph follicles of the white pulp in our study. Moreover, MCs were not seen in the splenic cords. While MCs, which were found individually in the white pulp, were predominantly seen in the red pulp, either alone or groups.

When the groups were evaluated, the least number of MCs was detected in the control group, whereas the highest number of MCs was observed in the diabetes group. The MC numbers in the S. nigra and diabetes + S. nigra groups were close. The application of S. nigra to diabetic rats resulted in a considerable reduction in the number of MCs, which was a notable finding in our investigation. Table 1 shows the mean number of MCs in each group after staining toluidine blue (Figure 1).

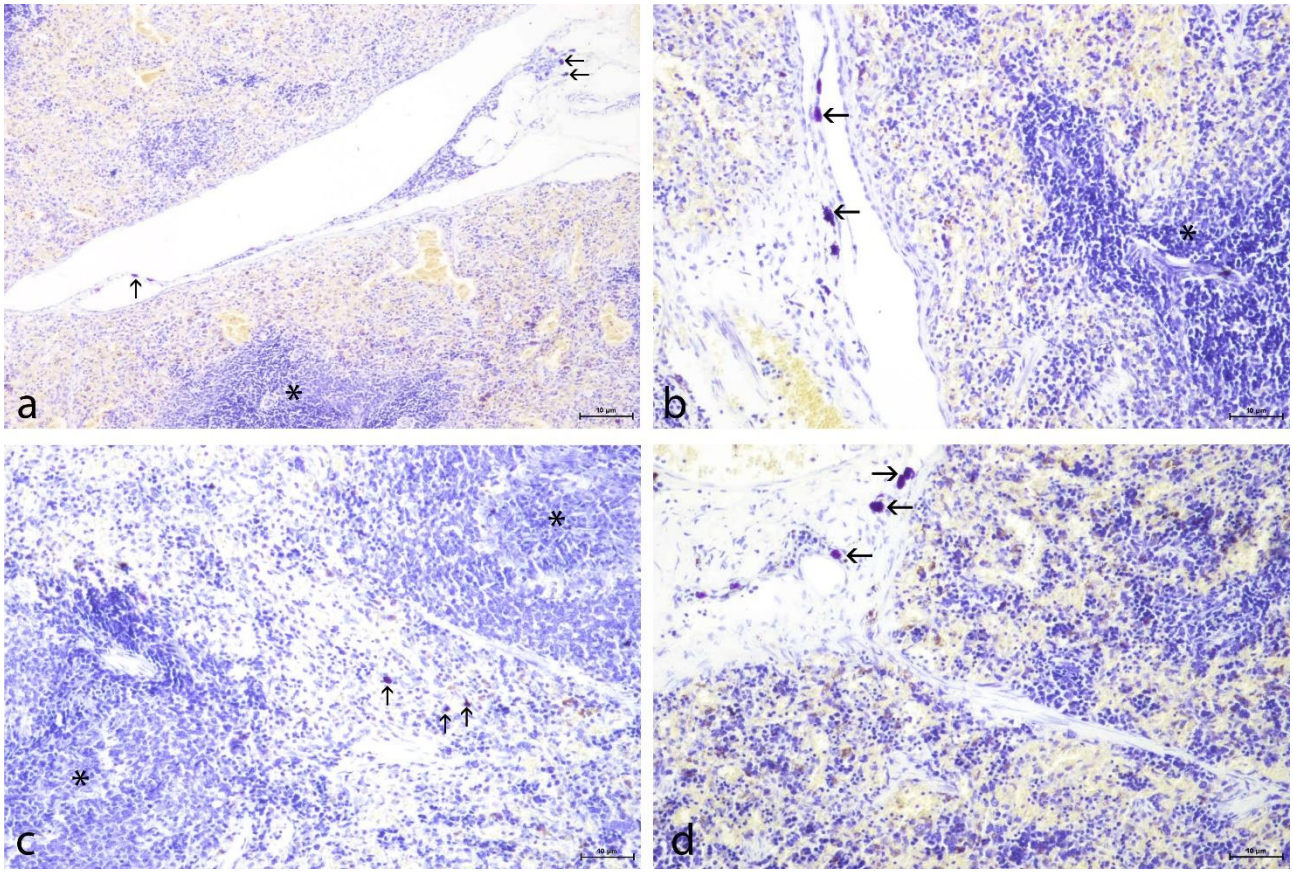


Figure 1. Toluidine blue staining. a. Control group, b. Diabetes group, c. S. nigra group, d. Diabetes + S. nigra group, (arrow): metachromatic mast cells, (asterix): white pulp, range bar, 10 µm.

Table 1. Mean count of MCs in groups after staining with toluidine blue.

Group	Mast cell count (× ±Sx /mm^2)	Minimum	Maximum
Control group	**20.45±0.73**	**17.20**	**21.40**
Diabetes group	**30.52±0.93[a]**	**26.40**	**32.40**
S. nigra gruop	**23.60±0.71[a]**	**20.20**	**25.20**
Diyabetes+S. nigra group	**24.72±1.17[a, b]**	**21.20**	**26.60**

[a]p<0.001 compared with the control group and [b]p<0.001 compared with the diabetes group (n=8).

Immunohistochemical findings

The density of VEGF-positive cells was evaluated semiquantitatively. Table 2 shows the immunohistochemical reactions in detail as well as a comparison of the groups. The positive

brown color VEGF immune-positive cells in the spleen tissue of all groups were characterized by different sizes, round or oval shapes. Intracytoplasmic stained VEGF immune-positive cell expression was observed in the spleen capsule, between lymph follicles and lymphatic cords in the red pulp areas. VEGF-positive cells were found in the red pulp, particularly in clusters. Positive cells were observed around the edges of lymph follicles and/or inside lymph follicles in the white pulp of the spleen on a very occasional basis.

When the groups were evaluated in detail among themselves, it was determined that the number of immune-positive VEGF cells increased only in the diabetes group. VEGF expression was similar in the control group, S. nigra group, and diabetes + S. nigra group. As a remarkable finding in our study, it was observed that the application of S. nigra caused a decrease in the number of immune-positive VEGF cells in diabetic rats (Figure 2).

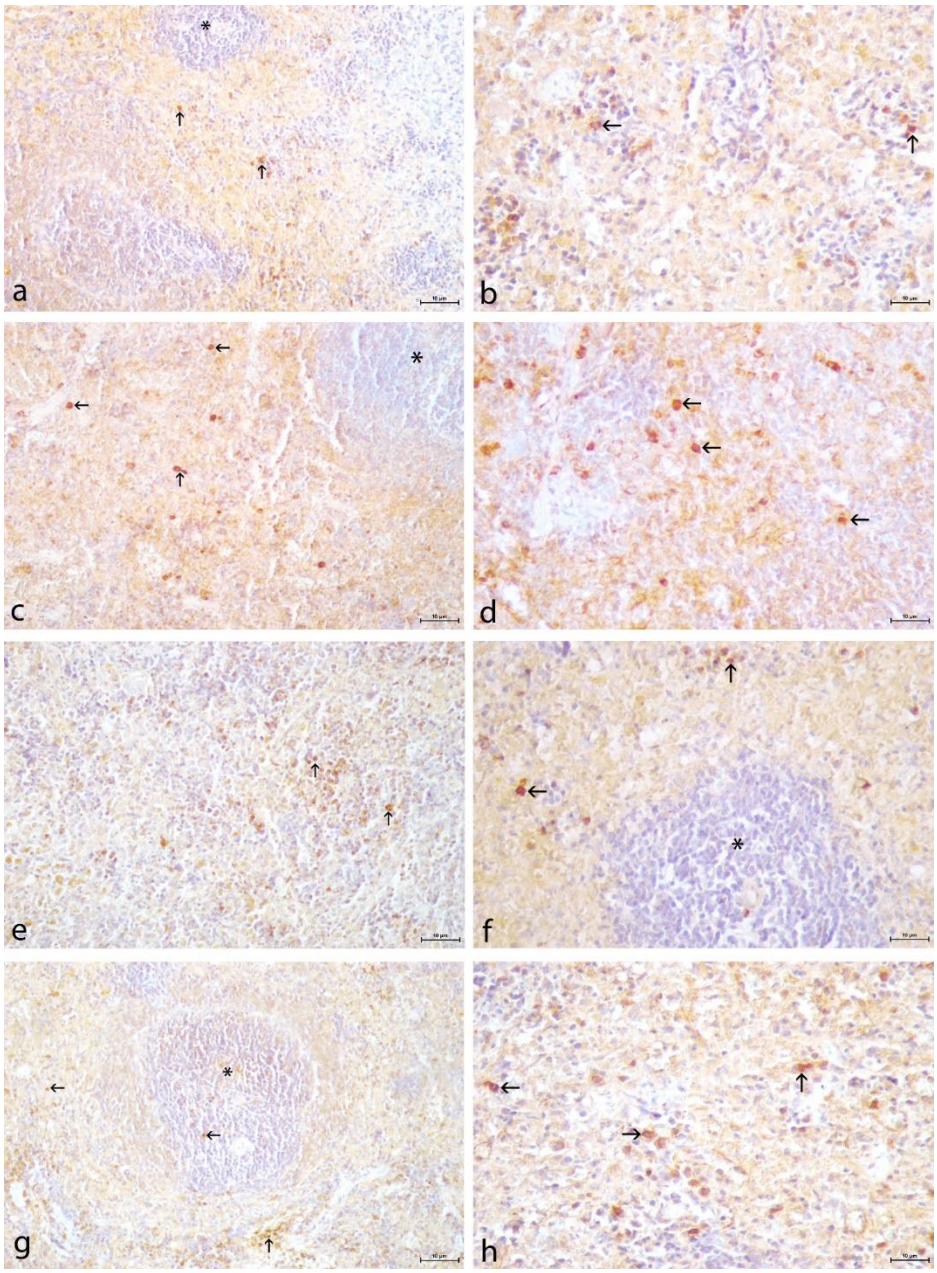


Figure 2. VEGF immunostaining. a-b. Control group, c-d. Diabetes group, e-f. S. nigra group, g-h Diabetes + S. nigra group, (arrow): VEGF immune-positive cell, (asterix): white pulp, range bar, 10 µm.

Table 2. Semiquantitative immunostaining score of VEGF positive cell reactivity in different groups' spleen tissue.

Group	VEGF immune-positive cells
Control group	++
Diabetes group	+++
S. nigra gruop	++
Diyabetes+S. nigra group	++

Note: no stained cells in the scanned area no positive cell (–), 1-2 cells (±), 3-4 cells (+), 5-6 cells (++), 7 and more cells (+++).

DISCUSSION

It has been suggested that increased IgE levels in the bloodstream of diabetic patients and IgE-mediated activation of MCs may play a role in the pathogenesis of diabetes (Svensson et al., 2012). In all layers of the oral mucosa and tongue, particularly in the lamina propria and around blood vessels, there has reportedly been a significant increase in MC after STZ administration in rats compared to control tissue (Batbayar et al., 2003). Çetin et al. (2013) showed that diabetic rats had a statistically significant increase in the number of MC in their heart tissue compared to non-diabetic rats. Martino et al. (2015) have shown that a greater number of MC infiltrates into pancreatic islets in samples from donors with diabetes than in models without diabetes in their study on human pancreatic tissue samples. Researchers have also investigated the effect of insulin on MC activation in rats with experimental diabetes. This study revealed an increase in the number of MCs in the lungs in the diabetes group and a decrease in the MCs in the lungs in the insulin-supplemented group (Cavalher-Machadove et al., 2004). The role of MCs in STZ-induced diabetes has been investigated using MC-deficient mice (W/W^v). The study found that W/W^v mice hyperglycemia accelerated, and 100% of the mice became diabetic compared to healthy mice. In addition, in this study, MC adaptive transfer before STZ administration has been observed to impart resistance to diabetes (Carlos et al., 2015). In a study, it was reported that natural polyphenols in S. nigra can modulate specific and non-specific immune defense in insulin deficiency diabetes (Badescu et al., 2015). The present study reports for the first time the potential effects of S. nigra on MC number and distribution in the spleens of diabetic rats. In this study, in parallel with the studies mentioned above, an increase in the number of MCs was observed in the spleen tissue of the rats whose diabetes model was formed. Consequently, the present study showed that S. nigra statistically decreased the increases in spleen MC numbers of diabetic rats. However, the number of MCs increased in the S. nigra group compared to the control group. The data of our study suggest that S. nigra, whose immunomodulatory effects are known, may influence MC number and distribution.

VEGF is a potent regulatory cytokine that promotes vascular endothelial cell proliferation, differentiation, and migration (Apte et al., 2019). It has been specified that hyperglycemia-induced oxidative stress activates and increases VEGF expression, an angiogenic factor (Ozdemir et al., 2014). The role of VEGF in the pathophysiology of tissue dysfunction in diabetes was investigated, and for this purpose, STZ-induced diabetic rats were treated with monoclonal anti-VEGF antibodies. As a result of this study, it has been observed that inhibition of VEGF reduces tissue damage caused by diabetes (De Vriese et al., 2001). In a study conducted by Mıcılı et al. (2012), it was reported that there was an increase in VEGF expression in the glomerular and tubular areas of kidney tissue in rats with experimental diabetes. It has been shown that VEGF expression is increased in the retinal layers of rats with diabetic retinopathy (De Meloa et al., 2020). In the present study, we observed that

diabetes increased VEGF expression in the rat spleen. Our results are similar to previous studies. Additionally, in our research, it was also determined that S. nigra did not change the number of VEGF immune-positive cells in the spleen tissue. However, when the diabetes group and the S. nigra group were compared, it was observed that S. nigra semiquantitatively reduced VEGF expression. As a result, although the precise mechanisms remain unknown, these results suggest that S. nigra may reduce the possible increase in diabetes-induced VEGF immune-positive cells in spleen tissue.

MCs are reported to increase in number in their angiogenesis regions, and products such as histamine, fibroblast growth factor, VEGF, heparin, and tryptase secreted from their granules promote angiogenesis (Crivellato et al., 2010). As a result of a study on human dengue fever, it has been reported that the number of MCs and the levels of VEGF and MC-specific proteases tryptase and chymase in the circulation increased significantly compared to healthy individuals (Furuta et al., 2012). Liang et al. (2012) found an increase in the number of MCs in tissue sections stained with toluidine blue in pterygium syndrome compared to normal conjunctiva. Also, they have detected using the double immunostaining method that tryptase MCs and VEGF were expressed from the same cell. Tissue samples from the oral mucosa with leukoplakia lesions and healthy oral mucosa tissue samples were compared in a study. According to the findings, it has been reported that there was a parallel increase in the number of MCs and VEGF expression in the lesioned areas (Michailidou et al., 2012). In an experimental study with STZ, it has been found that both MC number and VEGF expression increased in mice groups with diabetes compared to healthy control groups in skin tissues (Nishikori et al., 2014). According to Gyurkovics et al., (2016) in experimental diabetes, it was observed that the number of MCs in gingiva increased significantly in rats. Furthermore, in this study, it has been shown that the expression of VEGF secreted from these MCs was also increased. A synergistic relationship was observed between MC number and VEGF expression in our study, especially in diabetes + S. nigra group. These findings suggest that MCs can be both a source and a target of VEGFs. In addition, the fact that MCs are strategically located in tissues, especially around vessels that have VEGF cytokine expression, suggests that they may play an essential role in angiogenic and tissue hemostasis.

CONCLUSION

In conclusion, the increased number of MCs and VEGF immune-positive cells in the spleens of experimental diabetic rats allowed us to suggest that MCs can synthesize and store VEGF in their granules. In addition, since there is not enough literature information about the effects of S. nigra on diabetes and especially on spleen tissue, it is thought that this study will contribute to the literature on this subject.

ACKNOWLEDGMENT

This study was supported by the Scientific Research Projects Project Programs, Ondokuz Mayis University (Project Number: PYO.VET.1904.20.020).

This article is summarized from the master's thesis of the same name.

Ethical approval:

Ondokuz Mayıs University Animal Experiments Ethics Committee 11.03.2020-2020/15

Conflict of interest: The author declared no conflict of interest.

REFERENCES

Apte, R. S., Chen, D. S., & Ferrara, N. (2019). Vegf in signaling and disease: beyond discovery and development. *Cell*, *176*(6), 1248-1264.

Badescu, M., Badulescu, O., Badescu, L., & Ciocoiu, M. (2015). Effects of Sambucus nigra and Aronia melanocarpa extracts on immune system disorders

within diabetes mellitus. *Pharmaceutical Biology*, 53(4):533-539.

Batbayar, B., Somogyi, J., Zelles, T., & Fehér, E. (2003). Immunohistochemical analysis of substance P containing nerve fibres and their contacts with mast cells in the diabetic rat's tongue. *Acta Biologica Hungarica*, *54*(3-4), 275-283.

Bidian, C., Mitrea, D. R., Tatomir, C., Perdeschrepler, M., Lazăr, C., Chetan, I., Bolfa, P., David, L., Clichici, S., Filip, G. A., Mureșan, M., & Micle, O. (2021). Vitis vinifera l. and sambucus nigra l. extracts attenuate oxidative stress and inflammation in femoral ischemia. *Farmacia*, *69*(1), 59-67.

Carlos, D., Yaochite, J. N. U., Rocha, F. A., Toso, V. D., Malmegrim, K. C. R., Ramos, S. G., Jamur, M. C., Oliver, C., Camara, N. O., Andrade, M. V. M., Cunha, F. Q., & Silva, J. S. (2015). Mast cells control insulitis and increase Treg cells to confer protection against STZ-induced type 1 diabetes in mice. *European Journal of Immunology*, *45*(10), 2873-2885.

Cavalher-Machado, S. C., de Lima, W. T., Damazo, A. S., Carvalho, V. de F., Martins, M. A., Silva, P. M. R., & Sannomiya. P. (2004). Down-regulation of mast cell activation and airway reactivity in diabetic rats: role of insulin. *European Respiratory Journal*, *24*(4), 552-558.

Çetin, A., Vardı, N. & Orman, D. (2013). Deneysel diyabetin sıçan kalp dokusunda meydana getirdiği histolojik değişiklikler üzerine aminoguanidinin iyileştirici etkileri. *İnönü Üniversitesi Sağlık Hizmetleri Meslek Yüksek Okulu Dergisi, 1*, 17-26.

Crivellato, E., Travan, L., & Ribatti, D. (2010). Mast cells and basophils: a potential link in promoting angiogenesis during allergic inflammation. *International Archives of Allergy and Immunology*, *151*(2), 89-97.

De Vriese, A. S., Tilton, R. G., Elger, M., Stephan, C. C., Kriz, W., & Lameire, N. H. (2011). Antibodies against vascular endothelial growth factor improve early renal dysfunction in experimental diabetes. *Journal of the American Society of Nephrology*. *12*(5), 993-1000.

De Meloa, M. F., Ferreiraa, C. G. M., Souza, E. H. L. S., Almeida, L. L., de Sáa F. B., Neto, C. J. C. L., de Castroa, M. V. P., Teixeira, V. W., & Teixeira, A. A. C. (2020). Melatonin regulates the expression of inflammatory cytokines, VEGF and apoptosis in diabetic retinopathy in rats. *Chemico-Biological Interactions*, *327*, 109183.

Darenskaya, M. A., Kolesnikova, L. I., & Kolesnikov, S. I. (2021). Oxidative stress: pathogenetic role in diabetes mellitus and ıts complications and therapeutic approaches to correction. *Bulletin of Experimental Biology and Medicine*, *171*(2), 179-189.

Dwyer, D. F., Barrett, N. A., Austen, K. F., & Immunological Genome Project Consortium. (2016). Expression profiling of constitutive mast cells reveals a unique identity within the immune system. *Nature Immunology*, *17*(7), 878-887.

Enerback L. (1966). Mast cells in rat gastrointestinal mucosa: II. Dye-binding and metachromatic properties. *Acta pathologica et microbiologica Scandinavica*. *66*(3), 303-312.

Ertuğrul T, Tutuncu Ş, Ozdemır B, & Delıce N. (2021). Possible effect of thymoquinone on mast cell number and chymase, IL-4 and IFN-γ expression in rat spleen. *Medycyna Weterynaryjna*, *77*(10), 484-490.

Ferreira, S. S., Silva, A. M., & Nunes, F. M. (2020). Sambucus nigra l. fruits and flowers: chemical composition and related bioactivities. *Food Reviews International*, *30*(3), 1-29.

Galli, S. J., Borregaard, N., & Wynn, T. A. (2011). Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nature Immunology*, *12*(11), 1035-1044.

Furuta, T., Murao, L. A., Lan, N. T. P., Huy, N. T., Huong, V. T. Q., Thuy, T. T., Tham, V. D., Nga, C. T. P., Ha, T. T. N., Ohmoto, Y., Kikuchi, M., Morita, K., Yasunami, M., Hirayama, K., & Watanabe, N. (2012). Association of mast cell-derived VEGF and proteases in Dengue shock syndrome. *PLOS Neglected Tropical Diseases*, *6*(2), e1505.

Gray, A. M., Abdel-Wahab, Y. H., & Flatt, P. R. (2000). The traditional plant treatment, Sambucus nigra (elder), exhibits insulin-like and insulin-releasing actions in vitro. *Journal of Nutrition*, *130*(1), 15-20.

Gyurkovics, M., Nagy, I., Bodor, C., Székely, A. D., Dinya, E., Rosivall, L., & Lohinai, Z. (2016). Expression of vascular endothelial growth factor has a regulatory role in gingival venules in experimental diabetes. *Journal of Periodontology*, *87*(3), e27-34.

Harding, J. L., Pavkov, M. E., Magliano, D. J., Shaw, J. E., & Gregg, E. W. (2019). Global trends in diabetes complications: a review of current evidence. *Diabetologia*, *62*(1), 3-16.

Huang, S., Yang, N., Liu, Y., Hu, L., Zhao, J., Gao, J., Li, Y., Li, C., Zhang, X., & Huang, T. (2012). Grape seed proanthocyanidins inhibit angiogenesis via the downregulation of both vascular endothelial growth factor and angiopoietin signaling. *Nutrition Research*, *32*(7), 530-536.

John, P. W. M. (1971). *Statistical Design and Analysis of Experiments: 1st Edition*. (pp. 59-87). Macmilllan.

Kumar, V., & Sharma, A. (2010). Mast cells: emerging sentinel innate immunecells with diverse role in immunity. *Molecular Immunology*, *48*(1-3), 14-25.

Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., & Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*, *246*(4935), 1306-1309.

Liang, K., Jiang, Z., Zhao, B., Shen, J., Huang, D., & Tao, L. (2012). The expression of vascular endothelial growth factor in mast cells promotes the neovascularisation of human pterygia. *British Journal of Ophthalmology*, *96*(9), 1246-1251.

Martino, L., Masini, M., Bugliani, M., Marselli, L., Suleiman, M., Boggi, U., Nogueira, T. C., Filipponi, F., Occhipinti, M., Campani, D., Dotta, F., Syed, F., Eizirik, D. L., Marchetti, P., & De Tata, V. (2015). Mast cells infiltrate pancreatic islets

in human type 1 diabetes. *Diabetologia, 58*(11), 2554-2562.

McHale, C., Mohammed, Z., & Gomez, G. (2019). Human skin-derived mast cells spontaneously secrete several angiogenesis-related factors. *Frontiers in Immunology*, 25(10), 1445.

Mıcılı, S., Ergür, B., Özoğul, C., Sarıoğlu, S., Bağrıyanık, A., Tuğyan, K., Pekçetin, Ç., Sönmez, Ü., Tekmen, İ., Erbil, G., Akdoğan, G. G., Özyurt, D., & Buldan, Z. (2012). Deneysel hipertansiyon ve diyabet modeli oluşturulan sıçanlarda böbreğin immunohistokimyasal olarak incelenmesi. *Dokuz Eylül Üniversitesi Tıp Fakültesi Dergisi, 26*(2), 91-101.

Michailidou, E. Z., Markopoulos, A. K., & Antoniades, D. Z. (2012). VEGF expression from human dysplastic or malignant oral epithelium may be related to mast cell density and the subsequent angiogenetic phenomena. *International Journal of Oral and Maxillofacial Surgery, 41*(12), 1467-1473.

Nishikori, Y., Shiota, N., & Okunishi, H. (2014). The role of mast cells in cutaneous wound healing in streptozotocin-induced diabetic mice. *Archives of Dermatological Research, 306*(9), 823-835.

Ozdemir, G., Ergün, Y., Bakariş, S., Kilinc, M., Durdu, H., & Ganiyusufoğlu, E. (2014). Melatonin prevents retinal oxidative stress and vascular changes in diabetic rats. *Eye, 28*(8), 1020-1027.

Sismanopoulos, N., Delivanis, D. A., Alysandratos, K. D., Angelidou, A., Therianou, A., Kalogeromitros, D., & Theoharides, T. C. (2012). Mast cells in allergic and inflammatory diseases. *Current Pharmaceutical Design, 8*(16), 2261-2277.

Svensson, J., Eising, S., Mortensen, H. B., Christiansen, M., Laursen, I., Lernmark, A., Nilsson, A., Simonsen, L. B., Carstensen, B., Pociot, F., Johannesen, J., & Danish Childhood Diabetes Registry. (2012). High levels of immunoglobulin E and a continuous increase in immunoglobulin G and immunoglobulin M by age in children with newly diagnosed type 1 diabetes. *Human Immunology, 73*(1), 17-25.

True, L. D. (1990). *Principles of immunohistochemistry.* (pp. 12-26). Gower Medical Publishing.

Yang, C., Zhang, W., Dong, X., Fu, C., Yuan, J., Xu, M., Liang, Z., Qiu, C., & Xu, C. (2020). A natural product solution to aging and aging-associated diseases. *Pharmacology & Therapeutics, 216*, 107673.

Ege bölgesi neonatal kuzu ölümlerinde *Escherichia coli* septisemisinin ve antibiyotik duyarlılığının araştırılması

Investigation of *Escherichia coli* septicemia and antibiotic susceptibility in neonatal lamb deaths in the Aegean region

Research Article

Volkan Özavcı[1a]
Çağatay Nuhay[2b]
Mehmet Özden[2c]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Dokuz Eylül University, Kiraz, Izmir, Türkiye
[2]İzmir Bornova Veterinary Control Institute, Bacteriology Laboratory, 35040, Izmir, Türkiye

ORCİD-
[a]0000-0003-3511-3008
[b]0000-0002-1475-3041
[c]0000-0001-9989-2624

ÖZET

Bu çalışmada 2019-2021 yılları arasında Ege bölgesine ait 7 ilde (Aydın, Denizli, İzmir, Kütahya, Manisa, Muğla, Uşak) görülen neonatal (0-28 gün) kuzu ölümlerinde *Escherichia coli* (*E. coli*) septisemisi araştırıldı ve etkenin antimikrobiyal duyarlılıkları tespit edildi. Araştırmanın materyalini 150 adet kuzu viseral organ ve dokusu (akciğer, karaciğer, dalak, lenf, kemik iliği ve barsak) oluşturdu. Örnekler, nutrient broth 37°C'de 24 saat aerobik şartlarda inkube edildi ve daha sonra %7 koyun kanlı agara ve MacConkey agara ekimleri yapılarak 37ºC'de 24-48 saat inkubasyona bırakıldı. Kanlı agarda grimsi S tipli, MacConkey agarda pembe, mukoid olmayan koloni oluşturan Gram negatif basiller *E. coli* olarak değerlendirildi ve Vitek 2 sistemi ile de doğrulandı. Örneklerin %88,66 (133/150)'sında *E. coli* etkeni tespit edildi. Etken izolasyonu en çok İzmir (31/133; %23,30) ve Aydın (25/133; %18,80)'da yapıldı. Diğer illerde bulgular birbirine yakın seyir gösterdi. Antibiyotik duyarlılık testinde amoksisilin-klavulanik asit (30 µg), sefoperazon (30 µg), eritromisin (15 µg), penisilin G (10 units), gentamisin (10 µg), tetrasiklin (30 µg), trimetoprim-sulfametoksazol (25 µg) ve enrofloksasin (5 µg) ticari diskleri kullanıldı. İzolatların 110'u (%73,33) gentamisine, 80'i sefoperazona (%53,33) ve 70'i (%46,66) amoksisilin-klavulanik asite duyarlı bulundu. Ek olarak, izolatların tüm'ü penisilin G'ye (%100), 146'sı (%97,33) eritromisine, 122'si tetrasikline (%81,33) ve 119'u (%79,33) sulfametaksazol-trimethoprime dirençli bulundu. Sonuç olarak Ege bölgesinde iç organlar tutulumu ile karakterize *E. coli* septisemisi görülmektedir. Mortalite'nin antibiyogram ile akılcı antibiyotik kullanımı, doğru tedavi yaklaşımları ve koruyucu hekimlik uygulamaları ile azalma göstereceği ve ekonomiye olumlu katkı sağlayacağı düşünülmektedir.

Anahtar Kelimeler: Antimikrobiyal direnç, *Escherichia coli*, ishal, kuzu, neonatal.

ABSTRACT

In this study, *Escherichia coli* (*E. coli*) septicemia was investigated in neonatal (0-28 days) lamb deaths in 7 provinces of the Aegean region (Aydın, Denizli, İzmir, Kütahya, Manisa, Muğla, Uşak) between 2019-2021, and antimicrobial susceptibility was determined. The material of the study consisted of 150 lamb visceral organs and tissues (lung, liver, spleen, lymph, bone marrow and intestine). The samples were incubated in nutrient broth under aerobic conditions and then inoculated on 7% sheep blood agar and MacConkey agar, and incubated at 37°C for 24-48 hours. Gram-negative bacilli that form greyish S-type on blood agar and pink, non-mucoid colony on MacConkey agar were evaluated as *E. coli* and were also confirmed by the Vitek 2 system. *E. coli* agent was detected in 88.66% (133/150) of the samples. Agent isolation was most common in İzmir (31/133; 23.30%) and Aydın (25/133; 18.80%). Findings in other provinces showed a similar trend. In antibiotic susceptibility test, amoxicillin-clavulanic acid, cefoperazone, erythromycin, penicillin G, gentamicin, tetracycline, trimethoprim-sulfomethoxazole and enrofloxacin commercial discs were used. of the isolates, 110 (73.33%) were sensitive to gentamicin, 80 (53.33%) to cefoperazone, and 70 (46.66%) to amoxicillin-clavulanic acid. In addition, all of the isolates were resistant to penicillin G (100%), erythromycin (97.33%), tetracycline (81.33%), and sulfamethoxazole-trimethoprim (79.33%). As a result, *E. coli* septicaemia characterized by visceral involvement is seen in the Aegean region. It is thought that mortality will decrease with the right treatment approaches and rational antibiotic use and will contribute positively to the economy.

Keywords: Antimicrobial resistance, *Escherichia coli*, diarrhoea, lamb, neonatal.

Correspondence
Volkan ÖZAVCI
volkan.ozavci@deu.edu.tr

Article info
Submission: 24-06-2022
Accepted: 11-11-2022
Online First: 23-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

How to cite this article
Özavcı, V., Nuhay, Ç., Özden M. (2022). Investigation of *Escherichia coli* septicemia and antibiotic susceptibility in neonatal lamb deaths in the Aegean region. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 305-312. https://doi.org/10.31797/vetbio.1135125

GİRİŞ

Neonatal kuzularda görülen bakteriyal ishaller, *Pasteurella multocida, Staphylococcus aureus, Clostridium perfringes, Escherichia coli, Campylobacter, Salmonella* sp. ve *Listeria* sp. gibi patojen etkenlerden kaynaklanmaktadır (Gökçe ve Erdoğan, 2009; Özçelik, 2018). Kuzularda neonatal döneme ait (0-28 gün) diyareal infeksiyon ile ilişkili en yaygın organizmalar arasında patojenik *E. coli* bulunmaktadır. Ayrıca, yüksek morbidite ve mortalite ile çiftlikler için büyük bir ekonomik tehdit de oluşturmaktadır (Munoz vd., 1996; Sharif vd., 2005; Zhao vd., 2021). Patojenik olarak tanınan dört ana intestinal *E. coli* patotip kategorisi bulunur. Bunlar, enterotoksijenik *E. coli* (ETEC), enteropatojenik *E. coli* (EPEC), enteroinvaziv *E. coli* (EIEC) ve enterohemorajik *E. coli* (EHEC) olarak temsil edilir (Wani vd., 2004). Patojenik *E. coli* 'ler hem insanlarda hem de hayvanlarda çeşitli bağırsak ve bağırsak dışı kolibasillozlara neden olabilir (Tenaillon vd., 2010). Özellikle hayvan orjinli *E. coli* 'nin halk sağlığı açısından zoonotik önemi de mevcuttur. Örneğin, sığır, koyun, kuzu ve keçi gibi küçük ruminantların shiga-toksijenik *E. coli* (STEC), enterohemorajik *E. coli* (EHEC) gibi suşlar için ana rezervuar konakçılar olduğu ve insanlarda da hastalık oluşturduğu bilgisi de verilmektedir (Bélanger vd., 2011).

Koyun sürülerinde *E. coli* 'nin identifikasyonu daha çok ishal vakalarında yapılmaktadır (Abdou vd., 2021). Postpartum 3. saatten itibaren ishal görülebilmektedir ve yaşamın ilk 4 haftasını tehdit edebilmektedir. Ölümler içinde en sık tanımlanan nedenlerden birisi de bulaşıcı olan septisemidir (Uetake, 2013; Holmøy vd., 2017). Kuzularda ve oğlaklarda yeni doğan ölüm oranının (4 haftalık yaşta) %3,2 olduğu gösterilmiştir. Bununla birlikte, 48 saat içinde görülen erken neonatal ölüm oranının yaklaşık %2 olduğu ve genel olarak ölümlerin %62'sini temsil ettiği de belirtilmiştir (Sharif vd., 2005). 0-7 günlük kuzuların %48,78 'inde, 8-14 günlük kuzuların %17,07 'sinde, 15-21 ve 22-28 günlük kuzuların %10,97 'sinde ölümlerin yaygın görüldüğü bildirilmiştir (Ahmed vd., 2010b). Kuzu ölüm oranlarının %50 'si ilk 24 saatte, %30'u 1-3 gün içinde, %11'i 4.-7. günlerde ve %4'ünün de 1. hafta sonrası olduğu tespit edilmiştir (Özçelik vd., 2018). Gökçe vd. (2013), neonatal ölümlerin %84,6'sının ilk hafta içerisinde olduğunu vurgulamıştır. Dünya çapında kuzu ölüm oranı ortalama %9-20'dir (Mousa-Balabel, 2010). Açlık, annesiz kalma, kolostrum alamama, güç doğum, doğum sonrası pasif bağışıklık immunoglobulin düzeyinin yetersiz olması ve yoğun patojenlerle kontaminasyon ölümlerde etkili olmakla birlikte iki ana faktör olarak enteritis ve septisemi daha çok ön planda olmaktadır (Chaarani vd., 1991; Mahboub vd., 2013; Brien vd., 2014). Örneğin, ishalin etiyolojisinde *E. coli* patojenik suşların %10-63,2 oranında etkili olduğu bildirilmiştir (Gökçe vd., 2010).

Türkiye'de kuzu ölüm oranları ırk seçimi ve üretim 'e bağlı olarak %9,50-14,43 oranları arasında değişmekte ve %75 seviyelerine kadar çıkabilmektedir. Ayrıca, gebeliğin son döneminde 4 hafta ara ile yapılan iki aşılama ile kuzuların *E. coli* 'ye karşı önemli oranda koruma sağladığı da tespit edilmiştir (Ünal vd., 2018). Ülkemizde koyun sayısı bir önceki yıla göre %7,2 oranında artış göstermiştir (45 milyon 178 bin baş) ve küçükbaş hayvanların değeri de %30,60 oranında artmıştır (Tüik, 2022). Hayvancılık endüstrisinde antibiyotiklerin bilinçsiz ve sık kullanımı *E. coli* 'nin antimikrobiyal direnç spektrumunu genişletmekte, direnç oranını artırmakta ve hastalığın sağaltım başarısını azalmaktadır (Lee, 2009). Bu çalışmada, ülkemiz Ege bölgesi 7 ilinde görülen neonatal dönem (0-28 gün) *E. coli* septisemisi kaynaklı ölümlerin incelenmesi ve antibiyotik duyarlılıklarının belirlenmesi amaçlandı.

MATERYAL VE METHOD

Örnek toplama

2019-2021 yılları arasında Ege bölgesine ait 7 il ‘de (Aydın, Denizli, İzmir, Kütahya, Manisa, Muğla, Uşak) görülen kuzu ölümleri sonrası Bornova Veteriner Kontrol ve Araştırma Enstitüsüne kısa zaman içerisinde gönderilen (1-4 saat) 150 adet ölü kuzuya ait viseral organlar ve dokulardan (akciğer, karaciğer, dalak, lenf düğümü, kemik iliği ve barsak) alınan örnekler bakteriyoloji laboratuvarında çalışıldı (Tablo 1).

Tablo 1. İllerden toplanan viseral örneklerin yıllara göre dağılımı

Örnek Toplanan İller	Toplanan (akciğer, karaciğer, dalak, lenf, kemik iliği ve barsak) Örneklerinin Yıllara Göre Dağılımı			Örneklerin İllere Göre Dağılımı
	2019	2020	2021	
Aydın	8	9	8	**25**
Denizli	4	6	5	**15**
İzmir	9	12	10	**31**
Kütahya	5	8	7	**20**
Manisa	6	9	7	**22**
Muğla	5	8	6	**19**
Uşak	5	8	5	**18**
Genel Toplam	**42**	**60**	**48**	**150**

Bakteriyolojik testler

Alınan akciğer, karaciğer, dalak ve lenf düğümü örnekleri %7 koyun kanlı agara ve MacConkey agara, barsak içerikleri de nutrient brotha ekilip 37^{o}C aerobik ortamda 24-48 saat inkube edildi. Nutrient broth'un inkubasyonu sonrası MacConkey agar'a pasajı yapıldı. Daha sonra kanlı agarda grimsi, S tipli, katalaz pozitif, oksidaz negatif, MacConkey agarda mukoid olmayan pembe renkli, Gram negatif, çomak özellik gösteren kolonilerden Eosin Methylene-blue Lactose Sucrose Agar (EMB) (Oxoid) ve Tryptone Bile X-glucuronide (TBX) (Liofilchem) agara pasajlamalar yapıldı. TBX agarda mavi-yeşil koloniler, EMB agar'da menekşe renkli ve metalik refle veren koloniler *E. coli* olarak değerlendirildi (Quinn vd., 1994). Elde edilen izolatlar ayrıca Vitek 2 sistemi ile doğrulandı.

Antimikrobiyal duyarlılık testi

İzolatlara Mueller-Hinton agar besiyeri kullanılarak, Kirby-Bauer disk difüzyon yöntemine göre antibiyogram testi yapıldı

(Bauer vd., 1966). Bu amaçla, amoksisilin-klavulanik asit (30 µg), sefoperazon (30 µg), eritromisin (15 µg), penisilin G (10 units), gentamisin (10 µg), tetrasiklin (30 µg), sulfametaksazol-trimethoprim (25 µg) ve enrofloksasin (5 µg) ticari diskleri kullanıldı.

BULGULAR

Kuzulara ait toplam 150 adet organ ve doku örneğinin mikrobiyoloji laboratuvarında yapılan izolasyon ve identifikasyon çalışmaları sonucunda 133/150 (% 88,66) *E. coli* etkeni saptandı. Elde edilen izolatların yıllara ve illere göre dağılımları Tablo 2'de verilmiştir.

Tablo 2 Pozitif izole edilen *Escherichia coli* sayısı ve illere göre dağılımı.

Örnek Toplanan İller	Numune Sayısı (n)	*Escherichia coli* İdentifikasyonu		
		2019	2020	2021
Aydın	**25**	7	8	7
Denizli	**15**	4	6	5
İzmir	**31**	8	10	8
Kütahya	**20**	5	7	6
Manisa	**22**	5	8	6
Muğla	**19**	4	7	6
Uşak	**18**	5	7	4
Toplam İdentifikasyon Sayısı	**150**	**38**	**53**	**42**
			133	

Elde edilen izolatların İzmir (31/133; %23,30) ve Aydın (25/133; %18,80) ilinde sayıca daha fazla olduğu görülmektedir. Bu bulguları Manisa (22/133; %16,54), Kütahya (20/133; %15,03) ve Muğla (19/133; %14,25) illeri takip etmiştir. İzolatların 110'u (110/150; %73,33) gentamisine, 80'i sefoperazona (80/150; %53,33) ve 70'i (70/150; %46,66) amoksisilin-klavulanik asite duyarlı bulundu. Ek olarak, izolatların tümü penisilin G' ye (150/150; %100), 146'sı (146/150; %97,33) eritromisine, 122'si tetrasikline (122/150; %81,33) ve 119'u (119/150; %79,33) sulfametaksazol-trimethoprime dirençli bulundu. Belirlenen inhibisyon zon çapları Clinical Laboratory Standards Institute standartlarına göre değerlendirildi (CLSI, 2012; CLSI, 2016). İzole edilen *Escherichia coli* izolatlarına ait antibiyotik duyarlılık sonuçları Tablo 3' de verilmiştir.

Tablo 3. İzole edilen Escherichia coli 'nin antibiyotik duyarlılık sonuçları

Antibakteriyel İlaçlar	Escherichia coli (n, %)		
	Dirençli (R)	Orta Duyarlı (I)	Duyarlı (S)
Amoksisilin-klavulanik asit (30 µg)	35/150 (23,33)	45/150 (30)	70/150 (46,66)
Sefoperazon (30 µg)	22/150 (14,66)	48/150 (32)	80/150 (53,33)
Eritromisin (15 µg)	146/150 (97,33)	4/150 (2,66)	-
Penisilin G (10 units)	150/150 (100)	-	-
Gentamisin (10 µg)	32/150 (21,33)	8/150 (5,33)	110/150 (73,33)
Tetrasiklin (30 µg)	122/150 (81,33)	-	28/150 (18,66)
Sulfametaksazol-trimethoprim (25 µg)	119/150 (79,33)	26/150 (17,33)	5/150 (3,33)
Enrofloksasin (5 µg)	65/150 (43,33)	22/150 (14,66)	63/150 (42)

S: duyarlı; I: Orta-Duyarlı; R: dirençli, Amoksisilin-Klavulanik Asit (30 µg), Sefoperazon (30 µg), Eritromisin (15 µg), Penisilin G (10 units), Gentamisin (10 µg), Tetrasiklin (30 µg), Sulfametaksazol-Trimethoprim (25 µg), Enrofloksasin (5 µg)

TARTIŞMA

Escherichia coli bakterisi kuzularda neonatal dönemde 3 ay'a kadar görülen ishal vakalarına, yüksek morbitide'ye ve mortalite'ye sebep olmaktadır (Wani vd., 2004; Ghanbarpour vd., 2017; Özçelik, 2018). Araştırmamızda Ege

bölgesine ait 7 il'de neonatal (0-28 gün) kuzulara ait organ ve dokulardan *E. coli* izolasyon oranı %88,66 olarak tespit edilmiştir. Bulgularımız, kuzularda 0-4 haftalık yaşta neonatal ölüm oranının %3,2 olduğu değerlendirmesi ile benzerlik göstermektedir (Sharif vd., 2005). 0-28 günlük ishalli kuzu gaita örneklerinde *E. coli* %9 oranında tespit edilmiştir (Özçelik vd., 2018). Kuzuların enteritis vakalarının %18,5-63,2'sinin *E. coli* yönünden pozitif olduğu tespit edilmiştir (Chaarani vd., 1991). Nitekim, ishalli kuzuların %26-36'sında *E. coli* varlığı da bildirilmiştir (Munoz vd., 1996; Blanco vd. 2003; Martins vd., 2016). Gökçe ve Erdoğan (2010), yenidoğan kuzu morbidite oranının %48,6 olduğunu ve bu oranın %15,4'ünün ishalden kaynaklandığını belirtmiştir. Marmara bölgesinde 6 aylık kuzu ishal vakalarında %22,8 oranında *E. coli* tespit edilmiştir (Arslan vd., 2016). Ülkemizde enterotoksemi nedeniyle ölen koyun ve kuzuların mortalite oranı %38-86,6 olarak değerlendirilmiştir (Öztürk, 1996). Gülhan (2003) ishalli kuzulardan %77, Gökçe (2010) septisemiden ölen kuzulardan %42,5, Ahmed (2010a), Nasr ve Meghawery (2007) ishalli kuzulardan sırasıyla %36,84 ve %27,3 oranında *E. coli* izolasyonunu rapor etmiştir. Battisti (2006) *E. coli* oranını farklı olarak kuzularda ve koyunlarda %0-4 olarak düşük oranda tespit etmiştir. Araştırmamızda elde edilen sonuçlar genel manada diğer çalışmalarda elde edilen verilerle uyumlu ve mevcut bulguları doğrular niteliktedir. *E. coli* kontamine yiyecek, su tüketimi ile ilişkili fekal-oral yolla insanlara bulaşan zoonotik bir mikroorganizmadır ve insanlara bulaşmasında, koyunlar ve kuzular önemli rezervuarlardır (Blanco vd., 2021). Çiftlikte barınmanın ve hijyenik koşullarının yönetimi, ağıl ve içme suyu hijyeni, uygun hayvan yoğunluğu, biyogüvenlik önlemleri ve işletmenin etkin sanitasyonu septisemi görülmesinde önemli sayılan faktörlerdir (Le Jeune vd., 2021). Bu bilgiler ışığında, Ege bölgesinde yüksek oranda etken izole edilmesi *E. coli*' nin kuzuların neonatal ölümlerinde bölgesel bazda çok etkin olduğunu, halk sağlığı noktasında da dikkate alınması gerektiğini vurgulamaktadır. *E. coli* infeksiyon ishalleri genellikle antibiyotiklerle tedavi edilir, ancak hayvanlardaki dirençli suşlar nedeniyle tedavi bazen başarısız olabilir (Kolar vd., 2008). Kumar (2022) *E. coli* izolatlarının büyük çoğunluğunun penisiline tam (%100) direnç gösterdiğini ve bunu ampisilin (%93), tetrasiklin, (%68,8) ve gentamisin (%37,4) direncinin takip ettiğini rapor etmiştir. *E. coli* izolatları üzerinde yapılan testlerde penisilin (%77-93) (Haulisah vd., 2021) ve ampisilin (%43,6), enrofloksasin (%34,2), gentamisin (%20,4) dirençlilikleri saptanmıştır (Zhao vd., 2021). Ampisilin, tetrasiklin, trimetoprim-sülfametoksazol ve eritromisine karşı bir diğer çalışmada %100 antimikrobiyal dirençliliğin geliştiğine dikkat çekilmiştir (Acosta-Dibarrat vd., 2021). Yapılan birçok araştırmada *E. coli* izolatları amoksisilin, eritromisin, gentamisin, enrofloksasin, tetrasiklin, ampisilin ve sülfametoksazol-trimetoprime karşı en az >%70 oranda dirençli bulunmuştur (Kidsley vd., 2018; Kiponza vd., 2019; Musa vd., 2020; Fouad vd., 2022; Khawaskar vd., 2022; Sowunmi vd., 2022; Yun vd., 2022). Çalışmamızda *E. coli* için en yüksek ve %100 dirençlilik penisilin için (150/150; %100) diğer çalışma bulgularıyla uyumlu olarak tespit edilmiştir. Bunu eritromisin (146/150; 97,33), tetrasiklin (122/150; %81,33) ve sulfametaksazol-trimethoprim (119/150; %79,33) izlemiştir ve yukarıda verilen bulguları net bir şekilde doğrulamaktadır. Ayrıca, en yüksek duyarlılık gentamisin (110/150; %73,33) için tespit edilmiş ve genel olarak dirençliliğin yüksek oranda gelişmediği yorumlarıyla bulgu uyumludur. Aydın'da farklı olarak buzağı işletmelerinde bulunan ishalli vakalardan identifiye edilen *E. coli* izolatlarının penisilin G ve eritromisine %100, tetrasiklin ve trimetoprim-

sulfometoksazole %80, kanamisine ise %76,5 oranlarına dirençli olduğu saptanmıştır (Aşçı, 2016). Kuzulardan elde edilen bulgularda da penisilin, eritromisin ve tetrasiklin ile birlikte sülfametoksazol-trimetoprimde direnç artışının görülmesi veteriner saha tedavisinde bu ilaçların yaygın olarak kullanılması hususunu akla getirebilmektedir.

Bir diğer önemli konu, *E. coli* 'nin konakçı bağışıklık sisteminden kaçmasına ve tekrar hastalığa neden olmasına yardımcı olan çok sayıda virülans faktörü içermesidir. Tür, antijenik bileşimi temelinde serolojik olarak serogruplara ve serotiplere ayrılır (*E. coli* somatik (O), flagellar (H) ve kapsül (K) antijenleri). Kapsül, *E. coli* 'nin bazı suşlarına özgü kapsüler (K) antijeni içerir ve patojene karşı serum direnci sağlar. Polisakarit tabakası, komplemana karşı bağlanma yerlerini bloke ederek antikorları yerinden eden O-antijenden oluşur. Diyare, patojenik bakteriler tarafından çoklu virülans belirleyicilerinin koordineli bir ifadesidir. Enterotoksijenik *E. coli* (ETEC) infeksiyonu buzağılarda en yaygın kolibasilloz türüdür. ETEC 'in ana virülans özellikleri, çoğunlukla büyük plazmitlerde genlerle düzenlenen bağırsak epiteline yapışmayı sağlayan fimbrialar, afimbrial adezinler ve enterotoksin üretimidir. Enteropatojenik *E. coli* (EPEC), ETEC 'e benzer şekilde sulu ishali indükler, ancak aynı kolonizasyon faktörlerine sahip değildirler ve stabil toksin (ST) veya labil (LT) toksinleri üretmezler. Bunlar, yapışmanın son aşamalarına aracılık eden, orta derecede invaziv ve bazı Shiga benzeri toksinler olarak bildiren dış zar proteini intimin üretirler. Virülens genlerin patojen tarafından kazanılması, hastalık yükünü artıran antimikrobiyal maddelere direnç gösteren izolatların ortaya çıkmasına izin verir. (Fouad vd., 2022; Orole vd., 2022). *E. coli* 'de virülans faktörlerini (virotipler) kodlayan genlerin sıklığını ve bu virotiplerin veya diğer ishale neden olan patojenlerin, ishal olan veya olmayan buzağılardaki morfolojik bağırsak değişiklikleri ile ilişkisinin araştırılması da önem arz edebilir (Ngeleka vd., 2019).

SONUÇ

Sonuç olarak, Ege bölgesinde neonatal kuzuların ishal vakalarında *Escherichia coli* suşlarının izolasyon oranlarının yüksek olduğu tespit edilmiştir. Bununla birlikte, diğer illerde de direnç profillerinin belirlenmesine yönelik çalışmaların yapılması bölgesel verilerin elde edilmesinde faydalı olacaktır. Yüksek antibiyotik direncini ve yeni dirençli izolatların gelişimini engellemek için sağaltımda akılcı antibiyotik tercihi yapılmalıdır. Buzağılarda şiddetli veya ölümcül enterit ile ilgili durumun iyileştirilmesi için genetik virülens faktörlerin de ayrıca araştırılması önerilmektedir. Neonatal ishal etkenlerinin teşhisi/tedavisi ile ilgili diğer bölgelerle karşılaştırmalı araştırılma sayılarının artması ülkemizde kuzu ölümlerine karşı gerekli tedbirlerin alınmasında kullanılacak faydalı bilgilerin edinimine katkı sağlayacaktır.

AÇIKLAMALAR

Araştırmamızda çalışma olanaklarını sağlayan Bornova Veteriner Araştırma Enstitüsü Bakteriyoloji bölümüne teşekkürlerimizi sunarız.

Etik beyan: Sunulan çalışmada İzmir Bornova Veteriner Kontrol Enstitüsü' ne teşhis amacıyla gönderilmiş atık materyalleri kullanılmış olduğundan 15 Şubat 2014 tarih ve 28914 sayılı Resmî Gazetede yayınlanan "Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esaslarına Dair Yönetmeliğin 8.madde (k) bendinde yer alan hükümlere göre Hayvan Deneyleri Yerel Etik Kurulu Onayına gerek bulunmamaktadır.

Çıkar çatışması: Yazarlar olarak bu makale için bir çıkar çatışması olmadığını beyan ederiz.

KAYNAKLAR

Abdou, N., Majeed, Q., El-Azazy, O., Tahrani, L., AlAzemi, M. S., & Alajmi, A. (2021). Risk factors of diarrhea in small ruminants in Kuwait. *Iranian Journal of Veterinary Research, 22*(2), 146–149.

Acosta-Dibarrat, J., Enriquez-Gómez, E., Talavera-Rojas, M., Soriano-Vargas, E., Navarro, A., & Morales-Espinosa, R. (2021). Characterization of Diarrheagenic *Escherichia Coli* Strains Isolated from Slaughtered Sheep in México (Doctoral dissertation, Universidad Nacional Autonoma de Mexico).

Aşçı, İ.B. (2016). *Buzağı ishallerinde rol oynayan enterotoksijenik Escherichia coli türlerinin araştırılması ve antibiyotik duyarlılıklarının belirlenmesi/ Investigation of enterotoxigenic Escherichia coli species which take role in calf diarrhea and detection of their antimicrobial susceptibility* [Yüksek Lisans Tezi, Adnan Menderes Üniversitesi Sağlık Bilimleri Enstitüsü Mikrobiyoloji Programı]

Ahmed, A., Egwu, G.O., Garba, H.S., & Magaji, A.A. (2010a). Studies on risk factors of mortality in lambs in Sokoto, Nigeria. *Nigerian Veterinary Journal*, *31*(1).

Ahmed, A., Egwu, G.O., Garba, H.S., & Magaji, A.A. (2010b). Prevalence of bacterial pathogens and serotyping of E. coli isolates from diarrhoeic lambs in Sokoto state, Nigeria. *Sokoto Journal of Veterinary Sciences*, *8*(1&2).

Arslan, S., Öncel, T., Malal, M.E., Satır, E., Sait, A., Baca, A.Ü., & Aydoğan, D.Y. (2016). Bacteriological, Virological and Parasitological Etiology in Diarrhea Cases in Determined in Post-mortem Lambs and Kids in Marmara Region. *Van Veterinary Journal*, *27*(3).

Battisti, A., Lovari, S., Franco, A., Di Egidio, A., Tozzoli, R., Caprioli, A., Morabito, S. (2006). Prevalence of *Escherichia coli* O157 in lambs at slaughter in Rome, central Italy. *Epidemiology and Infection*, 134, 415-419.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M. (1966). Antibiotic susceptibilty testing by a standardized single disk method. *American Journal of Clinical Pathology*, *45*, 493.

Bélanger, L., Garenaux, A., Harel, J., Boulianne, M., Nadeau, E., & Dozois, C.M. (2011). Escherichia coli from animal reservoirs as a potential source of human extraintestinal pathogenic E. coli. *FEMS Immunology and Medical Microbiology*, *62*(1), 1-10.

Blanco Crivelli, X., Bonino, M.P., Sanin, M.S., Petrina, J.F., Disalvo, V.N., Massa, R., ... & Bentancor, A. (2021). Potential Zoonotic Pathovars of Diarrheagenic *Escherichia coli* Detected in Lambs for Human Consumption from Tierra del Fuego, Argentina. *Microorganisms*, *9*(8), 1710.

Brien, F.D., Cloete, S.W.P., Fogarty, N.M., Greeff, J.C., Hebart, M.L., Hiendleder, S., ... & Miller, D.R. (2014). A review of the genetic and epigenetic factors affecting lamb survival. *Animal Production Science*, *54*(6), 667-693.

Chaarani, B., Robinson, R.A., & Johnson, D.W. (1991). Lamb mortality in Meknes province (Morocco). *Preventive Veterinary Medicine*, *10*(4), 283-298.

Clinical and Laboratory Standards Institute (CLSI), (2012). Performance standards for antimicrobial disk susceptibility tests, approved standard-ninth Edition. Wayne, PA: M2-A9.

Clinical and Laboratory Standards Institute (CLSI), (2016). Performance standards for antimicrobial susceptibility testing; Twenty-sixth informational supplement. Wayne, PA: M100-S26.

Fouad, H., Saleh, H., Elazazy, H., Hamed, A., & Samir, S. (2022). Prevalence of pathogenic *E. coli* in diarrhoeic cattle calves and antibiotic resistance genes. *Kafrelsheikh Veterinary Medical Journal*, *20*(1), 12-18.

Ghanbarpour, R., Askari, N., Ghorbanpour, M., Tahamtan, Y., Mashayekhi, K., Afsharipour, N., & Darijani, N. (2017). Genotypic analysis of virulence genes and antimicrobial profile of diarrheagenic *Escherichia coli* isolated from diseased lambs in Iran. *Tropical Animal Health and Production*, *49*(3), 591-597.

Gökçe,E., Erdoğan, & H.M. (2009). An epidemiological study on neonatal lamb healty. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*, *15*, 225236.

Gökçe, E., Ünver, A., & Erdoğan, H. M. (2010). İshalli neonatal kuzularda enterik patojenlerin belirlenmesi. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*, *16*(5), 717-722.

Gökçe, E., Kırmızıgül, A. H., Erdoğan, H. M., & Çitil, M. (2013). Risk factors associated with passive immunity, health, birth weight and growth performance in lambs: I. effect of parity, dam's health, birth weight, gender, type of birth and lambing season on morbidity and Mortality. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*, *19* (Supplement A).

Gülhan, T. (2003). Sağlıklı görünen hayvanların dışkılarından izole edilen *Escherichia coli* suşlarının biyokimyasal, enterotoksijenik ve verotoksijenik özelliklerinin belirlenmesi. *Yüzüncü Yıl Üniversitesi Veteriner Fakültesi Dergisi*, *14*(1), 102-109.

Haulisah, N.A., Hassan, L., Bejo, S.K., Jajere, S.M., & Ahmad, N.I. (2021). High levels of antibiotic resistance in isolates from diseased livestock. *Frontiers in Veterinary Science*, 8, 652351.

Holmøy, I.H., Waage, S., Granquist, E.G., L'Abée-Lund, T.M., Ersdal, C., Hektoen, L., & Sørby, R. (2017). Early neonatal lamb mortality: postmortem findings. *Animal*, *11*(2), 295-305.

Khawaskar, D.P., Sinha, D.K., Lalrinzuala, M.V., Athira, V., Kumar, M., Chhakchhuak, L., ... & Thomas, P. (2022). Pathotyping and antimicrobial susceptibility testing of *Escherichia coli* isolates from neonatal calves. *Veterinary Research Communications*, *46*(2), 353-362.

Kidsley, A.K., Abraham, S., Bell, J.M., O'Dea, M., Laird, T.J., Jordan, D., ... & Trott, D.J. (2018). Antimicrobial susceptibility of *Escherichia coli* and *Salmonella* spp. isolates from healthy pigs in Australia: results of a pilot national survey. *Frontiers in Microbiology*, 9, 1207.

Kiponza, R., Balandya, B., Majigo, M.V., & Matee, M. (2019). Laboratory confirmed puerperal sepsis in a national referral hospital in Tanzania: etiological

agents and their susceptibility to commonly prescribed antibiotics. *BMC Infectious Diseases*, *19*(1), 1-7.

Kolář, M., Bardoň, J., Vagnerova, I., Sauer, P., Koukalova, D., Petrželová, J., ... & Pospíšil, R. (2008). Resistance to antibiotics in strains of *Staphylococcus* spp., *Enterococcus* spp. and *Escherichia coli* isolated from rectal swabs of pigs. *Acta Veterinaria Brno*, *77*(1), 103-110.

Kumar, K., Sharma, N.S., Kaur, P., & Arora, A.K. (2022). Molecular Detection of Antimicrobial Resistance Genes and Virulence Genes in *E. coli* Isolated from Sheep and Goat Faecal Samples. *Indian Journal of Animal Research*, *56*(2), 208-214.

Lee, J. H. (2009). Antimicrobial resistance of *Escherichia coli* O26 and O111 isolates from cattle and their characteristics. *Veterinary microbiology*, *135*(3-4), 401-405.

LeJeune, J. T., Zhou, K., Kopko, C., & Igarashi, H. (2021). FAO/WHO Joint Expert Meeting on Microbiological Risk Assessment (JEMRA): Twenty Years of International Microbiological Risk Assessment. *Foods*, *10*(8), 1873.

Mahboub, H.D., Ramadan, S.G., Helal, M. A., & Aziz, E.A. (2013). Effect of maternal feeding in late pregnancy on behaviour and performance of Egyptian goat and sheep and their offspring. *Global Veterinaria*, *11*(2), 168-176.

Martins, F. H., Guth, B. E., Piazza, R. M., Elias, W. P., Leão, S. C., Marzoa, J., ... & Pelayo, J. S. (2016). Lambs are an important source of atypical enteropathogenic *Escherichia coli* in southern Brazil. *Veterinary microbiology*, 196, 72-77.

Mousa-Balabel, T.M. (2010). The relationship between sheep management and lamb mortality. *World Academy of Science, Engineering and Technology*, 41, 1201-1206.

Munoz, M., Alvarez, M., Lanza, I., & Carmenes, P. (1996). Role of enteric pathogens in the aetiology of neonatal diarrhoea in lambs and goat kids in Spain. *Epidemiology & Infection*, *117*(1), 203-211.

Musa, L., Casagrande Proietti, P., Branciari, R., Menchetti, L., Bellucci, S., Ranucci, D., ... & Franciosini, M. P. (2020). Antimicrobial susceptibility of *Escherichia coli* and ESBL-producing *Escherichia coli* diffusion in conventional, organic and antibiotic-free meat chickens at slaughter. *Animals*, 10(7), 1215.

Nasr, E.M., & Meghawery, M.A. (2007). Studies on diarrhea in calves with emphasis on the role of clostridium perfringens and *Escherichia coli*. *Research Journal of Animal and Veterinary Sciences*, 2, 28-33.

Ngeleka, M., Godson, D., Vanier, G., Desmarais, G., Wojnarowicz, C., Sayi, S., Huang, Y., Movasseghi, R., Fairbrother, J.M. (2019). Frequency of *Escherichia coli* virotypes in calf diarrhea and intestinal morphologic changes associated with these virotypes or other diarrheagenic pathogens. *Journal of Veterinary Diagnostic Investigation*, *31*(4), 611-615.

Orole, O.O., Gambo, S.M., Fadayomi, V.S. (2022). Characteristics of Virulence Factors and Prevalence of Virulence Markers in Resistant *Escherichia coli* from Patients with Gut and Urinary Infections in Lafia, Nigeria. *Microbiology Insights*, 15.

Özçelik, A. (2018). *Elazığ yöresinde ishalli neonatal kuzu ve oğlakların bazı ishal etkenlerinin hızlı test kitleri ile teşhisi/Diagnosis of some diarrhea factor with rapid test kits in lambs and kids with neonatal diarrhea in Elazig province* [Yüksek Lisans Tezi, Fırat Üniversitesi].

Öztürk, G. (1996). Etiopathology of Enterotoxaemia in Small Ruminants in Elazig and Surrounding Cities I. The Group in Which *Cl. perfringens* was Isolated from Enterotoxaemia Suspected Cases. *Turkish Journal of Veterinary & Animal Sciences*, *20*(1), 63-68.

Quinn, P.J. Carter ME, Markey B, Carter G.R. (1994). Clinical Veterinary Microbiology. pp. 21-67. Mosby-Wolfe, London, U.K.

Sharif, L., Obeidat, J., & Al-Ani, F. (2005). Risk factors for lamb and kid mortality in sheep and goat farms in Jordan. *Bulgarian journal of veterinary medicine*, *8*(2), 99-108.

Sowunmi, K., Olamiji, O.M., Adesola, D.Y., Lawal, A.A., Adejoke, O.K., Emmanuel, K.A., ... & Seun, O.O. (In press) (2022). Antimicrobial Resistance of *Escherichia coli* Isolated from a Variety of Meats in Sabo Market, Ikorodu, Lagos, Nigeria. *Research Square*. doi:10.21203/rs.3.rs-1350579/v1.

Tadesse, D.A., Zhao, S., Tong, E., Ayers, S., Singh, A., Bartholomew, M.J., & McDermott, P. F. (2012). Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950–2002. *Emerging infectious diseases*, *18*(5), 741.

Tenaillon, O., Skurnik, D., Picard, B., & Denamur, E. (2010). The population genetics of commensal *Escherichia coli*. *Nature reviews microbiology*, *8*(3), 207-217.

Tüik, (2022, Şubat 09). *Hayvansal Üretim İstatistikleri*. https://data.tuik.gov.tr/Bulten/Index?p=Hayvansal-%C3%9Cretim-%C4%B0statistikleriAral%C4% B1k-2021-45593&dil=1

Uetake, K. (2013). Newborn calf welfare: A review focusing on mortality rates. *Animal Science Journal*, *84*(2), 101-105.

Ünal, H., Taşkın, T., & Kandemir, Ç. (2018). Housing and Husbandry Practices to Reduce Mortality Rate of Offspring in Small Ruminant Production. *Journal of Animal Production*, *59*(2), 55-63.

Wani, S.A., Bhat, M.A., Samanta, I., Ishaq, S.M., Ashrafi, M.A., & Buchh, A.S. (2004). Epidemiology of diarrhoea caused by rotavirus and *Escherichia coli* in lambs in Kashmir valley, India. *Small ruminant research*, *52*(1-2), 145-153.

Yun, J., Mao, L., Li, J., Hao, F., Yang, L., Zhang, W., ... & Li, W. (2022). Molecular characterization and antimicrobial resistance profile of pathogenic *Escherichia coli* from goats with respiratory disease in eastern China. *Microbial pathogenesis*, 166, 105501.

Zhao, X., Lv, Y., Adam, F.E. A., Xie, Q., Wang, B., Bai, X., ... & Yang, Z. (2021). Comparison of antimicrobial resistance, virulence genes, phylogroups, and biofilm formation of *Escherichia coli* isolated from intensive farming and free-range sheep. *Frontiers in microbiology*, *12*, 1660.

Evaluation of the effects of ozone therapy on the treatment of cutaneous wounds with tissue-loss in dogs and cats

Research Article

Osman Aydın[1a]
Ali Kumandaş[1b]

[1] Department of Surgery, Faculty of Veterinary Medicine, Kırıkkale University, Kırıkkale, Türkiye

ORCİD-
[a]0000-0001-6457-0983
[b]0000-0002-7679-2126

Correspondence
Ali KUMANDAŞ
alikumandas@kku.edu.tr

ABSTRACT

The aim of this study was to investigate the effects of ozone treatments on the rapid and uncomplicated recovery of the wound or the shortening of the time of suture placement. This study was carried out on a total of 40 patients who were admitted to surgery clinic with 20 cats and 20 dogs of different ages, races and genders. These animals were divided into 4 groups. According to the statistics obtained; The patients in group 1 (ozonated oil) had the best reduction in wound size and clinical efficacy. This group was followed by group 3, which was supported by treatment with ozonated water. There was no statistically significant difference in both ozone bagging and control groups. As a result, it was observed that in addition to the treatment of skin wounds with tissue loss, ozonated oil or ozonated water accelerated healing by 50% on average.

Keywords: Ozone bagging, Ozonated oil, Ozonated water, Skin wound with tissue-loss.

INTRODUCTION

Researching methods and materials that will help heal wounds with tissue loss that can be seen in different parts of the body, and wounds that are infected or prone to infection, especially in animals, is a topic that remains up-to-date in the field of human and veterinary medicine (Engin, 2004; Güzel et al., 2011; Gornicki and Gutsze, 2000; Kim et al., 2009; Kirsner and Eaglsterin, 1993; Kutlubay et al., 2010; Nogales et al., 2008; Polat, 2009; Shinuzuka et al., 2008).

Especially in infected wounds, the infection must be eliminated for healing to take place. For this purpose, systemic and parenterally intensive antibiotics are used. In addition, it is used locally in skin wounds with tissue loss, together with pomades, in solutions with antiseptic properties. These applications require regular maintenance and dressing. Especially when pets are considered, the importance of care and dressing increases even more due to the care conditions and natural behaviors. The aim of wound care and dressing is to create a suitable environment for healing to occur, stimulate the onset of healing, control the exudate, minimize pain with mechanical support, and prevent contamination (Beanes et al., 2003; Rigler, 1997; Theoret, 2004; Wilmink and van Weeren, 2004; Witte and Burbul, 1997).

Wound healing is a complex process. These; inflammation, cell migration, angiogenesis, matrix synthesis, new collagen formation, and reepithelialization stages (Glenn and Thomas 1995).

Article info
Submission: 18-04-2022
Accepted: 02-11-2022
Online First: 24-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

How to cite this article
Aydın, O., Kumandaş A. (2022). Evaluation of the effects of ozone therapy on the treatment of cutaneous wounds with tissue-loss in dogs and cats. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 313-320. https://doi.org/10.31797/vetbio.1104760

Healing of the wound, that is, the repair of the tissue that has lost its integrity, is an important biological response for the continuity of life (Dinçer et al., 1996; Li et al., 2007). After injury, local and systemic immune responses are somehow stimulated. Disruption of tissue integrity for any reason initiates a series of physiological events that will restore the functional and morphological characteristics of the wound area (Li et al., 2007; Philips, 2000; Rigler, 1997). The most important feature in wound healing is that as long as a phase is not completed, the next phase does not start and the wound healing stops (Anderson, 1996;Diegelmann and Evans, 2004; Engin, 2000; Philips, 2000).

The aim of this study is to investigate the effects of ozonated oil, ozone bagging and ozonated water, which are the main application methods of ozone therapy in tissue-loss skin wounds, on the healing of tissue-loss skin wounds in dogs and cats.

MATERIAL and METHOD

Animals

This study was carried out on a total of 40 cases, 20 cats and 20 dogs of different ages, genders, and breeds, who were brought to the Surgery clinic with the complaint of cutaneous defect wounds in various parts of the body.

Ozonated oil; Face Ozonated oil (Face Ozon®, Ankara, Turkiye) was used, which was obtained as a result of exposure of specially prepared 0 acid cold-pressed 500 ml olive oil to ozone for 48 hours by a device producing 1 gr/hour ozone.

Ozone Bagging; Bags made of thick nylon prepared according to the extremity of the area to be applied. The ozone gas produced by the 500 mg ozone device supplied by the company (Refresh Ozon Ankara, Turkiye) is sent into the bag via the serum set and lasts for an average of 10 minutes. The wound area was exposed to ozone gas for a period of time.

Ozonated Water; Ozonated water was obtained by applying ozone gas for 10 minutes with a 500 mg/h ozone device to 0.5 lt mains water in a glass jar (Refresh O_3, Ankara, Turkiye). This ozonated water was applied to the wound within 5 min.

After obtaining anamnesis information from the owners for the cases included in this study, detailed information was given verbally about the patient's condition, the severity of the event, the treatment to be performed, and the study, and verbal consent was obtained from the owners. In these patients, after shaving around the wound, the wound was washed with saline and cleaned with chlorhexidine gluconate (Biohand Chx Scrub®, Tosel İlaç Sanayi, Turkiye) solution. Wound dimensions were measured and recorded as mm^2 (Figure 1). One of the ozone therapy methods was chosen according to the region of the wounds and the condition of the wound in the animals included in the study. Ozone applications were made in addition to routine wound care in all groups. After the wound was cleaned, ozone was applied according to the group of the patient. The dressing was applied to these patients. Beta-lactamase group antibiotics were administered to these patients as the antibiotic group. Antibiotic administration was applied for 7 days and antibiotic administration was terminated.

Figure1. Planimetry for calculation of wound area. A- Wound area calculation in a cat, B- Wound area calculation in a dog.

Parenteral antibiotic administration and local ointment were applied to the cases included in the study as a routine treatment. The pomades

used; A mixture of nitrofuran (Furacin®, Zentiva, Turkiye), Centella Asiatica (Madecassol®, Bayer, Turkiye), biyo rivoderm (Biyoteknik®, Turkiye), and rifamycin (Rif®, Koçak Farma, Turkiye) was applied on wounds with deep tissue loss, and zinc oxide (Oxyde de zinc®, Biyoteknik, Turkiye) pomade was applied on more superficial open wounds.

In the study groups, the wound sizes of the animals included in the whole study were measured and recorded on the 0th, 4th, 7th, 10th, 14th, and 21st days before, during, and after the treatment. The findings were recorded in the prepared wound follow-up form.

Patients, consisting of 20 cats and 20 dogs, who applied to the clinic with the complaint of cutaneous wounds with tissue loss, were divided into 4 groups, 10 cases in one group. Some of these were classified as full-thickness, while others were classified as superficial. In addition to the routine treatment protocol, ozone oil was applied to 5 cats and 5 dogs in the 1st group. In the second group, ozone bagging was applied to 5 cats and 5 dogs in addition to the routine treatment protocol. In addition to the routine treatment protocol, ozonated water was applied to 5 cats and 5 dogs in the 3rd group. In the 4th group, only routine treatment was applied to 5 cats and 5 dogs as the control group. In wounds with excessive tissue loss, granulation, contraction and epithelialization conditions of the tissue were evaluated after the discharge from the wound was stopped, and sutures were applied to the appropriate ones. In cases where sutures were applied, the size of the wound just before suturing was recorded by drawing on millimetric paper and was accepted as the last measurement of the case.

Statistical Analysis

The efficacy of ozone therapy was determined by evaluating the findings clinically and statistically. 5 animals from each group were used for statistical data. In the statistical evaluation, the data obtained on the 1st, 4th, 7th, 10th, 14th and 21st days were used. After statistical analysis of the data, it was determined that there was no normal distribution. Cats and dogs were evaluated separately. Differences between groups were determined by the Kruskal-Wallis test, which is a non-parametric test. Significance levels were evaluated with the Mann-Whitney test. Pearson Correlation analysis was performed to determine the relationship between wound size and wound closure rate in the study groups. Independent variables t-test was used to determine the relationship between infection and wound closure. SPSS 25.0 statistical package program was used for statistical evaluations. $P<0.05$ was accepted to determine the significance levels.

RESULTS

The distribution of wounds in the animals included in the study showed great variation. These; bite wounds, burn wounds, injuries due to accidents and falls, leash wounds, wire cutting wounds, wounds with tissue loss due to scratching due to parasitic skin diseases, and wounds with tissue loss due to poor postoperative process management. In the animals included in the study, it was determined that the wounds mainly appeared in the neck region, back region, abdomen, and extremities as bitten wounds and incisional wounds caused by sharp objects.

The methods specified in the literature were used to evaluate the data on wound healing (Bohling et al. 2004). Wound healing was evaluated according to signs of infection control, wound contraction, epithelialization and granulation. Obtained data were evaluated separately in dogs and cats for each group in terms of wound closure times and closure rates. This was done to avoid the difficulty of comparing differences by species. Ozonated oil application was easier to obtain and applied more easily during application compared to other applications, and no symptoms of allergic

reaction or sensitivity were encountered (Figure 2).

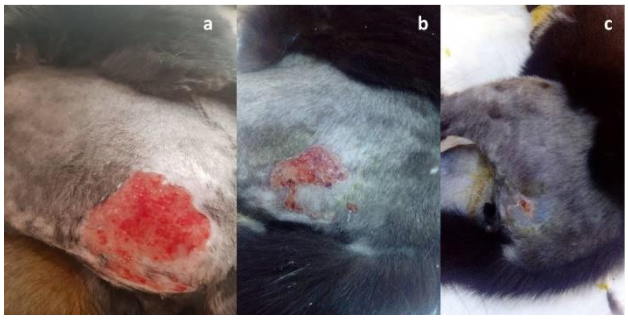


Figure 2. Ozonated oil treatment. a: Day 0 of treatment, b: Day 7of treatment, c: Day 14 of treatment.

It has been observed that the ozone bagging technique can be applied without any problems in animals with a calm temperament, however, there are difficulties in restraint in animals that are active and aggressive. It was observed that the dry wound line was irrigated after ozone bagging (Figure 3).

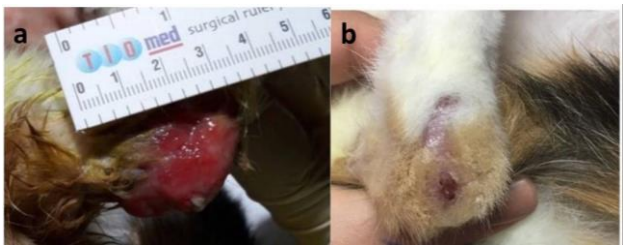


Figure 3. Ozone bagging treatment. a: Day 0 of treatment, b: Day 21of treatment.

No allergic reactions or sensitivities were observed in the patients. In all ozone applications, it was observed that granulation tissue protruding from the wound line was formed as a result of long-term ozone application. However, it was determined that this rate was slightly higher in ozonated water applications (Figure 4). Only routine treatment was applied to the patients in the control group.

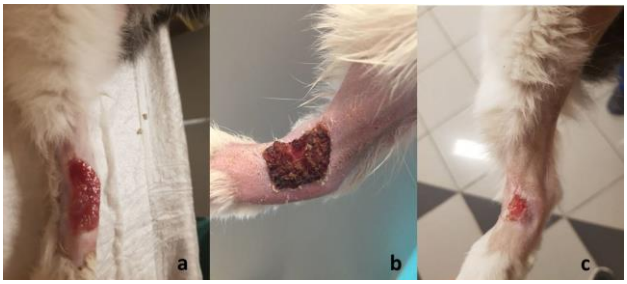


Figure 4: Ozonated water treatment. a: Day 0 of treatment, b: Day 7of treatment, c: Day 14 of treatment.

The clinical efficacy percentages of the groups were calculated according to the data obtained as a result of the follow-up of the animals included in the study during the recovery period (Table 1). In some animals, the wounds were closed by suturing on the 7th, 10th, and 14th days. The time of suturing was considered the recovery time in these animals. When evaluated as the healing time, it was determined that the fastest healing was in the wounds of the animals in the ozonated oil and ozonated water groups.

Table 1. Average of clinical efficacy percentages of study groups

Group	Animal	Day 0	Day 4	Day 7	Day 10	Day 14	Day 21	P
Ozonated oil	Dog	0	28,98	37,026	66,81[a]	78,04	91,76	0,040
	Cat	0	21,38[c]	30,74	77,50	*	-	0,056
Ozone bagging	Dog	0	17,49	19,16	25,44[a]	48,98[b]	88,86	0,013[a]- 0,016[b]
	Cat	0	9,85[c]	14,48[d]	14,09	11,11	57,76	0,041[c]- 0,027[d]
Ozonated water	Dog	0	30,94	32,48	44,40	76,01[b]	*	0,010
	Cat	0	32,13	37,92[d]	51,70	71,96	93,85	0,027
Control	Dog	0	16,29	33,52	45,65	61,41[b]	88,12	0,016
	Cat	0	8,49[c]	13,42[d]	16,16	28,92	35,32	0,021[c]-0,013[d]

(*)There is no data to participate in the measurement because the suture was applied or the wound closure was completed. Data are expressed as mean (n=40). [a]:Significant difference in clinical efficacy parameters between ozonated olive oil group and other groups on 10th day in dogs. [b]:Significant difference in clinical efficacy parameters between ozonated olive oil group and other groups on 14th day in dogs. [c]:Significant difference in clinical efficacy parameters between ozonated olive oil group and other groups on 4th day in cats. [d]:Significant difference in clinical efficacy parameters between ozonated olive oil group and other groups on 7th day in cats.

When wound closure times and percentages were examined in statistical evaluations, a negative correlation was found between the ozonated oil group and both the control group and ozone bagging group. A positive correlation was determined between antibiotic use and wound healing time in all study groups. Wound healing and closure rate was determined at the highest level, especially in the groups in which ozonated oil was applied. There was no statistically significant relationship between ozone bagging and the control group as a local application (Figure 5).

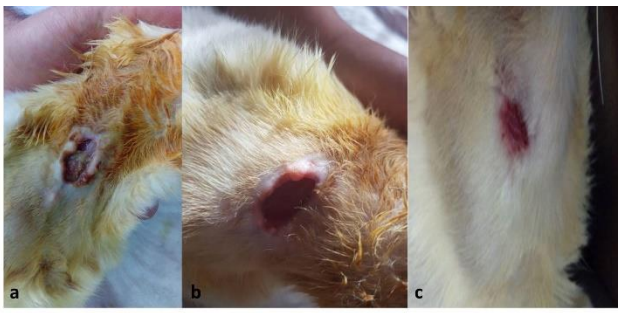


Figure 5. Routine application in the Control group. **a:** Day 0 of treatment, **b:** Day 14 of treatment, **c:** Day 21 of treatment.

The two techniques with the highest recovery percentage were determined as Ozonated oil and Ozonated water. In the statistical evaluation of the dogs included in the study, it was determined that there was a significant difference between the ozone oil application and both the control and ozone bagging methods P=0,040. In the cat groups, although the values were close in terms of clinical efficacy, statistically marginal significance was determined at P=0,056. There was no significant difference between ozone bagging and control groups in terms of healing and wound closure times and percentages of clinical efficacy.

DISCUSSION

Wounds and wound healing have been of great importance for all living things since the beginning of life. Therefore, many studies have been conducted on wound healing. Studies on wound healing in veterinary medicine are mostly performed on porcine and rodents. Especially in the field of veterinary medicine, proper and rapid wound healing is of great importance due to the care conditions and natural behavior of animals. Delayed healing in infected wounds or wounds with infected tissue loss causes patients to be affected systemically. For this purpose, it is necessary to apply drugs that will not harm the organism with its strong disinfectant feature for the purpose of healing infected wounds. In this context, the use of O_3 gas, one of the natural disinfectants, can be considered an innovative approach. The wound may close spontaneously by contraction and epithelialization. In wounds with large tissue loss, contraction is not sufficient, but if granulation is good and there is no infection, it can be closed in a shorter time by suturing. In this study, comparisons were made in terms of the contribution of different ozone therapy methods to skin wound healing, self-closing, or suturing time in dogs and cats.

In a study by Valacchi et al. (2005), ozone application was applied to patients with chronic ischemia using the autohemotherapy method and they concluded that it had a positive effect on healing. In this study, since it was thought that pure ozone gas could have a negative effect on ozone applications, it was applied in the form of an O_3/O_2 mixture. However, according to the statistical information obtained at the end of the study, it was observed that the recovery was not adversely affected in the cases where the ozone bagging method was applied, but it did not provide a significant contribution.

In another study conducted on patients who received ozone therapy, it was found that the patients who received ozone therapy had a positive effect, and it was concluded that the aspect that positively affected the healing in patients was the increase in tissue oxygenation. In addition, it has been determined that ozone does not have a stored structure and cannot show its effect very much because it cannot

penetrate when applied to the skin (Bocci, 2006). He concluded that ozonated oil is the best penetrating oil to the skin in ozone applications and it has a more positive effect on the patients who are applied. In this study, which was carried out in parallel with this information, it was determined that the recovery was faster in cases where ozonated oil and ozonated water were applied.

Kim et al. (2009) created full-thickness skin wounds in rats. They applied pure olive oil and ozonated oil to these wounds. It was determined that the healing was faster in the cases where ozonated oil was applied, and the amount of fibroblast growth factor was higher on the 7th day. Compared with this information, it was determined that among the ozone groups in the study, ozonated oil accelerated recovery by 50% and ozonated water by 40%.

Travagli et al. (2010) examined the effectiveness of ozone gas and ozonated oil applications on skin diseases and reviewed the recovery stages. In the study, they reported that the penetration of ozone oil on the skin is better, and that ozone gas does not have a significant positive effect on healing as a result of the lack of penetrating ability. The result obtained in our study is similar to the results in the literature. It has been observed that it has a positive effect on recovery in animals using ozonated oil. In the ozone bagging group, it had the same effect as the control group in terms of its contribution to healing.

Khadre et al. (2001) evaluated the antimicrobial effect of ozone in a study they conducted. As a result of this study, they concluded that ozone gas has a very strong antimicrobial effect and it would be appropriate to use it in foods since it does not leave any residue after use. In this study, it was observed that the infection regressed in a shorter time compared to other groups and was brought under control, especially in cases where ozonated oil and ozonated water were applied to skin wounds with infected tissue loss.

Bocci et al. (2005) also investigated the autohemotherapy method they performed in patients with tumor tissues, which supports the positive effect of ozone on tissue oxygenation, and they supported that ozone increases tissue oxygenation.

In order for the tissue to heal and repair itself, it is necessary to increase oxygenation and improve blood flow (Clavo et al., 2004). In this study, like other researchers, it has been observed that it has a positive effect on tissue oxygenation and healing, especially in tissue loss skin wounds where ozone oil and ozonated water are used.

Bette et al. (2006) applied intraperitoneal O_3/O_2 mixture in a study they conducted on rats with peritonitis and observed that the survival rate increased by 33% in rats with peritonitis due to increased oxygenation and decreased bacterial load. In this study on skin wounds with tissue loss, it was observed that the infection was reduced and prevented at a faster rate in ozone groups of infected wounds.

Zamora et al. (2007) also divided 192 Wistar male rats with experimental septic shock into 16 groups of 12 animals in their study. As a result of the research carried out, the use of ozone alone does not have any effect on recovery. However, they observed that when used as an adjunct treatment, it had a positive effect on recovery. In a study by Shinuzka et al. (2008), it was determined that bacteria exposed to ozone gas had a significant reduction in toxin release and thus minimized the harmful effect it created. In this study, it was observed that the infection recovered faster and tissue regeneration increased in all ozone groups.

Kuroda et al. (2015) also investigated the effect of ozonated water on tumor tissues. As a result of this research, it was determined that ozonated water increased tissue oxygenation

and positively affected healing. At the same time, since direct inhalation of O_3 gas will have a toxic effect on the lungs, treatment with ozonated water has been tried and it has been determined that it has a positive effect on the treatment. In this study, it was determined that the recovery was the fastest after ozone oil in cases where ozonated water was used.

CONCLUSION

As a result, it is a known fact that ozone is used for therapeutic purposes with very different methods. Especially in the field of veterinary surgery, literature information about its use in skin wounds with tissue loss is rare. In this study, ozone oil, ozonated water and ozone bagging techniques, which are the main local applications of ozone therapy in the treatment of tissue loss skin wounds, were compared in terms of their contribution to clinical healing. The findings obtained after the study were compared with the medical literature due to the scarcity of studies in the veterinary field. It has been determined from the data obtained that ozone therapy contributes to wound healing. However, among these study groups, it was determined that the most effective technique was ozone oil application, ozonated water and ozone bagging method, respectively. It is thought that ozone oil can be used more comfortably with the dressing material clinically. In the light of the findings obtained from the study, it was concluded that the use of ozone oil will contribute 50% more to wound healing and this study will shed light on more detailed studies in both veterinary medicine and human medicine.

ACKNOWLEDGMENT

Ethical approval:

Kırıkkale University Animal Experiments Ethics Committee. Ethical Approval: Date: 2020- 2020/05 Number: HADYEK-2020-29

Conflict of interest: Conflict of interest: The authors stated that there is no conflict of interest

REFERENCES

Anderson, D. (1996). Wound management in small animal practice, *In Pract*, 18, 115-128.

Beanes, S.R., DANG, C., Soo, C., Ting, K. (2003). Skin repair and scar formation: the-central role of TGF-β, *Expert. Rev. Mol. Med*, *5* (21), 1-11.

Bette, M., Nusing, R.M., Mutters, R., Zamora, Z.B., Menendez, S., Schulz, S. (2006). Efficiency of tazobactam/piperacilin in lethal peritonitis is enhanced after preconditioning of rats with O_3/O_2-pneumoperitoneum, *Shock*, *25*, 23-29.

Bocci, V. (1999). Biological and clinical effects of ozone: has ozone therapy a future in medicine?, *British Journal of Biomedical Science*, *56*, 270-279.

Bocci, V. (2006). Scientific and medical aspects of ozone therapy. *State of the art. Archives of Medical Research*, *37*, 425–435.

Bocci, V., Larini, A., Micheli, V. (2005). Restorationnof normoxia by ozone therapy may control neoplastic growth: a review and a working hypothesis, *The Journal of Alternative and Complementary Medicine*, *11* (2), 257-265.

Bohling, M.W., Henderson, R.A., Swaim, S.F., Kincaid, S.A., Wright, J.C. (2004). Cutaneous wound healing in the cat: A macroscopic description and comparison with-cutaneous wound healing in the dog, *Vet. Surg*, *33*, 579-587.

Clavo, B., Perez, J.L., Lopez, L., Suarez, G., Lloret, M., Rodriguez, V., Marias, D., Santana, M., Hernandez, M.A., Martin-Oliva, R., Robaina, F. (2004). Ozone therapy for tumor oxygenation: a pilot study, *Evidence-based Complementary and Alternative Medicine*, 1(1), 93-98.

Diegelmann, R.F., Evans, M.C. (2004). Wound healing: an overview of acute, fibrotic-and delayed healing, *Front. Biosci*, *9*, 283-289.

Dinçer, S., Babul, A., Erdoğan, D., Özgül, L., Dinçer, S.L. (1996). Effect of-Taurine on Wound Healing, *Aminoacids*, *10*, 59-71.

Engin, A. (2004). Yara iyileşmesi, In İ. Sayek (Ed.), *Temel Cerrahi*, pp. (266-277). Güneş Kitabevi, Ankara,

Glenn, F.B., Thomas, A.M. (1995). Pharmacologic enhancement of wound healing, *Annu Rev. Med*, *46*, 467-481.

Gornicki, A., Gutsze, A. (2000). In vitro effects of ozone on human erythrocyte membranes: An EPR study. *Acta Biochimica Polonica*, *47*(4), 963-971.

Güzel, Ö., Yıldar, E., Erdikmen, D.O. (2011). Medikal ozon ve veteriner cerrahide kullanımı, *Vet. Fak. Derg. Med*., *37*(2), 177-184.

Khadre, M.A., Yousef, A.E., Kim J.G. (2001). Microbiological aspects of ozone applications in food:a review, *Journal of Food Scıence*, *66*(9), 1242-1252.

Kim, H.S., Noh, S.U., Han, Y.W., Kim, K.M., Kang, H., Kim, H.O., Park, Y.M. (2009). Therapeutic effects of topical application of ozone on acute

cutaneous wound healing. *Journal of Korean Medical Science 24*, 368-374.

Kirsner, R.S., Eaglsterin, W.H. (1993). The wound healing process, *Dermatol. Clin*, *11*(4), 629-640.

Kuroda, K., Azuma, K., Mori, T., Kawamoto, K., Murahata, Y., Tsuka, T., Osaki, T., Ito, N., Imagawa, T., Itoh, F., Okamoto, Y. (2015). The safety and anti-tumor effects of ozonated water in vivo, *International Journal of Molecular Sciences*, *16*, 25108-25120.

Kutlubay, Z., Engin, B., Serdaroğlu, S., Tüzün, Y. (2010). Dermatolojide ozon tedavisi, *DERMATOZ*, *1*(4), 209-216

Li, J., Chen, J., Kirsner, R. (2007). Pathophysiology of acute wound healing, *Clin. Dermatol*, *25*, 9-18.

Nogales, C.G., Ferrari, P.A., Kantorovich, E.O., Lage-Marques, J.L. (2008). Ozone therapy in medicine and dentistry, *The Journal of Contemporary Dental Practice*, *9*(4), 75-84.

Philips, S.J. (2000). Physiology of wound healing and surgical wound care, *ASAIO Journal*, *46*, 2-5.

Polat, H. (2009). Dezenfeksiyon amaçlı ozon kullanımı, *Yunus Araştırma Bülteni*, *9*(2), 14-15.

Rigler, D.J. (1997). Inflamation and repair, In T.C. Jones, R.D. Hunt, N.W. King, (Eds.), *Veterinary Pathology*, (pp.150-157).

Shinuzuka, Y., Uemetsu, K., Takagi, M., Taura, Y. (2008). Comparison of the amounts of endotoxin released from Escherichia coli after exposure to antibiotics and ozone: an In Vitro evaluation, *Journal of Veterinary Medical Science*, *70*(4), 419-422.

Theoret, C.L. (2004). Wound repair in the horse: Problems and proposed innovative-solutions, *Clin. Tech. Equine. Pract*, 3;134-140. doi:/10.1053/j.ctep.2004.08.010

Travagli, V., Zanardi, I., Valacchi, G., Bocci, V. (2010). Ozone and ozonated oils in skin diseases: *A review. Mediators of Inflammation*, doi:/10.1155/2010/610418

Valacchi, G., Fortino, V., Bocci, V. (2005). The dual action of ozone on the skin, *Br J Dermatol*, *153*, 1096-1100.

Wilmink, J.M., Van Weeren, P.R. (2004). Differences in wound healing between horses and-ponies: application of research results to the clinical approach of equine wounds, *Clin. Tech. Equine. Pract, 3*; 123-133.

Witte, M.B., Burbul, A. (1997). General principles of wound healing, *Surg. Clin. N.Amer*, *77*; 509-258.

Zamora, Z. B., Menéndez, S., Bette, M., Mutters, R., Hoffmann, S., & Schulz, S. (2003, August). Ozone Prophylactic Effect and Antibiotics as a Modulator of Inflamatory Septic process in Rats. In *Proceedings of the 16th International Ozone Association World Congress, Las Vegas, NV, USA* (p. 123). https://medicalozone.info/wp-content/uploads/Ozone-Therapy-Sepsis.pdf

Afyonkarahisar ilinde meme tümörü saptanan köpek ve kedilerde ırk ve yaş dağılımı

Breed and age distribution of dogs and cats with mammary tumors in Afyonkarahisar province

Research Article

Duygu Baki Acar[1a]
Ece Tunç[1b]

[1]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03200, Afyonkarahisar, Türkiye

ORCİD-
[a]0000-0002-6884-2621
[b]0000-0001-6913-4961

Correspondence
Ece TUNÇ
etunc@aku.edu.tr

ÖZET

Araştırmada Afyonkarahisar ilindeki köpek ve kedilerde rastlanan meme tümörü olgularının ırk ve yaş dağılımlarının değerlendirilmesi amaçlandı. Araştırmanın materyalini Afyon Kocatepe Üniversitesi Veteriner Fakültesi Hayvan Hastanesi ve özel veteriner kliniklerine meme tümörü şikayeti ile getirilen köpek ve kediler oluşturdu. Çalışmamızda meme tümörü şikayeti ile gelen 23 adet köpek ve 8 adet kedi değerlendirildi. Terrier ırkı köpeklerde %34,8 oranında meme tümörüne rastlanırken, melez ırklarda %21,7, Pointer ırkında %17,4, Cocker ırkında %13, Golden Retriever'larda %4,3, Alman Çoban Köpeği'nde %4,3 ve Belçika Malinois ırkında %4,3 oranında olduğu belirlendi. Kediler değerlendirildiğinde, 7 adet Tekir ırkı (%87,5) ve 1 adet melez ırk (%12,5) kedide meme tümörü olgusu ile karşılaşıldı. Meme tümörü gözlenen köpek ve kedilerin yaş dağılımları değerlendirildiğinde, köpeklerde yaş ortalamasının 8,08±3,46 olduğu tespit edilirken, kedilerde yaş ortalamasının 11,12±1,72 olduğu gözlendi. Bulgulara göre köpeklerde iki yaşından itibaren ileri yaşlara kadar her yaşta meme tümörü görülme riskinin olduğu, kedilerde meme tümörü olgularının ileri yaştaki hayvanlarda daha sık rastlandığı ortaya konulmuştur. Sonuç olarak, hasta sahiplerinin her yaş ve ırktaki kedi ve köpeklerinde meme tümörünün ilk tespit edildiği anda veteriner hekime başvurmalarının öneminin aktarılması ve hastalıktan korunma, tanı ve tedavi sürecine ilişkin detaylı bilgi verilmesinin önemli olduğu kanaatine varıldı.

Anahtar Kelimeler: Kedi, Köpek, Meme Tümörü, Irk, Yaş.

ABSTRACT

The present study investigated the breed and age distribution of mammary tumor cases in dogs and cats in Afyonkarahisar province. The materials of this study were the dogs and cats brought to Animal Hospital of Afyon Kocatepe University and private veterinary clinics with mammary masses. History of cases including breed and age information were recorded during the clinical examination. Totally 23 dogs and 8 cats with mammary tumors were evaluated in the study. The most frequent breed was Terrier (34.8%), mixed breed (21.7%), Pointer (17.4%), Cocker (13%), Golden Retriever (4.3%), German Shepherd (4.3%), and Belgium Malinois (4.3%) in dogs, respectively. In the cats, Tabby cat (87.5%) and a mixed breed (12.5%) were detected with mammary tumors. The age distribution was 8.08±3.46 as the mean age in dogs and 11.12±1.72 in cats. The results of this study revealed that dogs are at risk of mammary tumors as early as two years old. Nonetheless, the mammary tumors seen in cats are usually older. In conclusion, animal owners should be informed about each pet at every age and breed, detailed information about this pathology, and seek medical advice and treatment immediately.

Keywords: Age, Breed, Cat, Dog, Mammary Tumor.

Article info
Submission: 15-08-2022
Accepted: 08-12-2022
Online First: 25-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

How to cite this article
Baki Acar, D., Tunç E. (2022). Breed and age distribution of dogs and cats with mammary tumors in Afyonkarahisar province. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 321-326. https://doi.org/10.31797/vetbio.1162218

GİRİŞ

Tüm canlılarda hücre ve dokuların yenilenmesi, organ ve sistemlerin görevlerini yerine getirmesi, büyüme, gelişme, gerektiğinde büyümenin durdurulması ve apoptozis (programlı hücre ölümü) süreçleri homeostazis olarak adlandırılan bir düzen çerçevesinde sürmektedir. Homeostazisin herhangi bir aşamasında meydana gelen aksaklık, fizyolojik aşamaları olumsuz etkileyerek özellikle apoptozis sürecinde yaşanan sorunlar sonucunda hücrelerde hiperplazi veya neoplaziler ortaya çıkmaktadır (Cabadak, 2008). Meme dokusundan köken alan tümörler, zamanla farklı meme loblarına, bölgesel ve uzak lenf nodüllerine, akciğer ve karaciğer başta olmak üzere diğer organlara metastaz yapabilen neoplazik oluşumlardır. Köpeklerde görülme sıklığı kedilere oranla çok yüksektir. Ancak köpeklerde malign meme tümörü görülme oranı %41-53 arasında iken kedilerde bu oran %90'a ulaşmaktadır. Kedilerde malign meme tümörü görülme sıklığı yüksek olduğu için prognoz daha kötü olmakta, yaşam süresi ve yaşam kalitesinde belirgin düşüş görülmektedir (Alaçam, 2008; Johnson, 1993).

Meme tümörleri daha çok ileri yaştaki hayvanlarda görülürken, nadiren iki yaşın altındaki köpek ve kedilerde de rastlanmaktadır. Oluşumu henüz tam olarak ortaya konulamamış olsa da cinsiyet, yaş, ırk, vücut ağırlığı, endojen hormonlar ve büyüme faktörleri etkili olmaktadır. (Alaçam, 2008; Brodey vd., 1983). Meme tümörünün Poodle, İngiliz Spaniel Cocker, Dascshunds, Shih tzu gibi ırklarda görülme sıklığının yüksek olduğu bildirilirken (Kim vd., 2016; Zatloukal vd., 2005), ırk faktörünün bir etkisinin olmadığı da belirtilmektedir (Root Kustritz, 2010). Köpek ve kedilerde kısırlaştırmanın meme tümörü riskini önemli oranda azalttığı bilinmektedir. Özellikle ilk östrus belirtilerini göstermeden önce kısırlaştırılan hayvanlarda meme tümörü görülme oranı %0,5 iken, ilk östrusunu gösterdikten sonra yapılan kısırlaştırmalarda oran %8'e çıkmakta, ilerleyen yaşlarda yapılan kısırlaştırmalarda ise %26 ve üzerinde tespit edilmektedir (Alenza vd., 2000; Misdorp, 1988).

Sunulan bu araştırma ile Afyonkarahisar ilinde köpek ve kedilerde saptanan meme tümörü olgularında ırk ve yaş dağılımlarının ortaya konulması amaçlandı. Böylece mevcut risk faktörleri belirlenerek Afyonkarahisar'da yaşayan köpek ve kedi sahiplerine gerekli uyarılar ve önerilerin yapılması, köpek ve kedilerde meme tümörünün erken dönemde teşhisinin konulması, gerekli tedavilerin hızla uygulanması hedeflenmiştir.

MATERYAL VE METHOD

Araştırmanın materyalini iki yıllık süreçte değerlendirmeye alınan ve Afyon Kocatepe Üniversitesi Veteriner Fakültesi Hayvan Hastanesi ile Afyonkarahisar ilinde bulunan özel veteriner kliniklerine meme tümörü şikayeti ile getirilen köpek ve kediler oluşturmaktadır. Çalışmamız Afyon Kocatepe Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu (AKÜHADYEK) 49533702/120 numaralı izni ile gerçekleştirildi.

Tanı ve tedavi amacıyla kliniğe getirilen köpek ve kedilerin anamnez bilgileri, özellikle ırk ve yaş bilgileri kaydedildi. Ardından hayvanlara rutin meme tümörü teşhisinde kullanılan muayene yöntemleri yapıldı. Bu amaçla klinik muayene, akciğer ve abdominal radyografi, abdominal ultrasonografi, tam kan analizi, kan biyokimyasal analizleri uygulandı. Klinik muayene bulguları uygun olan olgulara tedavi prosedürü planlanarak rutin cerrahi meme tümörü tedavisi gerçekleştirildi. Klinik muayene sonuçlarına göre operatif tedaviye uygun olmayan hastalara preoperatif antibiyotik, nonsteroid antienflamatuar, sıvı/elektrolit sağaltımı gibi destek tedaviler

başlandı. Bu hastalarda klinik muayeneler tekrarlanarak klinik iyileşmeyi takiben meme tümörünün operatif tedavisi gerçekleştirildi. Tedavi sonrası tüm hastalar yakın takip altına alınarak izleyen dönemde gerekli kontrolleri gerçekleştirildi. Hayvan sahiplerinin talebi ve analiz maliyeti gibi faktörler nedeniyle olguların bir kısmında histopatolojik muayeneler gerçekleştirilemediği için araştırmaya kitlelerin histopatolojik değerlendirmeleri dahil edilmedi.

Çalışma sonucunda elde edilen veriler Windows SPSS 16.0 Paket Programı (SPSS Inc., Chicago, IL, USA) ile analiz edildi. Yaş ve ırk bulgularının ortalama ± standart sapma ve yüzde (%) değerleri hesaplandı.

BULGULAR

Çalışmamızda meme tümörü şikayeti ile gelen 23 adet köpek ve 8 adet kedi değerlendirildi. Buna göre kliniklere başvuran köpeklerden en sık Terrier ırkında (%34,8) meme tümörüne rastlanırken, melez ırk köpeklerde %21,7 oranında, Pointer ırkında %17,4 oranında, Cocker ırkında %13, Golden Retriever'larda %4,3 oranında, Alman Çoban Köpeği'nde %4,3 ve Belçika Malinois ırkında da %4,3 oranında belirlendi (Tablo 1). Kedilerde yapılan değerlendirmemizde ise 7 adet Tekir ırkı (%87,5) ve 1 adet melez ırk (%12,5) kedide meme tümörü olgusu ile karşılaşıldı (Tablo 2). Çalışmada kullanılan köpeklerin (tablo 3) ve kedilerin (tablo 4) yaş dağılımları tablo olarak sunulmuştur.

Meme tümörü gözlenen köpek ve kedilerin yaş dağılımları değerlendirildiğinde, köpeklerde yaş ortalamasının 8,08±3,46 olduğu tespit edilirken (Tablo 3), kedilerde yaş ortalamasının 11,12±1,72 olduğu gözlendi (Tablo 4).

Tablo 1. Çalışmada değerlendirilen meme tümörlü köpeklerde ırk dağılımı

Irk	Sayı	Yüzde (%)
Terrier	8	**34,8**
Melez	5	**21,7**
Pointer	4	**17,4**
Cocker	3	**13,0**
Golden Retriever	1	**4,3**
Alman Çoban Köpeği	1	**4,3**
Belçika Malinois	**1**	**4,3**

Tablo 2. Çalışmada değerlendirilen meme tümörlü kedilerde ırk dağılımı

Irk	Sayı	Yüzde (%)
Tekir	7	**87,5**
Melez	**1**	**12,5**

Tablo 3. Çalışmada değerlendirilen meme tümörlü köpeklerde yaş dağılımı

Yaş	Sayı	Yüzde (%)
2	1	**4,3**
3	1	**4,3**
4	1	**4,3**
5	3	**13,0**
6	3	**13,0**
8	6	**26,1**
10	2	**8,7**
11	2	**8,7**
13	2	**8,7**
14	**2**	**8,7**

Tablo 4. Çalışmada değerlendirilen meme tümörlü kedilerde yaş dağılımı

Yaş	Sayı	Yüzde (%)
8	1	**12,5**
10	2	**25,0**
11	1	**12,5**
12	2	**25,0**
13	**2**	**25,0**

TARTIŞMA

İnsanlarda ve evcil hayvanlarda meme tümörü insidansı dünya çapında artmaya devam etmektedir. Bu sürecin ciddiyetine ve yapılan çok sayıda çalışmaya rağmen, neoplazik yapıların karmaşık biyolojisinden dolayı bu alandaki mevcut tıbbi araştırmalar yetersiz

kalmakta, tanı ve tedavisi ile ilgili araştırmalar sürmektedir (Baştan vd., 2009; Özenç ve Baki Acar, 2018; Pastor vd., 2018; Pinho vd., 2012). Meme tümörleri köpeklerde yaygın görülmekte ve tüm tümör olgularının yaklaşık %25 ile %50'sini temsil etmektedir. Kedilerde meme tümörleri köpeklere oranla daha az rastlanmakta ve bu neoplazmalar tüm lenfoid olmayan tümörlerin yaklaşık %10'unu temsil etmektedir (Millanta vd., 2005). Meme tümörlerinin, kedilerde malign tümörlerin %12'sini ve dişi kedilerde tüm neoplazmaların %17'sini oluşturduğu belirtilmektedir. Kedilerde meme tümörlerinin lenfoma ve deri tümörlerinden sonra en sık görülen tümör olduğu bildirilmiştir (Novosad, 2003). Sunulan bu çalışma ile kedi ve köpeklerde sıklıkla rastlanan ve hayvanların yaşam kalitesini olumsuz etkileyen meme tümörü olguları, Afyonkarahisar ilinde iki yıllık süre zarfında ırk ve yaş dağılımı bakımından değerlendirildi.

Meme tümörü oluşumu açısından yüksek risk altında olduğu bildirilen köpek ırkları arasında Britanya Eponyölü, İngiliz Springer Spaniel, Labrador Retriever, İrlanda Setteri, Pointer, Pyrenees Çoban köpeği, Samoyed, Airedale Teriyeri, Minyatür Poodle ve Kaniş ırkı köpekler sayılabilmektedir (Novosad 2003). Yapılan bir meme tümörü çalışması ırk dağılımında otuz adet köpekten 15'inin Terrier (%50), 4 adedinin Poodle (%13,3), 3'ünün Melez ırk (%10), 2'sinin Alman Kurdu (%6,7), 2'sinin Pinscher (%6,7), 1'inin Cocker (%3,3), 1'inin Kangal (%3,3), 1'inin Papillon (%3,3) ve 1'inin de Setter (%3.3) olduğunu belirtmektedir (Baştan ve Zonturlu, 2002). Sunulan çalışmada, bildirilen araştırmalara benzer olarak en sık Terrier ırkındameme tümörüne rastlanırken, özellikle Pointer ve melez ırk köpekler olmakla birlikte birçok farklı ırkta da meme tümörü saptandı. Meme tümörlerinin çeşitli kedi ırklarında görüldüğü, ancak Siyam kedilerinde daha genç yaşlarda tümör gelişme riskine sahip olduğu bildirilmektedir (Novosad, 2003). Benzer şekilde bir diğer çalışmada da meme tümörlerinden tüm ırkların etkilenebileceği ancak Siyam ırklarının daha yatkın olduğu ve daha erken yaşlarda görülebileceği vurgulanmıştır (Zappulli vd., 2005). Overley vd., (2005) çalışmalarında kedideki meme tümörü vakalarının %8'inin Domestic Long Hair, %79'unun Domestic Short Hair, %3'ünün Persian, %7'sinin Siyam ve %3'ünün de diğer ırklara ait olduğunu bildirmiştir. Ito vd. (1996) meme tümörü teşhisi konulan elli üç kediden yirmi yedisinin Japanese Domestic, on sekiz adedinin Siyam ve dört adedinin de Persian ırkı olduğunu belirtmiştir. Kedilerde yapılan değerlendirmemizde ise 7 adet Tekir ırkı (%87,5) ve 1 adet melez ırk (%12,5) kedide meme tümörü olgusu ile karşılaşıldı. Çalışmanın gerçekleştirildiği iki yıllık süre boyunca Afyonkarahisar ilinde sadece 8 adet kedide meme tümörü olgusunun saptanması nedeniyle, ilerleyen dönemde daha uzun süren ve daha kapsamlı bir araştırma ile ilimizde kedilerde meme olgularının araştırılmasının uygun olacağı kanaatine varıldı.

Ežerskytė vd. (2011) meme tümörü belirlenen seksen adet köpek ile yaptığı çalışmada 1-5 yaş arası köpeklerde %4 oranında, on bir yaş üzeri köpeklerde %40 oranında ve en yüksek oranın (%56) 5-10 yaş arası köpeklerde olduğunu saptamıştır. Banchi vd. (2022)'de iki yüz yirmi beş köpek ile yaptığı çalışmada meme tümörlü köpeklerin yaş ortalamasını 9,8 olarak tespit etmiştir. Millanta vd. (2005) tarafından gerçekleştirilen bir çalışmada meme tümörü olan kırk yedi adet köpeğin yaş ortalamasını 8,9±2,4 olarak bildirmektedir. Alçığır vd. (2018)'de on sekiz olguyu değerlendirdiği bir çalışmada meme tümörü gelişen köpeklerin yaş ortalamalarının 11 olduğunu ve olguların%50'sini melez ırk köpeklerin, %22'sini Terrier ırkı köpeklerin oluşturduğunu bildirmektedir. Çalışmamızda ise yirmi üç adet köpeğin yaş ortalaması 8,08±3,46 bulunurken, yaşlarının iki ile 14 yaş arasında değiştiği gözlendi.

Seixas vd. (2011)'de meme tümörlü kedilerde yaptığı çalışmada kedilerin yaş ortalamasının 12 olduğunu, bir başka çalışmada ise 68 adet kedinin yaş ortalaması 9,6±2,8 olarak bildirilmiştir (Millanta vd., 2005). Sunulan çalışmada meme tümörü olan kedilerin %25'inin 10 yaşında olduğu ve %62,5'inin ise yaşının on bir yaş ve üzerinde olduğu tespit edildi.

SONUÇ

Çalışmamızdan elde edilen bulgular sonucunda Afyonkarahisar ilinde meme tümörü şikayeti ile kliniklere getirilen köpek ve kedilerde ırk ve yaş dağılımları değerlendirildi. Buna göre, genel kanının tersine köpeklerin erken yaşlarda da meme tümörü riski altında olduğu, hasta sahiplerinin köpeklerinin meme dokusu çevresinde herhangi bir kitle fark etmeleri halinde zaman kaybetmeden veteriner hekime başvurmalarının ve gerekli tedavinin uygulanması hakkında bilgilendirmenin önemi ortaya konuldu. Kedi ve köpek sahiplerinin, ırk ve yaşın meme tümörüne etkisi bakımından aydınlatılmasının hayvanlarının sağlıklı bir yaşam geçirebilmesini sağlamak açısından katkı sağlayacağı kanısına varıldı.

AÇIKLAMALAR

Bu çalışma Afyon Kocatepe Üniversitesi Bilimsel Araştırma Projeleri Koordinasyon Birimi (BAPK) tarafından desteklenmiştir (Proje Numarası: 17.KARİYER.146).

Etik beyan: Sunulan çalışma Afyon Kocatepe Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu (AKÜHADYEK) 49533702/120 numaralı izni ile gerçekleştirildi.

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

KAYNAKLAR

Alaçam, E. (2008). Meme Sorunları. Köpek ve Kedilerde Üreme Süreci ve Sorunları. Ed., AlaçamE. Medisan Yayınevi, Ankara.

Alçığır, M.E., Anadol, E., Gültiken, N., Alkan, K.K., Alkan, H., & Kanca, H. (2018). Mikst orjinli köpek meme tümörlerinde cxc chemokine ligand 12 ve g protein coupled receptor 30 ekspresyonları. *Kocatepe Veterinary Journal. 11*(2), 104-112.

Alenza, D.P., Pena, L., Del Castillo, N., & Nieto, A.I. (2000). Factors influencing the incidence and prognosis of canine mammary tumors. *J. Small Anim. Pract. 41*, 287-291.

Banchi, P., Morello, E.M., Bertero, A., Ricci, A., & Rota A. (2022). A retrospective study and survival analysis on bitches with mammary tumours spayed at the same time of mastectomy. *Veterinary and Comparative Oncology*. *20*(1), 172-178.

Baştan, A., & Zonturlu, A. (2002). Köpek meme tümörlerinde yaş, ırk, tümör tipi ve yerleşim yeri arasındaki ilişkinin araştırılması. *Ankara Üniversitesi Veteriner Fakültesi Dergisi.* 49(3), 203-206.

Baştan, A., Özenç, E., Pir Yağcı, İ. & Baki Acar, D. (2009). Ultrasonographic Evaluation of Mammary Tumors in Bitches. *Kafkas Üniv Vet Fak Derg*. *15* (1), 81-86.

Brodey, R.S., Goldscmidt, M.H., & Roszel, J.R. (1983). Canine mammary gland neoplasms. *J. Am. Anim. Hosp. Assoc*. *19*, 61-90.

Cabadak, H. (2008). Hücre siklusu ve kanser. *ADÜ Tıp Fak Derg. 9*(3), 51-61.

Ežerskytė, A., Zamokas, G., Grigonis, A., & Juodžiukynienė, N. (2011). The retrospective analysis of mammary tumors in dogs. *Vet Med Zoot*. *53*(75), 3-8.

Ito, T., Kadosawa, T., Mochizuki, M., Matsunaga, S., Nishimura, R., & Sasaki, N. (1996). Prognosis of malignant mammary tumor in 53 cats. *Journal of veterinary medical Science*. *58*(8), 723-726.

Johnson S.D. (1993). Reproductive Systems. In: Slatter, D. Editor. Textbook of Small Animal Surgery, 2nd edition. Saunders, Philadelphia.

Kim, H.W., Lim, H.Y., Shin, J.I., Seung, B.J., Ju, J.H., & Sur, J.H. (2016). Breed- anda ge- related differences in canine mammary tumors. *The Canadian J Vet Res*. *80*, 146-155.

Millanta, F., Calandrella, M., Bari, G., Niccolini, M., Vannozzi, I., & Poli, A. (2005). Comparison of steroid receptor expression in normal, dysplastic, and neoplastic canine and feline mammary tissues. *Research in veterinary science*. *79*(3), 225-232.

Misdorp, W. (1988). Canine mammary tumors: Protective effect of late ovariectomy and stimulating effect of progestins. *Vet Q. 10*, 26-33.

Novosad, C.A. (2003). Principles of treatment for mammary gland tumors. *Clinical techniques in small animal practice*. *18*(2), 107-109.

Overley, B., Shofer, F. S., Goldschmidt, M. H., Sherer, D., & Sorenmo, K. U. (2005). Association between

ovarihysterectomy and feline mammary carcinoma. *Journal of Veterinary Internal Medicine*, *19*(4), 560-563.

Özenç, E. & Baki Acar, D. (2018). Köpeklerde Meme Tümörlerinde B-Mod Ultrasonografik Değerlendirmeler. *Turkiye Klinikleri J Vet Sci Obstet Gynecol-Special Topics*. *4*(1), 50-54.

Pastor, N., Caballé, N.C., Santella, M., Ezquerra, L. J., Tarazona, R., & Duran, E. (2018). Epidemiological study of canine mammary tumors: age, breed, size and malignancy. *Austral journal of veterinary sciences*, *50*(3), 143-147.

Pinho, S.S., Carvalho, S., Cabral, J., Reis, C.A., & Gartner, F. (2012). Canine tumors: a spontaneous animal model of human carcinogenesis. *Transl Res 159*, 165-72.

Root Kustritz, M.V. (2010). Should bitches be spayed at the time of removalof a mammary mass? In: Root Kustritz, MV. Editor. Clinical Canine and Feline Reproduction Evidence Based Answers. Blackwell Publishing, Iowa.

Seixas, F., Palmeira, C., Pires, M.A., Bento, M.J., & Lopes, C. (2011) Grade is an independent prognostic factor for feline mammary carcinomas: a clinicopathological and survival analysis. *The Veterinary Journal*.; *187*(1), 65-71.

The inhibitory effects of tyrosol on clinical *Candida glabrata* planktonic and biofilm cells

Research Article

Zarifeh Adampour[1a]
Betül Yılmaz Öztürk[2b]
İlknur Dağ [2,3c]

[1]Institute of Science, Biotechnology and Biosafety Department, Eskisehir Osmangazi University, Eskisehir, Türkiye
[2]Central Research Laboratory Application and Research Center, Eskisehir Osmangazi University, Eskisehir, Türkiye
[2,3]Vocational Health Services High School, Eskisehir Osmangazi University, Eskisehir, Türkiye

ORCİD-
[a]0000-0001-6505-4999
[b]0000-0002-1817-8240
[c]0000-0002-7352-8653

Correspondence
İlknur DAĞ
ildag@ogu.edu.tr

Article info
Submission: 04-08-2022
Accepted: 13-12-2022
Online First: 25-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

ABSTRACT

Biofilm formation is an important problem in the healthcare industry and veterinary medicine and is very common in natural, industrial or hospital environments. Microorganisms can become very resistant to antimicrobials and environmental factors by biofilm forming on biotic or abiotic surfaces. There is a need to develop new, effective and specific antimicrobials that can reduce pathogenicity in biofilm formation that threatens public health due to their role in medical device-related or infectious diseases. *Candida* species are opportunistic pathogenic yeasts and can cause superficial or disseminated infections. Especially *C. glabrata* is one of the most common microorganisms causing fungal infections in immunocompromised patients and drug resistance is observed when associated with biofilm. Tyrosol (2-[4-hydroxyphenyl] ethanol) can act as both a quorum sensing molecule and an exogenous agent on *Candida* species. In this study, the antifungal activity of tyrosol against a clinical *C. glabrata* isolate was investigated on both planktonic and biofilm forms. Broth microdilution test results demonstrated the inhibitory effect of tyrosol on *C. glabrata*. Transmission electron microscopic findings showed that tyrosol affected the planktonic *C. glabrata* cells in a multi targeted manner, and in the groups treated with tyrosol, significant damage was observed in the cell wall, cell membrane, cytoplasm, nucleus and mitochondria. Also, scanning electron microscopic images confirmed biofilm reduction in pre-/post-biofilm applications as a result of tyrosol treatment. In conclusion, tyrosol may be a potential alternative candidate for reducing the C. *glabrata* biofilm.

Keywords: Biofilm, *C. glabrata*, Tyrosol, Electron Microscopy.

INTRODUCTION

Microorganisms form biofilms on host tissues, domestic/industrial surfaces, biomaterials such as prostheses and catheters, and can cause highly resistant infections relative to their planktonic form (Lison et al., 2022; Sharma et al., 2019). The robust architecture of the biofilm contributes to resistance by both reduced metabolic activity and the presence of the extracellular matrix. There are several steps in the formation of a typical microbial biofilm, such as attachment to living or non-living surfaces, maturation and dispersion. Therefore, biofilms can act as a reservoir for pathogenic cells, and their release can cause septicemia, leading to disseminated systemic infections of organs and tissues. It is reported in the literature that approximately 80% of microbial infections are associated with biofilms and show high mortality rates (Srinivasan et al., 2017; Chen and Wen, 2011). Thus, there is an urgent need for new anti-biofilm approaches to control and eradicate these infections.

How to cite this article
Adampour, Z., Yılmaz Öztürk, B., Dağ İ. (2022). The inhibitory effects of tyrosol on clinical *Candida glabrata* planktonic and biofilm cells. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 327-337. https://doi.org/10.31797/vetbio.1153190

It is stated in the literature that most of the devices with medical applications can cause biofilm infections. *Candida* is prominent in biofilms formed by clinically important fungi. Especially *C. albicans* can form a biofilm in almost every medical device and can cause infection by passing into the bloodstream from where it is located. Although *C. albicans* is the most frequently isolated *Candida* species, there has been a significant increase in the frequency of non-*albicans* species such as *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. krusei* in recent years due to various reasons such as immunosuppression, various diseases, prematurity, exposure to broad-spectrum antibiotics. (Ramage et al., 2005).

One of the interesting and effective methods proposed in the biofilm management in recent years is the use of Quorum sensing (QS) molecules. When the cell density in the biofilm reaches a certain level, microorganisms secrete QS molecules and various physiological activities are regulated by the modification of target genes. Tyrosol is one of the major QS molecules isolated from *C. albicans*. Aromatic alcohol tyrosol is known to induce yeast-hyphae transition, but studies on its antibiofilm activity are very limited. However, it is reported that tyrosol has a biofilm-reducing effect when used in combination with some known antifungals (Arias et al., 2016). On the other hand, exogenously applied tyrosol stimulates hyphae production in the early stages and before some cells start hyphal development (1-6 hours). This condition shows that tyrosol acts as a QS molecule on both planktonic cells and biofilm (Rodriguez and Cernakova, 2020). However, information on the antibiofilm effects of tyrosol on *C. glabrata* is very limited in the literature.

To study fungal biofilms, improvable *in vitro* models are needed. The effects of various inhibitory substances on these models can be studied at different concentrations or the effects of different growth environments can be investigated. Especially, the oral cavity, which contains thousands of bacterial and nearly a hundred fungal species, attracts attention as a valuable research area for biofilm models. In this study, it was aimed to investigate the effectiveness of *C. glabrata* on planktonic and biofilm forms as a result of exogenous application of tyrosol, a newly defined QS molecule. In addition, acrylic resin was used to mimic the oral biofilm with an *in vitro* model. Microbiological and electron microscopic techniques were used in the study.

MATERIAL and METHOD

Preparation of yeast suspension

The clinical *C. glabrata* 1744 isolate used in the study was obtained from Eskisehir Osmangazi University, Faculty of Medicine, Department of Microbiology and was selected among the strains with strong biofilm characteristics. This isolate was maintained on glycerol/SDB (20/80 v/v) at −80 °C in Eskisehir Osmangazi University Central Research Laboratory, Application and Research Center (ARUM), Biotechnology Laboratory. *C. glabrata* 1744 isolate from the stock culture was activated in YPD (Yeast Extract Peptone Dextrose) medium at 37 °C, and then incubated in RPMI 1640 broth and 37 °C for 24 hours. The sample was then taken up in 5 ml of 0.85% saline. The turbidity of the suspension was adjusted to 0.5 McFarland (1-5 x 10^6 cells/ml). The prepared initial suspensions were diluted 1/50 with sterile saline and then 1/20 with RPMI 1640, and a concentration of 1-5 x 10^3 cells/ml was obtained.

Susceptibility of planktonic C. glabrata to tyrosol

The determination of the Minimum Inhibition Concentration (MIC) of tyrosol ((2-[4-hydroxyphenyl]) (Sigma Chemical Co., USA)) was based on the Clinical and Laboratory Standards Institute Microdilution Reference

Method (CLSI M27-A3). Briefly, cell suspensions of isolates were adjusted to the 0.5 McFarland standard in saline solution and diluted in Roswell Park Memorial Institute (RPMI 1640, Sigma Aldrich) medium. Tyrosol was first diluted in deionized water and then diluted in RPMI 1640 medium to obtain concentrations ranging from 600-1.17 µg/ml. Then, 100 µl of each cell suspension and 100 µl of each tyrosol concentration were pipetted into 96-well microplates. Plates were incubated at 37 °C for 48 hours and MIC value was defined as the lowest drug concentration that inhibited the growth of microorganisms. Standard control antifungal Amphotericin B (Sigma, USA) (AppliChem A1907,0050) was dissolved in DMSO (dimethyl sulfoxide) and its final concentrations were adjusted to be 16-0.0313 µg/ml. After the microdilution process was carried out as stated above and the MIC of amphotericin B was defined as the lowest drug concentration that complete inhibition of visible growth (CLSI M27-A3). All the experiments were performed in triplicate and results were averaged (Dağ et al., 2018).

Transmission electron microscopy (TEM)

In the study, TEM was used to reveal the ultrastructural effects of tyrosol on planktonic *C. glabrata*. The medium and tyrosol solutions prepared at MIC and ½ MIC concentrations were added to sterile 6-well tissue culture plates. Microorganism suspensions were also inoculated at 200 µl per well. A control group without tyrosol was also prepared on the same plate, and absorbance measurements at 490-600 nm were made after 24 hours of incubation at 37°C.

For the routine TEM procedure of the samples, the cell suspensions taken into sterile centrifuge tubes were centrifuged for 15 minutes, the supernatant was removed, and the obtained pellet was washed with PBS buffer 3 times for 10 minutes and taken into 2.5% glutaraldehyde (in PBS) medium for primary fixation (24 hours at +4 °C). After washing with PBS 3 times for 15 minutes, the samples were left in osmium tetraoxide in the dark for 1 hour with the help of a rotator, and then washed again with PBS 3 times at the end of the period. Samples embedded within 1.5 % agar were dehydrated in a series of ethanol at increasing concentrations after block staining with 1% uranyl acetate. After clarification with propylene oxide and embedding in resin, the samples were polymerized in an incubator at 60 °C for 48 hours, 60 nm thick sections were taken on copper grids with the help of an ultramicrotome (Leica UltraCutR) and stained with uranyl acetate+lead citrate. Then samples were analyzed with TEM device (Hitachi HT 7800), (Yapıcı et al., 2021; Öztürk et al., 2020).

Scanning electron microscopy (SEM)

Prebiofilm study

The effect of tyrosol on biofilm formation was tested by SEM and the biofilms were developed on the surface of the acrylic resin support materials (Acrystone, 8 x 4 mm) placed in 24-well plates. Acrylic resin samples were sterilized under UV before the study. To study the prebiofilm effects, tyrosol were applied to the cells at the beginning (0 h) of the study and at the three different ranges. For this purpose, acyrilic resin pieces were replaced in 24 well plates. Tyrosol was prepared in RPMI 1640 at MIC concentration and dilutions were added to each well. The turbidity of the yeast suspension was adjusted to 0.5 Mc Farland. Each well of 24 well plates was inoculated with standardized cell cells. Inoculated RPMI 1640 without tyrosol was also included to study. Amphotericin B was used as control antifungal. Chlorhexidine gluconate (Pharmactive, %0,12) was also used as positive control. Then the samples were taken into the routine SEM procedure (Yapici et al., 2021).

Postbiofilm study

The effectiveness of tyrosol on the formed biofilm was used and analyzed by SEM device,

as it could give an idea about the therapeutic properties of tyrosol. For this purpose, biofilms were developed by inoculating standardized cell suspensions developed in RPMI 1640 medium on acrylic resin surfaces placed in 24-well tissue culture plates, and then incubated for an additional 24 hours by adding MIC concentration of tyrosol. The samples were then subjected to the routine SEM procedure (Yapıcı et al., 2021)

SEM analysis

After the samples were incubated in 2.5% glutaraldehyde (in PBS) for 24 hours at +4 °C, they were washed 3 times with PBS for 15 minutes to remove the fixative. The samples, which were taken into osmium tetroxide for secondary fixation, were kept in the dark for 1 hour with the help of a rotator and were washed 3 times with PBS again. After the samples were dehydrated with increasing concentrations of ethanol, they were were dried on aluminum stubs, coated with gold-palladium (Polaron SC7620 Sputter Coater) and analysed with SEM device (HITACHI Regulus 8230), (Öztürk et al., 2022; Seneviratne et al., 2009).

RESULTS

According to the results of *in vitro* antifungal susceptibility test results of *C. glabrata* 1744 isolate, MIC value of tyrosol was found as 18.75 µg/ml. The MIC value of Amphotericin B, which is widely used in the treatment of fungal diseases, was found to be lower than tryosol (0.78 µg/ml).

The data obtained by TEM revealed the ultrastructural changes in the tyrosol treated groups compared to the control group. Untreated control *C. glabrata* cells exhibited typical and healthy *Candida* morphology. The nucleus is prominent and centrally located, the cytoplasm is regular, the cell wall and cytoplasmic membrane structure were observed as a whole. Mitochondria and cristae structures were also clearly observed (Figure 1A-C). In cells exposed to tyrosol at ½ MIC concentration, a marked enlargement between the wall and the membrane was remarkable. Also, deformations in the cells, occasional cytoplasmic ruptures, severe nuclear damage, cytoplasmic lysis, structural disruptions in the cell membrane, and a small number of ghost cells were detected (Figure 1D-F). Cells exposed to MIC concentration of tyrosol similarly showed cytoplasmic lysis, cell wall and membrane damage, nuclear damage, and mitochondrial greying (Figure 1G-I).

In this study, the effects of tryosol on *C. glabrata* biofilm were investigated by SEM. In the control group samples, smooth-surfaced and healthy-appearance biofilm cells were found on the acrylic surface (Figure 2A). When the cells were exposed to tyrosol at MIC concentration before the biofilm formation (prebiofilm study), some bleb formations and cell shrinkages were observed (Figure 2B). Cells were exposed to tyrosol at MIC concentration after the biofilm formation (postbiofilm study), cellular deformities appeared in many different ways. Damages in the form of indentations, fused cells or shape deformities were determined (Figure 2C). In the samples treated with antifungal AMB at MIC concentration, a significant decrease was observed in the cells compared to the control, but it was determined that the cell morphologies were largely preserved (Figure 2D). In the chlorhexidine-administered groups, the number of cells decreased compared to the control, and these few cells were also damaged (Figure 2E).

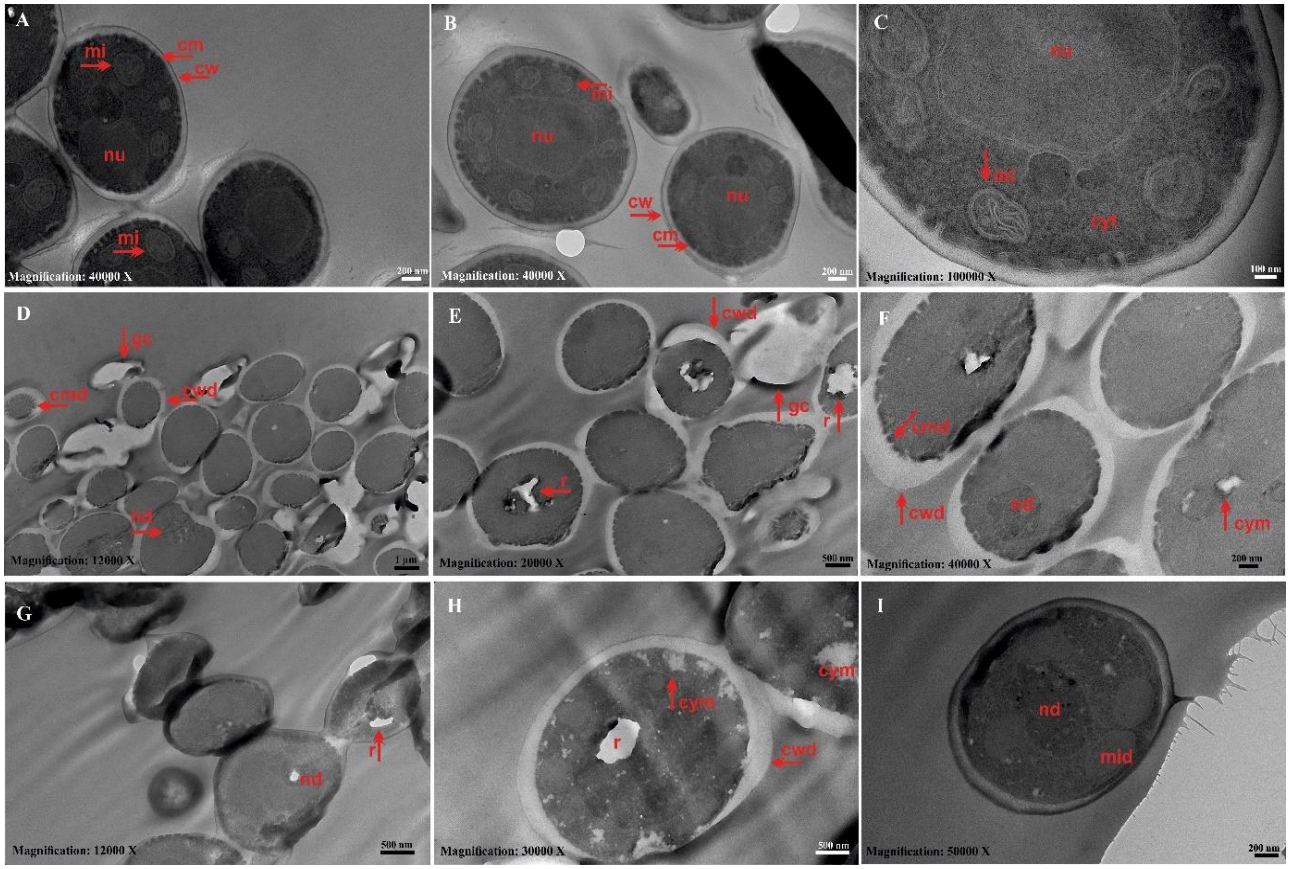


Figure 1. Demonstrative TEM images of *C. glabrata* 1744 cells before and after exposure to tyrosol, showing ultrastructural changes. (A–C) Untreated control cells revealed normal cell morphology with centrally located nucleus, regular cytoplasm, intact cell wall and cytoplasmic membrane structures and healthy mitochondria (A and B= 200 nm, C = 100 nm scales); (D-I) Cells treated with tyrosol at subinhibitor concentration (1/2 MIC) showed some irregularities and damages with marked enlargement between the wall and the membrane structure, membrane deformations, cytoplasmic ruptures, severe nuclear damage, cytoplasmic lysis and ghost cells (D= 1µm, E= 500 nm, F = 200 nm scales); (G-I) Cells treated with tyrosol at subinhibitor concentration MIC) exhibited cytoplasmic lysis, cell wall and membrane damage, nuclear damage, and mitochondrial greying (G and H= 500 nm, I = 200 nm scales) Abbreviations: mi mitochondria, nu nucleus, cvm cytoplasmic meltings, nd nuclear damage, md membrane damage, mid mitochondrial damage, r ruptures, gc ghost cell, cwd cell wall damage

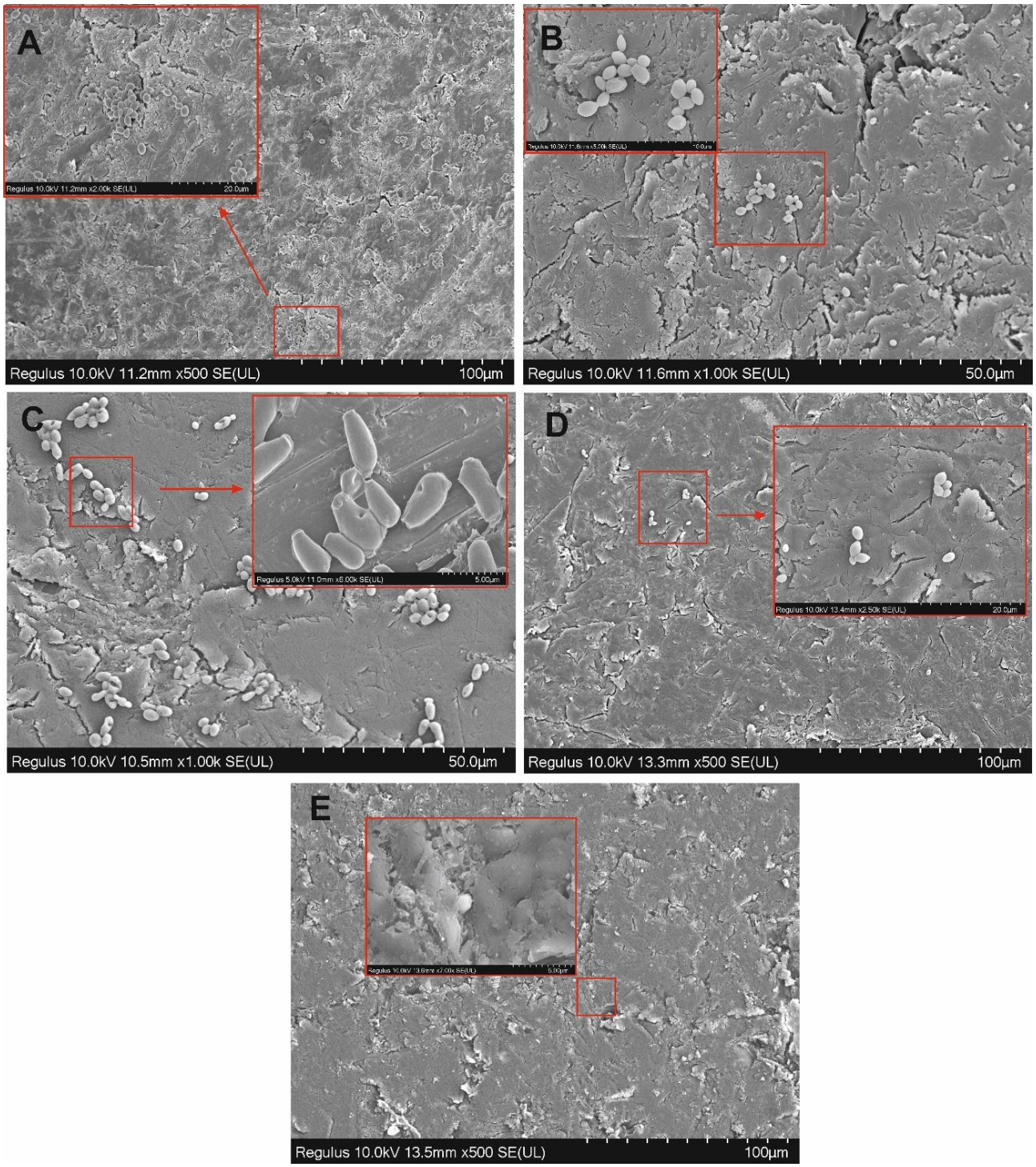


Figure 2. Demonstrative SEM images of *C. glabrata* 1744 biofilm with and without tyrosol on acyrlic resin surfaces, showing morphological changes. (A) Untreated control group biofilm dense biofilm and EPS structure (A= 100 µm and larger magnification of same image at the upper left corner 20 µm); (B) Cells treated with tyrosol at MIC concentration before the biofilm formation (prebiofilm study) revealed some bleb formations and cell shrinkages (B= 50 µm and larger magnification of same image at the upper left corner 10 µm); (C) Cells treated with tyrosol at MIC concentration after the biofilm formation (postbiofilm study), cellular deformities with indentations, fused cells or shape deformities (C= 50 µm and larger magnification of same image at the upper right corner 5 µm); (D) Cells treated with amphotericin B at MIC concentration showed significant decrease in biofilm cells but intact cell structures (D= 100 µm and larger magnification of same image at the upper right corner 20 µm); (E) Cells treated with chlorhexidine gluconate showed decreased biofilm and few damaged cells (E= 100 µm and larger magnification of same image at the upper left corner 5 µm).

DISCUSSION

Biofilm formation is a mechanism that allows many microorganisms to survive in the host environment and plays a very important role in antimicrobial resistance. Biofilms, also called microbial communities that adhere to surfaces, organize under the control of QS signaling molecules (Zhou et al., 2020). This event is a communication mechanism by synchronized gene expression in response to the cell density population (Sharma et al., 2019). Biofilm-associated infections, which can develop on the surface of biomaterials such as indwelling devices and in various tissues, are considered an important and increasingly common source of morbidity and mortality in the healthcare system. Biofilm eradication is difficult and often requires removal of the infected indwelling device, and biofilm is the main source of many persistent infections. Therefore, much more research is needed in terms of biofilm prevention and management.

The QS event in fungi is relatively new and only started to be understood after the discovery of farnesol, which discovery the filamentation process in *C. albicans*. After this discovery, tyrosol was also described as a QS molecule in *C. albicans*, and its roles on growth, morphogenesis and biofilm formation began to be elucidated. In *Candida*, farnesol induces the transition from yeast to hyphae and biofilm formation, while tyrosol has the opposite effect. On the other hand, such QS molecules can also be applied exogenously and act as an antimicrobial agent against various fungal and bacterial pathogens (Monteiro et al., 2017). Currently, fungal QS research is in initial level and studies on the effects of tyrosol on non-albicans *Candida* are very limited (Rodriguez and Cernakova, 2020). In this study, the *in vitro* inhibitory effects of tyrosol on a clinical *C. glabrata* isolate were investigated.

In the literature, it is seen that the MIC values obtained for QS molecules such as tyrosol and farnesol against microorganisms such as *Candida* and *Streptococcus mutans* show differences (40 µmol l^{-1} - 5 mmol l^{-1}). Cordeiro et al. (2015) investigated the effect of high concentrations of exogenous tyrosol on planktonic cells and biofilms of *C. albicans* (n = 10) and *C. tropicalis* (n = 10) isolates, and evaluated whether tyrosol was synergistic with antifungals targeting cellular ergosterol. The authors studied concentration ranges of 0.04 - 22 mM in the study, which is approximately 2x - 1000x times the concentration reached endogenously in *C. albicans*. According to the MIC data obtained as a result of broth microdilution, planktonic cells exhibited varying MIC values of 2.5 - 5.0 mM. In addition, a high rate of synergy was found in combinations of tyrosol with AMB, itraconazole and fluconazole. In another study by Monteiro et al. (2015), the effect of tyrosol on the adhesion of *C. albicans* and *C. glabrata* on acrylic surfaces was investigated. The authors reported that *C. albicans* isolate was more sensitive to tyrosol than *C. glabrata*. While the MIC value was 50 mM and the MFC value was 90 mM for *C. albicans*, the MIC value was 90 mM and the MFC value was 100 mM for *C. glabrata*.

In another study, the effectiveness of tyrosol on single and mixed biofilms of *C. albicans*, *C. glabrata* and *S. mutans* on acrylic resin and hydroxyapatite surfaces was investigated. The MIC values obtained for tyrosol in the study were found to be higher than these values reported in the literature (MIC for *C. glabrata*: 90 mmol l^{-1}, MFC: 100 mmol l^{-1}). The authors stated that the different MIC results in these studies can be explained by the different susceptibility profile in various strains. In the same study, tyrosol, especially used at a concentration of 200 mmol l^{-1}, led to a significant decrease in the number of CFUs and metabolic activity for all single and mixed biofilms formed on both surfaces. Cell damage after tyrosol exposure was also supported by SEM examinations (Arias et al., 2016). In our

study, the MIC value obtained for tyrosol was 18.75 µg/ml. This value is generally lower than the above-mentioned study data. However, as stated in the literature, MIC data show a wide range in studies related to tyrosol, and the differences may vary depending on the strain. Also, one of the limitations of our study is that we only studied with a single isolate, so it seems that there is a need for detailed studies on the subject.

When the effects of MIC and ½ MIC concentration of tyrosol exposure on planktonic *C. glabrata* cells were analyzed by TEM, it was determined that tyrosol had a multi-targeted effect. To our knowledge, there is no study in the literature examining the effects of tyrosol on *C. glabrata* at the ultrastructural level, but there are some studies examining the effects of farnesol on *Candida* species by TEM. In a previous study, the antibiofilm effects of farnesol on *Candida* species were investigated, and cell wall-membrane separations, ghost cell formations, cytoplasm damage, vacuol formation, some completely lyzed cells were detected in the TEM data obtained as a result of exposure to 37.5 µM farnesol on *C. albicans* ATCC 14053 (Yapıcı et al., 2021). In another study, a relatively regular morphology was observed in *C. albicans* cells treated with farnesol, and membrane and wall damage and electron density were observed in a small number of cells (Yenice Gürsu, 2020). Decanis et al (2011) also reported large and irregular cytoplasmic vacuoles and especially peripheral vacuoles regarding the ultrastructural effects of farnesol on *C. albicans*, and showed cell wall and membrane damage and cytoplasmic granulation findings. However, in our study, the effects of tyrosol on *C. glabrata* were significantly damaging and significant damage was observed at the level of cell wall, membrane, nucleus and mitochondria.

According to the data we obtained with SEM, it was determined that there were biofilm reduction and cellular damage as a result of both prebiofilm and post biofilm applications after tyrosol exposure. In a previous study, the effects of a combination of tyrosol and farnesol were investigated against *C. albicans* and *C. glabrata* isolates in planktonic or biofilm form. In the study, total biomass, metabolic activity and the amount of cultivable cells were determined; tyrosol and farnesol showed a synergistic effect against *C. glabrata* planktonic cells. In addition, in studies in which both single and dual biofilms were created, there was a significant reduction in total biomass and metabolic activity for *C. glabrata* biofilms, and the results were confirmed by SEM studies. The biofilm structure of *C. glabrata* was thinner and partially covered the surface in groups where the active substance was applied alone or in combination (Monteiro et al., 2017). In also another study, the effects of tyrosol and chlorhexidine gluconate in combination against planktonic or biofilm cells of *C. albicans*, *C. glabrata* and *S. mutans* were investigated. The authors classified the effect of the drug combination on planktonic cells as antagonistic for *C. albicans* and as indifferent for all other strains. The drugs showed indifferent effects on the tested biofilms. However, the drug combination showed a synergistic effect in reducing the number of *C. albicans* hyphae (Do Vale et al., 2017). In also our study, the reducing effects of tyrosol on *C. glabrata* biofilm and damaging cells were observed. It has been reported in the literature that tyrosol can act by inhibiting the incipient production of the extracellular matrix. (Monteiro et al., 2015; Li et al., 2007). Although a strong decrease in biofilm was observed in the groups treated with AMB and chlorhexidine compared to the control, it was determined that the morphology was better preserved in a small number of cells on the surface. It is known that chlorhexidine has important effects in the fight against biofilm-related oral infections, and it is used as the main component in toothpastes,

mouthwashes or gels. In our study, strong effects of chlorhexidine on the *C. glabrata* biofilm on the acrylic surface were observed. The EPS structure observed in the biofilm structure could not be seen clearly in the SEM images in our study, but it is thought that the dehydration processes applied during electron microscopic follow-up reduce EPS. In addition, the curved nature of acrylic resin created some difficulties in SEM examinations, such as charging or loss of clarity in some areas.

C. albicans is a polymorphic yeast that can form pseudohyphae and true hyphae. *C. glabrata* is a species that grows only as yeast, and the MIC and MFC values obtained for this species are higher than *C. albicans*. Although *C. albicans* is the main pathogen in cases of denture stomatitis, the lower susceptibility of *C. glabrata* proves that this species is resistant to conventional antifungals, and this issue stands out as an important health problem (Meşeli et al., 2019; Hannah et al., 2017). While the microbiota in the oral cavity protects the human body against infectious diseases, various oral infections may develop due to reasons such as immunodeficiency, malnutrition, poor oral hygiene or the use of improperly fitting removable dentures. Denute stomatitis is a chronic oral fungal infection that significantly affects denture wearers. This infection has often been associated with microbial biofilms on the acrylic base and prosthesis-bearing mucosa. Removable acrylic resin prostheses provide adequate physical, mechanical and aesthetic properties, but the resinous nature of these materials makes them vulnerable. Local factors such as mismatched dentures, rough or cracked denture surfaces, weak saliva, mucous discharge, or proliferation of *Candida* species on the denture surface can lead to the development of denture stomatitis (Bajunaid et al., 2022., Monteiro et al., 2015; Bianchi et al., 2016). In our study, acrylic resin tooth samples were used to imitate the oral biofilm model and it was observed that tryrosol had reducing effects on *C. glabrata* biofilms growing on the surfaces.

CONCLUSION

In conclusion, investigating the effects of QS molecules as one of the new antifungal strategies targeting biofilm formation and growth in *Candida* is a promising topic. The obtained data can give an idea about prophylactic approaches, especially in providing oral hygiene. Tyrosol showed a significant inhibition effect on both planktonic and biofilm forms of *C. glabrata*. On the other hand, since the problem of antimicrobial resistance to existing antifungals is a very important health problem, the combined use of new antifungal candidate agents such as tyrosol with antifungals may produce stronger effects. However, it is necessary to verify the data by making detailed studies on this subject.

ACKNOWLEDGMENT

This work has been supported by Eskisehir Osmangazi University Scientific Research Projects Coordination Unit under grant number FDK-2022-2228

This study was presented as an oral presentation at the 7th International Congress on Advances in Veterinary Sciences & Technics (ICAVST) on July 25-29, 2022.

Also, we thank Eskisehir Osmangazi University Central Research Laboratory Application and Research Center (ARUM) for allowing all facilities and infrastructure to use.

Ethical approval:

We declare as the authors of the study that the approval of the Ethics committee is not required within the scope of the presented study.

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

REFERENCES

Arias, L. S., Delbem, A. C. B., Fernandes, R. A., Barbosa, D. B., & Monteiro, D.R.(2016). Activity of tyrosol against single and mixed-species oral biofilms. *Journal of applied microbiology*, *120*(5), 1240-1249. https://doi.org/10.1111/jam.13070.

Bajunaid, S. O., Baras, B. H., Weir, M. D., & Xu, H. H. (2022). Denture Acrylic Resin Material with Antibacterial and Protein-Repelling Properties for the Prevention of Denture Stomatitis. *Polymers*, *14*(2), 230. https://doi.org/10.3390/polym14020230.

Bianchi, C.M.P.D.C.; Bianchi, H.A.; Tadano, T.; De Paula, C.R.; Hoffmann-Santos, H.D.; Leite, D.P.L.; Hahn, R.C. (2016). Factors related to oral candidiasis in elderly users and non-users of removable dental prostheses. Revista do Instituto de Medicina Tropical de São Paulo, 58, e17. https://doi.org/10.1590/S1678-9946201658017

Chen, L., Wen, Y. M. (2011). The role of bacterial biofilm in persistent infections and control strategies. *International Journal of Oral Science*, *3*(2), 66-73.

Clinical Laboratory Standards Institute, (CLSI). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved Standard-Third Edition, CLSI document M27-A3. Wayne, PA: Clinical and Laboratory Standards Institute; 2008.

Cordeiro, R. D. A., Teixeira, C. E., Brilhante, R. S., Castelo-Branco, D. S., Alencar, L. P., de Oliveira, J. S., Monteiro A.J., Bandeira T.J.P.G., Sidrim J.J.C, Moreira, J.L.B., Rocha, M. F. (2015). Exogenous tyrosol inhibits planktonic cells and biofilms of Candida species and enhances their susceptibility to antifungals. *FEMS Yeast Research*, *15*(4), fov012. https://doi.org/10.1093/femsyr/fov012.

Dağ, İ., Yenice Gürsu, Bükay., Dikmen, G., Ulken, Z. (2018). Influence of Carvacrol on the Planktonıc and Biofılm Forms of Salmonella spp And Listerıa Monocytogenes. *Fresenius Environmental Bulletin*, 27(11), 7270-7277.

Décanis, N., Tazi, N., Correia, A., Vilanova, M., & Rouabhia, M. (2011). Farnesol, a fungal quorum-sensing molecule triggers *Candida albicans* morphological changes by downregulating the expression of different secreted aspartyl proteinase genes. *The Open Microbiology Journal*, *5*: 119-126. doi: 10.2174/1874285801105010119.

Do Vale, L. R., Delbem, A. C. B., Arias, L. S., Fernandes, R. A., Vieira, A. P. M., Barbosa, D. B., Monteiro, D. R. (2017). Differential effects of the combination of tyrosol with chlorhexidine gluconate on oral biofilms. *Oral Diseases*, *23*(4), 537-541. doi:10.1111/odi.12648.

Gursu, B. Y., Dag, İ., & Dikmen, G. (2022). Antifungal and antibiofilm efficacy of cinnamaldehyde-loaded poly (DL-lactide-co-glycolide)(PLGA) nanoparticles against Candida albicans. *International Microbiology*, *25*(2), 245-258. https://doi.org/10.1007/s10123-021-00210-z.

Hannah, V. E., O'Donnell, L., Robertson, D., & Ramage, G. (2017). Denture stomatitis: causes, cures and prevention. *Primary dental journal*, *6*(4), 46-51. https://doi.org/10.1308/205016817822230175.

Li, L., Redding, S., Dongari-Bagtzoglou, A. (2007). *Candida glabrata*, an emerging oral opportunistic pathogen. *Journal of Dental Research*, *86*(3), 204-215. https://doi.org/10.1177/154405910708600304

Lisoń, J., Taratuta, A., Paszenda, Z., Szindler, M., & Basiaga, M. (2022). Perspectives in Prevention of Biofilm for Medical Applications. *Coatings*, *12*(2), 197. https://doi.org/10.3390/coatings12020197

Meşeli, S. E., Genç, G. E., Çolakoğlu, G., Pelit, S., Esra, K. O. Ç., & Tarçın, B. G. (2019). The evaluation of the colonization and biofilm formation capacity of the Candida species isolated from denture wearer patients. *Atatürk Üniversitesi Diş Hekimliği Fakültesi Dergisi*, *29*(3), 387-393. https://doi.org/10.17567/ataunidfd.566861

Monteiro, D. R., Feresin, L. P., Arias, L. S., Barão, V. A. R., Barbosa, D. B., & Delbem, A. C. B. (2015). Effect of tyrosol on adhesion of Candida albicans and Candida glabrata to acrylic surfaces. *Medical Mycology*, *53*(7), 656-665. https://doi.org/10.1093/mmy/myv052

Monteiro, D. R., Arias, L. S., Fernandes, R. A., Deszo da Silva, L. F., de Castilho, M. O. V. F., Da Rosa, T. O., Vieria, A.P.M., Straioto, F.G., Barbosa , D.B., Delbem A.C.B. (2017). Antifungal activity of tyrosol and farnesol used in combination against *Candida* species in the planktonic state or forming biofilms. *Journal of Applied Microbiology*, *123*(2), 392-400. https://doi.org/10.1111/jam.13513

Öztürk, B. Y., Gürsu, B. Y., & Dağ, İ. (2020). Antibiofilm and antimicrobial activities of green synthesized silver nanoparticles using marine red algae *Gelidium corneum*. *Process Biochemistry*, *89*, 208-219. https://doi.org/10.1016/j.procbio.2019.10.027

Yılmaz Öztürk, B., Yenice Gürsu, B., & Dağ, İ. (2022). In vitro effect of farnesol on planktonic cells and dual biofilm formed by Candida albicans and Escherichia coli. *Biofouling*, 1-12.

Ramage, G., Saville, S. P., Thomas, D. P., Lopez-Ribot, J. L. (2005). *Candida* biofilms: an update. *Eukaryotic cell*, *4*(4), 633-638. DOI: https://doi.org/10.1128/EC.4.4.633-638.2005

Rodrigues, C. F., Černáková, L. (2020). Farnesol and tyrosol: secondary metabolites with a crucial quorum-sensing role in *Candida* biofilm development. *Genes*, *11*(4), 444. https://doi.org/10.3390/genes11040444

Sharma, D., Misba, L., & Khan, A. U. (2019). Antibiotics versus biofilm: an emerging battleground in microbial communities. *Antimicrobial Resistance & Infection Control*, *8*(1), 1-10. https://doi.org/10.1186/s13756-019-0533-3

Seneviratne, C. J., Silva, W. J., Jin, L. J., Samaranayake, Y. H., Samaranayake, L. P. (2009). Architectural analysis, viability assessment and growth kinetics of *Candida albicans* and *Candida glabrata* biofilms. *Archives of oral biology*, *54*(11), 1052-1060. https://doi.org/10.1016/j.archoralbio.2009.08.002

Sharma, D., Misba, L., & Khan, A. U. (2019). Antibiotics versus biofilm: an emerging battleground in microbial communities. *Antimicrobial Resistance & Infection Control*, *8*(1), 1-10. https://doi.org/10.1186/s13756-019-0533-3

Srinivasan, A., Torres, N. S., Leung, K. P., Lopez-Ribot, J. L., Ramasubramanian, A. K. (2017). nBio chip, a lab-on-a-chip platform of mono-and polymicrobial biofilms for high-throughput downstream applications. *Msphere*, *2*(3), e00247-17. DOI: https://doi.org/10.1128/mSphere.00247-17

Yapıcı, M., Gürsu, B. Y., & Dağ, İ. (2021). In vitro antibiofilm efficacy of farnesol against *Candida* species. *International Microbiology*, *24*(2), 251-262. https://doi.org/10.1007/s10123-021-00162-4

Yenice Gürsu, B. (2020). Potential antibiofilm activity of farnesol-loaded poly (DL-lactide-co-glycolide)(PLGA) nanoparticles against *Candida albicans*. *Journal of Analytical Science and Technology*, *11*(1), 1-10. https://doi.org/10.1186/s40543-020-00241-7

Zhou, L., Zhang, Y., Ge, Y., Zhu, X., & Pan, J. (2020). Regulatory mechanisms and promising applications of quorum sensing-inhibiting agents in control of bacterial biofilm formation. *Frontiers in microbiology*, *11*, 589640. https://doi.org/10.3389/fmicb.2020.589640

Çeşitli gıda örnekleri ve kesimhanelerden izole edilen bazı patojen bakterilerin biyofilm oluşturma yeteneğinin araştırılması

Evaluation of biofilm forming ability of some pathogenic bacteria isolated from various food samples and slaughterhouses

ÖZET

Biyofilmler, bakterilere güçlü tolerans ve uygun yaşam ortamları sağlayan, ekstraselüler polimerik maddelere gömülü mikroorganizmalar topluluğudur. Çoğu patojen özellikteki mikroorganizma, uygun koşullar oluştuğunda gıdalarda ve gıda ile temas eden yüzeylerde biyofilm oluşturarak gıda endüstrisinde ve halk sağlığı açısından sorunlara yol açmaktadır. Bu çalışmada, daha önceki çalışmalarla çeşitli gıda örnekleri ve kesimhane ortamından izole edilen ve moleküler yöntemlerle identifiye edilmiş, toplam 120 *Salmonella* Virchow, *Salmonella* Infantis, *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium* izolatı materyal olarak kullanıldı. Biyofilm oluşumunun kalitatif olarak tespiti amacıyla Kongo Kırmızısı Agar kullanıldı. Yapılan analizler doğrultusunda, toplam 120 izolattan 15'inin (%12,5) kalitatif olarak biyofilm ürettiği tespit edildi. Kalitatif olarak biyofilm oluşturduğu tespit edilen suşların biyofilm oluşturma gücü mikroplak yöntemiyle kantitatif olarak araştırıldı. Mikroplak yöntemine göre 2 *E. faecium* ve 1 *E. faecalis* suşu güçlü biyofilm üreticisi; 2 *L. monocytogenes* (serotip 1/2a) suşu orta düzey biyofilm üreticisi; 4 *E. coli*, 4 *S. aureus* ve 2 *L. monocytogenes* (serotip 1/2a ve serotip 4b) suşu zayıf biyofilm üreticisi olarak belirlendi. *Salmonella* spp. suşlarının tümü biyofilm üretimi açısından negatif olarak belirlendi. Sonuç olarak; süt, mandıra ürünleri, tüketime hazır gıdalar ve kesimhanelerden izole edilen bakterilerde biyofilm üretiminin hem halk sağlığı hem de gıda işletmeleri için önemli olduğu düşünülmektedir. Bu nedenle, gıda işleme tesislerinde, üretim hattı boyunca temizlik ve dezenfeksiyon işlemlerinin gerçekleştirilmesinde HACCP, İyi Üretim Uygulamaları gibi gıda sanitasyon sistemlerine riayet edilmesinin biofilm oluşumunun önüne geçilmesinde faydalı olacağı öngörülmektedir.

Anahtar Kelimeler: Biyofilm, gıda, *Enterococcus*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus.*

ABSTRACT

Biofilms are ensemble of microorganisms embedded in extracellular polymeric substances that providing bacteria strong tolerance and suitable habitats. In this study, a total of 120 *Salmonella* Virchow, *Salmonella* Infantis, *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium* isolates, which were isolated from various food samples and slaughterhouse environment and identified by molecular methods, were used as material. Congo Red Agar was used for the qualitative detection of biofilm formation. According to results, it was determined that 15 (12,5%) out of 120 isolates produced biofilm qualitatively. Biofilm forming levels of qualitative biofilm producers were later quantitatively investigated by microplate method. According to the microplate method, 2 *E. faecium* and 1 *E. faecalis* strains are strong biofilm producers; 2 strains of *L. monocytogenes* (serotype 1/2a) are moderate biofilm producers; 4 strains of *E. coli*, 4 *S. aureus* and 2 *L. monocytogenes* (serotype 1/2a and serotype 4b) were determined as weak biofilm producers. All *Salmonella* spp. strains were negative for biofilm production.

How to cite this article
Uyanık, T., Bölükbaş, A., Gücükoğlu, A., Çadirci Ö. (2022). Evaluation of biofilm forming ability of some pathogenic bacteria isolated from various food samples and slaughterhouses. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 338-345.
https://doi.org/10.31797/vetbio.1194207

Research Article

Tolga Uyanık[1a]
Ayşegül Bölükbaş[1b]
Ali Gücükoğlu[1c]
Özgür Çadirci[1d]

[1]Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Türkiye

ORCİD-
[a]0000-0002-3181-3878
[b]0000-0002-5516-3689
[c]0000-0002-8465-7768
[d]0000-0003-2018-2545

Correspondence
Tolga UYANIK
tolga.uyanik@omu.edu.tr

Article info
Submission: 25-10-2022
Accepted: 13-12-2022
Online First: 26-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

As a result; it is thought that biofilm production in bacteria isolated from milk, dairy products, ready-to-eat foods and slaughterhouses is important for both public health and food industry. It is anticipated that, in the cleaning and disinfection of food processing facilities, compliance with food sanitation systems such as HACCP and Good Manufacturing Practices will be beneficial to prevent biofilm formation.

Keywords: Biofilm, *Enterococcus*, *Escherichia coli*, food, *Listeria monocytogenes*, *Staphylococcus aureus*

GİRİŞ

Biyofilmler, bakterilere güçlü tolerans ve uygun yaşam ortamları sağlayan, ekstraselüler polimerik maddelere gömülü mikroorganizmalar topluluğudur. Leeuwenhoek tarafından 1684 yılında ilkel ışık mikroskobu kullanılarak yapılan incelemelerin, dış yüzeylere tutunan sabit mikroorganizmalar fikrini ortaya atarak mikrobiyal biyofilmlerin ilk gözleminin temelini ortaya attığı kabul edilmektedir. Sonraki yıllarda yapılan çalışmalarda, gemi gövdelerinde toplanan deniz kökenli bakterilerin yüzeyde korozyona yol açtığını gözlemlenmiştir (Shi ve Zhu, 2009). Yüzeylerde bakteriyel yapışma, kümelenme ve çoğalma anlamına gelen 'film' terimi, 1930'lu yıllarda mikrobiyolojide yapışan sabit bakterileri serbest haldeki planktonik organizmalardan ayırmak için kullanılmıştır. 1970'li yıllarda yapılan araştırmalarda, enfekte kistik fibroz hastalarından alınan akciğer doku örneklerinde *Pseudomonas aeruginosa* etkeninin ortamdaki yığınlarının gözlemlenmesiyle biyofilm yapısı hakkında detaylı bilgiler edinilmeye başlanmıştır (Høiby, 2017).

Bakterinin hayatta kalması için uygun koşulları sağlayan biyofilmler, bünyesinde çoğunlukla bakteri hücrelerini ve ekstraselüler matrisi içermektedir. Hücrelerin başta ekzopolisakkaritleri olmak üzere, ekstraselüler DNA'sı ve proteinleri, salgılanan ekstraselüler polimerik maddelere gömülü olarak bulunmaktadır. Bazı ortamlarda, biyofilmlerin ekstraselüler polimerik maddelerinde polisakkaritler, proteinler, fosfolipidler, teikoik asit ve nükleik asit gibi ana bileşenlerin yanı sıra, mineral kristalleri, silt parçacıkları ve kan bileşenleri de bulunmaktadır (Donlan, 2002). Biyofilm toplulukları, bir veya daha fazla bakteri türünü içerebilen tek katmanlı veya üç boyutlu yapılardır. İçerisinde Gram negatif bakterilerin baskın olduğu biyofilmler nötral veya polianyonik özellikteyken, gram pozitif bakterilerin baskın olduğu biyofilmlerde katyonik matrikslerin oluştuğu bildirilmektedir (Costerton vd., 1995). Bu karmaşık biyofilm yapısının önemi ve amacı; oksijen seviyesinin düşmesi, besinlerin tükenmesi, kimyasal ve mekanik dezenfektanların ortamda bulunması gibi bazı olumsuz koşullara karşı bakterileri korumaktır. Ayrıca biyofilmlerin hücreler arası iletişim sinyallerini iletmede de rol oynadığı yapılan birçok çalışmada gösterilmiştir (De la Fuente-Núñez vd., 2013; Rahman vd., 2021).

Çoğu patojen özellikteki mikroorganizma, uygun koşullar oluştuğunda gıdalarda ve gıda ile temas eden yüzeylerde biyofilm oluşturarak gıda endüstrisinde ve halk sağlığı açısından sorunlara yol açmaktadır. Gıda işlendikten sonra geride kalan gıda kalıntıları uzaklaştırılmadığı taktirde, mikroorganizmalar ekipmanların çeşitli yerlerinde üreyebilmekte ve bu mikroorganizmaların bir araya toplanması biyofilm oluşumuna yol açabilmektedir (Fysun vd., 2019). Yüzeylere tutunan ve ortamdan uzaklaştırılamayan biyofilmlerin varlığı patojen mikroorganizmaların kontaminasyonunda rezervuar görevi görmektedir. Herhangi bir çapraz kontaminasyon durumunda, gıdanın mikrobiyel kalitesi etkilenerek hem raf ömrü azalabilmekte ve hem de bu gıdalar hastalıklara neden olabilmektedir (Flemming ve Wingender, 2010).

Gıda üretim hattı zengin besin varlığı nedeniyle mikroorganizmaların büyümesi, gelişmesi ve oldukça karmaşık olan biyofilm oluşumu için kaynak sağlamaktadır. Gıda endüstrisi, hammadde ve işlenmiş ürünün mikrobiyotasına bağlı olarak belirli bir biyofilm oluşturan mikroorganizma yüküne sahiptir. 1966 yılında *Salmonella* spp. tarafından oluşturulan biyofilmlerin gıda kaynaklı ilk bakteriyel biyofilm olarak bildirilmesinin ardından insanlar için patojen olan *Campylobacter jejuni, Yersinia enterocolitica, Listeria monocytogenes, Escherichia coli, Bacillus cereus, Staphylococcus aureus* suşlarının biyofilm üreten önemli mikroorganizmalar içerisinde yer aldığı yapılan çalışmalarda ortaya konmuştur (Gómez vd., 2016; Var ve Sağlam, 2017; Woo ve Ahn, 2013; Zhao vd., 2017). Öte yandan, gıda örneklerinden izole edilen *Enterococcus* spp. izolatlarında biyofilm oluşumu çeşitli çalışmalarda araştırılmıştır (Gürkan vd., 2021; Kankaya vd., 2017). Bu bilgiler ışığında, bu çalışmada 2012 ve 2021 yılları arasında çeşitli gıda örnekleri ve kesimhane ortamından izole ve identifiye edilmiş 120 izolatın biyofilm üretme yeteneklerinin kalitatif ve kantitatif yöntemlerle ortaya konması amaçlanmıştır.

MATERYAL VE METHOD

Bu çalışmada, daha önceki çalışmalardan izole edilen ve moleküler yöntemlerle identifiye edilmiş 120 izolat materyal olarak kullanıldı. Suşların tür bazında dağılımları ve izole edildikleri ortamlar Tablo 1'de gösterilmektedir.

Suşların biyofilm oluşturma yetenekleri kalitatif ve kantitatif yöntemlerle araştırıldı. Biyofilm oluşumunun kalitatif olarak tespiti amacıyla Kongo Kırmızısı Agar (Bileşimi; Brain Heart Infusion: 37 g/L, sükroz: 5 g/L, kongo red: 0,8 g/L, Agar-Agar: 15 g/L) kullanıldı. Bu amaçla Tryptic Soy Broth (TSB) içinde 37 °C'de 18-24 saat inkübe edilen bakteriyel suşlardan, inkübasyonun ardından bir öze dolusu alınarak Kongo Kırmızısı Agar'a geçiş yapıldı. Daha sonra petriler 37 °C'de 24 saat inkübe edildi. İnkübasyonun ardından siyah koloni oluşumu biyofilm oluşumu açısından pozitif olarak kabul edilirken, kırmızı-pembe kolonilerin oluşması biyofilm oluşumu açısından negatif olarak değerlendirildi (Freeman vd., 1989).

Tablo 1. Çalışmada kullanılan bakteri suşları

Adet	Bakteri türü	İzole edildiği ortam	Referans
30	*Escherichia coli*	Tüketime hazır sandviç	(Uyanik, 2022)
30	*Staphylococcus aureus*	Süt ve süt ürünleri	(Gücükoğlu vd., 2012)
22	*Salmonella* Infantis	Kanatlı eti	(Çadırcı vd., 2021)
6	*Listeria monocytogenes* (serotip 1/2a)	Tavuk parça etleri	(Çadırcı vd., 2018)
5	*Listeria monocytogenes* (serotip 1/2a)	Kesimhane zemini	(Çadırcı vd., 2018)
5	*Listeria monocytogenes* (serotip 1/2b)	Bıçak	(Çadırcı vd., 2018)
5	*Listeria monocytogenes* (serotip 1/2b)	Sığır karkası	(Çadırcı vd., 2018)
5	*Salmonella* Virchow	Kanatlı eti	(Çadırcı vd., 2021)
3	*Listeria monocytogenes* (serotip 1/2a)	Kanatlı eti	(Çadırcı vd., 2021)
3	*Listeria monocytogenes* (serotip 1/2c)	Kanatlı eti	(Çadırcı vd., 2021)
2	*Listeria monocytogenes* (serotip 4b)	Bıçak bileyicisi	(Çadırcı vd., 2018)
2	*Enterococcus faecium*	Süt	(Yayınlanmamış veri)
1	*Enterococcus faecalis*	Süt	(Yayınlanmamış veri)
1	*Listeria monocytogenes* (serotip 1/2a)	Sığır derisi	(Çadırcı vd., 2018)

Biyofilm oluşumunun kantitatif olarak tespiti mikroplak yöntemiyle gerçekleştirildi. Bu amaçla, 96 kuyucuklu polistren plakalar ve BioTek Synergy 4 mikroplak okuyucusu kullanıldı. Stepanović vd. (2007) tarafından önerilen bu yöntemin uygulanmasında bazı modifikasyonlar gerçekleştirildi ve aşağıdaki adımlar izlendi:

- Her suştan 200 µL (kriyoprezerve edilmiş stoklardan) alınarak 10 ml TSB'ye geçildi ve 37 °C'de 24 saat inkübe edildi
- İnkübasyondan sonra, her TSB'den 200 µL alınarak mikroplaka kuyucuklarına aktarıldı ve mikroplakalar 37 °C'de 48 saat inkübe edildi.
- İnkübasyondan sonra kuyucukların içeriği boşaltıldı. Her kuyucuk, non-adherent hücreleri uzaklaştırmak için 300 µL PBS ile üç kez yıkandı
- Daha sonra oluşan biyofilmlerin fiksasyonu için mikroplakalar 60 °C'de 1 saat inkübe edildi
- Yapışkan biyofilm tabakası 150 µL %2'lik kristal viyole ile oda sıcaklığında 15 dakika boyama işlemine tabi tutuldu. İnkübasyondan ardından, kuyucuklar PBS ile yıkandı
- Her kuyucuğa 150 µL %95'lik etanol eklendi ve oda sıcaklığında 10 dakika inkübe edildi
- Her kuyucuğun optik yoğunluğu (OD), BioTek Synergy 4 mikroplaka okuyucu kullanılarak 570 nm dalga boyunda ölçüldü.

Çalışmada sadece TSB içeren kuyucuklar negatif kontrol olarak kullanıldı. Tüm testler üç tekrarlı olarak gerçekleştirildi. Cutoff OD değeri (ODc), negatif kontrolün ortalama değeri olarak belirlendi. Buna göre biyofilm oluşumu;

OD≤ODc= biyofilm negatif

ODc<OD≤2×ODc= zayıf biyofilm üreticisi,

2×ODc<OD≤4×ODc= orta düzey biyofilm üreticisi,

OD>4×ODc=güçlü biyofilm

üreticisi olarak derecelendirildi.

BULGULAR

Yapılan analizler doğrultusunda, toplam 120 izolattan 15'inin (%12,5) Kongo Red Agar'da siyah üreme göstererek kalitatif olarak biyofilm ürettiği tespit edildi. Mikroplak yöntemiyle, kalitatif olarak biyofilm üreten suşların biyofilm üretme derecesi araştırıldı. Buna göre 2 adet *E. faecium* ve 1 adet *E. faecalis* suşu güçlü biyofilm üreticisi; 2 adet *L. monocytogenes* (serotip 1/2a) suşu orta düzey biyofilm üreticisi; 4 adet *E. coli*, 4 adet *S. aureus* ve 2 adet *L. monocytogenes* (serotip 1/2a ve serotip 4b) suşu zayıf biyofilm üreticisi olarak belirlendi. Kalitatif ve kantitatif olarak biyofilm ürettiği saptanan izolatların OD değerlerini içeren veriler Tablo 2'de gösterildi.

Tablo 2. Bakteri suşlarının biyofilm üretme dereceleri

Suş	Optik Dansite (ortalama)	Biofilm yoğunluğu	Suşun orjini
E. faecium	0,340	Güçlü	Çiğ süt
E. faecium	0,322	Güçlü	Çiğ süt
E. faecalis	0,317	Güçlü	Çiğ süt
E. coli	0,138	Zayıf	Sandviç
E. coli	0,131	Zayıf	Sandviç
E. coli	0,140	Zayıf	Sandviç
E. coli	0,126	Zayıf	Sandviç
S. aureus	0,115	Zayıf	Çiğ süt
S. aureus	0,142	Zayıf	Çiğ süt
S. aureus	0,128	Zayıf	Beyaz peynir
S. aureus	0,117	Zayıf	Beyaz peynir
L. monocytogenes (4b)	0,115	Zayıf	Bıçak bileyicisi
L. monocytogenes (1/2a)	0,119	Zayıf	Kesimhane zemini
L. monocytogenes (1/2a)	0,211	Orta	Sığır derisi
L. monocytogenes (1/2a)	0,227	Orta	Kanatlı eti
Negatif kontrol (Sadece TSB)	**0,075 (ODc değeri)**		

TARTIŞMA

Bu çalışmada analiz edilen bakteri suşlarının %12,5'inin (15/120) biyofilm ürettiği belirlenmiştir. Çalışmada kullanılan *Enterococcus* spp. suşlarının tümünün; *E. coli*, *S. aureus* ve *L. monocytogenes* suşlarının %13,3'ünün (her biri 4/30) çeşitli derecelerde

biyofilm ürettiği saptanmıştır. Buna karşın, *Salmonella* spp. suşlarının tümü biyofilm üretimi açısından negatif olarak belirlenmiştir. Ülkemizde gıda kaynaklı patojenlerin biyofilm üretimlerinin araştırıldığı çalışmalarda; et örneklerinden izole edilen *S. aureus* suşlarının %50'sinin (Gündoğan ve Ataol, 2012); süt, peynir ve etlerden izole edilen 60 *Enterococcus* spp. izolatının tümünün (Gürkan vd., 2021), çeşitli gıda örneklerinden izole edilen 8 *Salmonella* spp. ve 6 *L. monocytogenes* suşunun hepsinin biyofilm ürettiği (İset, 2016) belirlenmiştir. Bu bilgiler doğrultusunda, literatür taramalarından elde edilen sonuçlar, bu çalışmadan elde edilen verilerle değişkenlik göstermektedir. Bu farklılıkların bakterilerin biyofilm üretiminde rol oynayan sıcaklık, pH, tuz konsantrasyonu ve inkübasyon süresi gibi parametlerin değişkenliğinden dolayı kaynaklanabileceği düşünülmektedir.

Dünya genelinde yapılan çalışmalarda, gıda kaynaklı *Enterococcus* spp. izolatlarında güçlü biyofilm formasyonunun daha sıklıkla görüldüğünü bildiren çalışmalar ön plana çıkmaktadır (Barbosa vd., 2010; Diaz vd., 2016; Zarzecka vd., 2022). Öte yandan orta düzey ve zayıf biyofilm üretiminin daha yaygın olduğunu bildiren araştırmacılar da mevcuttur (El-Zamkan ve Mohamed, 2021). Yapılan çalışmalar, *E. faecalis* suşlarında güçlü biyofilm oluşumunun agregasyon substansının (*agg*) varlığı ile ilişkili olduğunu ortaya koymaktadır. Ayırca, sitolizin A negatif suşların zayıf biyofilm üretme olasılığının daha yüksek olduğu bildirilmektedir (Zheng vd., 2018). Diğer taraftan, Özkök vd. (2021), *E. faecalis* izolatlarının biyofilm oluşturma kapasitesinin *E. faecium*'dan daha yüksek olduğunu ve *esp* geninin biyofilm oluşumuyla ilgili olacabileceğini bildirmiştir.

E. coli suşlarının biyofilm üretimini ele alan çalışmalarda, zayıf biyofilm üretiminin *E. coli* suşlarında daha yaygın olduğu göze çarpmaktadır. Tajbakhsh vd. (2016) tarafından yapılan çalışmada 130 *E. coli* izolatının %56,25'inin zayıf biyofilm üreticisi olduğu raporlanmıştır. Buna paralel olarak, Flament-Simon vd. (2019) 394 *E. coli* izolatının yalnızca %10'unun güçlü biyofilm üretme yeteneğinde olduğunu bildirmişlerdir. Öte yandan, Wang vd. (2016) kanatlı orjinli *E. coli* izolatlarının %25,39'unun güçlü, %31,25'inin orta düzeyde, %28,90'ının zayıf biyofilm oluşturduğunu göstermiş ve izolatların %18,36'sının biyofilm oluşturmadığını bildirmiştir. *fimH, pap, sfa* ve *afa* genlerinin varlığının güçlü biyofilm üretiminde etkili olduğu ortaya konmuştur. Benzer şekilde, *S. aureus* izolatlarında da zayıf biyofilm üretiminin daha yaygın olduğu bildirilmektedir. Chen vd. (2020), 97 gıda kökenli *S. aureus* izolatından %54,64'ünün zayıf, %14,43 orta düzey ve %3,09'unun güçlü biyofilm ürettiğini saptarken, izolatların %28'inin biyofilm üretme yeteneğinde olmadığını bildirmiştir. Diğer yandan, Thiran vd. (2018) 24 adet mandıra kökenli *S. aureus* izolatından 13'ünün (%54) biyofilm üretmediğini, 11'inin (%45,8) zayıf biyofilm üreticisi olarak sınıflandırıldığını bildirmiştir. Metisilin dirençli *S. aureus* suşlarının güçlü biyofilm üretme olasılığının daha yüksek olduğu, *icaA* ve *icaD* genlerinin biyofilm oluşumuyla ilişkili olduğu bildirilmektedir (Manandhar vd., 2018).

Bu çalışmanın bulgularından farklı olarak, Díez-García vd. (2012) yapmış oldukları çalışmada kümes kökenli *S.* Infantis ve *S.* Virchow suşlarının zayıf biyofilm üretme yeteneğinde olduğunu bildirmiştir. Öte yandan Cufaoglu vd. (2021), kanatlı eti ve kesimhane kökenli *S.* Infantis suşlarının güçlü biyofilm üretme yeteneğinde olduğunu göstermiştir. Bu çalışmada ise analiz edilen tüm *Salmonella* spp. izolatları biyofilm üretimi açısından negatif olarak tespit edilmiştir. Açıkalın (2017), *S.* Infantis suşlarının biyofilm oluşturması için uygun şartların 72 saat inkübasyon, sıcaklık 20 °C, pH 6,6 ve tuz konsantrasyonu %0,5 olarak belirlemiştir. pH 4,5'te ve %0,5'in üzerindeki tuz konsantrasyonlarında ise tüm *Salmonella*

serotiplerinin biyofilm oluşturmadığını bildirmiştir. Ek olarak, biyofilm üretimi açısından *Salmonella* serotipleri arasında önemli farklılıkların olması, biyofilm üretim kapasitesinin suşa bağlı olabileceğini göstermektedir (Borges vd., 2018). Gıda kökenli serotipler arasında *S.* Agona, *S.* Heidelberg, *S.* Typhi ve *S.* Weltevreden güçlü biyofilm oluşturucuları olarak ön plana çıkmaktadır (Akinola vd., 2020; Díez-García vd., 2012).

Literatür taramalarında, *L. monocytogenes* 1/2a serotipinin, serotip 4b'ye göre daha fazla biyofilm ürettiği görülmektedir (Borucki vd., 2003; Kadam vd., 2013; Soni ve Nannapaneni, 2010). Ayrıca serotip 1/2a tarafından üretilen biyofilmlerin, serotip 4b'ye göre daha yüksek dansiteli olduğu bildirilmektedir (Pan vd., 2010). Diğer taraftan; kesimhane, kümes ve gıda orjinli *L. monocytogenes* izolatlarında orta düzey veya zayıf biyofilm formasyonunun çok daha yaygın olduğu bildirilmektedir (Doijad vd., 2015; Harvey vd., 2007; Moretro vd., 2013; Rodrigues vd., 2010). Bu kapsamda değerlendirildiğinde, bu çalışmadan elde edilen verilerin literatürle uyum içinde olduğu görülmektedir.

SONUÇ

Biyofilm içerisindeki patojen bakteriler gıda kontaminasyonlarının ve klinik enfeksiyonların bir kaynağı olarak görülmektedir. Biyofilm oluşumu sadece genetik temellere dayanan bir süreç olmayıp, aynı zamanda pH, sıcaklık ve besin bileşenleri gibi çevresel faktörlere bağlı olarak gerçekleşmektedir. Bu kapsamda, süt, mandıra ürünleri, tüketime hazır gıdalar ve kesimhanelerden izole edilen bakterilerde biyofilm üretiminin hem halk sağlığı hem de gıda işletmeleri için önemli olduğu düşünülmektedir. Bu çalışmada analiz edilen bakteri suşlarının %87,5'i biyofilm üretimi açısından negatif olarak tespit edilmiştir ancak özellikle *E. coli* ve *S. aureus* gibi gıda kaynaklı patojenlerde, karışık bakteri kültürlerinin sinerjik etkilerinin biyofilm üretimini artırabileceği bilinmektedir. Bu nedenle, gıda işleme tesislerinde, üretim hattı boyunca temizlik ve dezenfeksiyon işlemlerinin gerçekleştirilmesinde HACCP, İyi Üretim Uygulamaları gibi gıda sanitasyon sistemlerine riayet edilmesinin biofilm oluşumunun önüne geçilmesinde faydalı olacağı öngörülmektedir.

AÇIKLAMALAR

Etik beyan: Bu çalışmanın gerçekleştirilmesinde etik kurul onayının gerekmediğini beyan ederiz.

Çıkar çatışması: Yazarlar herhangi bir çıkar çatışması olmadığını beyan etmişlerdir.

KAYNAKLAR

Bampidis, V. A., & Robinson, P. H. (2006). Citrus by-products as ruminant feeds: A review. *Animal Feed Science and Technology*, *128*(3-4), 175-217. doi: 10.1016/j.anifeedsci.2005.12.002. **Açıkalın, D. (2017).** *Salmonella Infantis suşlarının oluşturduğu biyofilm üzerine çevresel ve genetik faktörlerin etkisinin araştırılması* [Yüksek Lisans Tezi, Ankara Üniversitesi, Sağlık Bilimleri Enstitüsü].

Akinola, S. A., Tshimpamba, M. E., Mwanza, M., & Ateba, C. N. (2020). Biofilm Production Potential of Serovars Isolated from Chickens in North West Province, South Africa. *Polish Journal of Microbiology*, *69*(4), 427-439.

Barbosa, J., Gibbs, P., & Teixeira, P. (2010). Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. *Food Control*, *21*(5), 651-656.

Borges, K. A., Furian, T. Q., de Souza, S. N., Menezes, R., de Lima, D. A., Fortes, F. B. B., Salle, C. T. P., Moraes, H. L. S., & Nascimento, V. P. (2018). Biofilm formation by Salmonella Enteritidis and Salmonella Typhimurium isolated from avian sources is partially related with their in vivo pathogenicity. *Microbial Pathogenesis*, *118*, 238-241.

Borucki, M. K., Peppin, J. D., White, D., Loge, F., & Call, D. R. (2003). Variation in biofilm formation among strains of Listeria monocytogenes. *Applied and Environmental Microbiology*, *69*(12), 7336-7342.

Chen, Q., Xie, S., Lou, X., Cheng, S., Liu, X., Zheng, W., Zheng, Z., & Wang, H. (2020). Biofilm formation and prevalence of adhesion genes among Staphylococcus aureus isolates from different food sources. *Microbiologyopen*, *9*(1), e00946.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial biofilms. *Annual Review of Microbiology*, *49*(1), 711-745.

Cufaoglu, G., Acar, B. O., Cengiz, G., Ayaz, N. D., & Goncuoglu, M. (2021). Mono-and Mixed-Species Biofilm Formation by Salmonella Infantis, Salmonella Kentucky, Enterococcus faecium, and Enterococcus faecalis. *Acta Veterinaria Eurasia*, *47*(3), 149-154.

Çadırcı, O., Gucukoglu, A., Gulel, G. T., Gunaydin, E., Uyanik, T., & Kanat, S. (2021). Determination and antibiotic resistance profiles of Salmonella serotypes isolated from poultry meat. *Fresenius Environmental Bulletin*, *30*, 4251-4261.

Çadırcı, Ö., Gücükoğlu, A., Terzi, G. T., Uyanik, T., & Alişarlı, M. (2018). The existence of Listeria monocytogenes in a cattle slaughterhouse and identification of serotypes by mPCR. *Ankara Üniversitesi Veteriner Fakültesi Dergisi*, *65*(3), 305-311.

De la Fuente-Núñez, C., Reffuveille, F., Fernández, L., & Hancock, R. E. (2013). Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Current Opinion in Microbiology*, *16*(5), 580-589.

Diaz, M., Ladero, V., Del Rio, B., Redruello, B., Fernández, M., Martin, M. C., & Alvarez, M. A. (2016). Biofilm-forming capacity in biogenic amine-producing bacteria isolated from dairy products. *Frontiers in Microbiology*, *7*, 591.

Díez-García, M., Capita, R., & Alonso-Calleja, C. (2012). Influence of serotype on the growth kinetics and the ability to form biofilms of Salmonella isolates from poultry. *Food Microbiology*, *31*(2), 173-180.

Doijad, S. P., Barbuddhe, S. B., Garg, S., Poharkar, K. V., Kalorey, D. R., Kurkure, N. V., Rawool, D. B., & Chakraborty, T. (2015). Biofilm-forming abilities of Listeria monocytogenes serotypes isolated from different sources. *PloS One*, *10*(9), e0137046.

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerging Infectious Diseases*, *8*(9), 881.

El-Zamkan, M. A., & Mohamed, H. M. (2021). Antimicrobial resistance, virulence genes and biofilm formation in Enterococcus species isolated from milk of sheep and goat with subclinical mastitis. *PloS One*, *16*(11), e0259584.

Flament-Simon, S.-C., Duprilot, M., Mayer, N., García, V., Alonso, M. P., Blanco, J., & Nicolas-Chanoine, M.-H. (2019). Association between kinetics of early biofilm formation and clonal lineage in Escherichia coli. *Frontiers in Microbiology*, *10*, 1183.

Flemming, H.-C., & Wingender, J. (2010). The biofilm matrix. *Nature reviews microbiology*, *8*(9), 623-633.

Freeman, D., Falkiner, F., & Keane, C. (1989). New method for detecting slime production by coagulase negative staphylococci. *Journal of Clinical Pathology*, *42*(8), 872-874.

Fysun, O., Kern, H., Wilke, B., & Langowski, H.-C. (2019). Evaluation of factors influencing dairy biofilm formation in filling hoses of food-processing equipment. *Food and Bioproducts Processing*, *113*, 39-48.

Gómez, N. C., Ramiro, J. M., Quecan, B. X., & de Melo Franco, B. D. (2016). Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of Listeria monocytogenes, Salmonella Typhimurium, and Escherichia coli O157: H7 biofilms formation. *Frontiers in Microbiology*, 863.

Gücükoğlu, A., Onur Kevenk, T., Uyanik, T., Çadirci, Ö., Terzi, G., & Alişarli, M. (2012). Detection of enterotoxigenic Staphylococcus aureus in raw milk and dairy products by multiplex PCR. *Journal of Food Science*, *77*(11), M620-M623.

G**ündoğan, N., & Ataol, Ö. (2012).** Et örneklerinden izole edilen Staphylococcus aureus ve koagülaz negatif stafilokok'ların biyofilm üretimi ve DNaz aktivitelerinin belirlenmesi. *Türk Hijyen ve Deneysel Biyoloji Dergisi*, *69*(3), 135-142.

Gürkan, T., Külahcı, M. B., & Çıtak, S. (2021). Gıda örneklerinden izole edilen Enterococcus türlerinin çeşitli virülans özellikleri, biyofilm oluşumu ve antibiyotik dirençliliklerinin belirlenmesi. *Avrupa Bilim ve Teknoloji Dergisi*(28), 924-932.

Harvey, J., Keenan, K., & Gilmour, A. (2007). Assessing biofilm formation by Listeria monocytogenes strains. *Food Microbiology*, *24*(4), 380-392.

Høiby, N. (2017). A short history of microbial biofilms and biofilm infections. *Apmis*, *125*(4), 272-275.

İset, Ş. (2016). *Çeşitli gıda örneklerinden izole edilen salmonella ve listeria monocytogenes suşlarının biyofilm oluşturma yeteneklerinin araştırılması ve elektron mikroskobik tekniklerle değerlendirilmesi* [Yüksek Lisans Tezi, Eskişehir Osmangazi Üniversitesi, Fen Bilimleri Enstitüsü].

Kadam, S. R., den Besten, H. M., van der Veen, S., Zwietering, M. H., Moezelaar, R., & Abee, T. (2013). Diversity assessment of Listeria monocytogenes biofilm formation: impact of growth condition, serotype and strain origin. *International Journal of Food Microbiology*, *165*(3), 259-264.

Kankaya, D. A., Tuncer, B. Ö., & Tuncer, Y. (2017). Gida kaynakli enterokoklarin potansiyel risk faktörleri. *Gıda*, *42*(1), 8-19.

Manandhar, S., Singh, A., Varma, A., Pandey, S., & Shrivastava, N. (2018). Biofilm producing clinical Staphylococcus aureus isolates augmented prevalence of antibiotic resistant cases in tertiary care hospitals of Nepal. *Frontiers in Microbiology*, *9*, 2749.

Moretro, T., Langsrud, S., & Heir, E. (2013). Bacteria on meat abattoir process surfaces after sanitation: Characterisation of survival properties of Listeria monocytogenes and the commensal bacterial flora.

Özkök, Z., Bilgin, K., Tanrıverdi Çaycı, Y., & Birinci, A. (2021). Investigation of biofilm formation of Enterococcus species isolated from blood by phenotypic and genotypic methods. *Turkish Bulletin of Hygiene and Experimental Biology*, *78*(3), 363-372.

Pan, Y., Breidt Jr, F., & Gorski, L. (2010). Synergistic effects of sodium chloride, glucose, and temperature on biofilm formation by Listeria monocytogenes

serotype 1/2a and 4b strains. *Applied and Environmental Microbiology*, *76*(5), 1433-1441.

Rahman, M. U., Fleming, D. F., Sinha, I., Rumbaugh, K. P., Gordon, V. D., & Christopher, G. F. (2021). Effect of collagen and EPS components on the viscoelasticity of Pseudomonas aeruginosa biofilms. *Soft Matter*, *17*(25), 6225-6237.

Rodrigues, L. B., Santos, L. R. d., Tagliari, V. Z., Rizzo, N. N., Trenhago, G., Oliveira, A. P. d., Goetz, F., & Nascimento, V. P. d. (2010). Quantification of biofilm production on polystyrene by Listeria, Escherichia coli and Staphylococcus aureus isolated from a poultry slaughterhouse. *Brazilian Journal of Microbiology*, *41*, 1082-1085.

Shi, X., & Zhu, X. (2009). Biofilm formation and food safety in food industries. *Trends in Food Science and Technology*, *20*(9), 407-413.

Soni, K. A., & Nannapaneni, R. (2010). Removal of Listeria monocytogenes biofilms with bacteriophage P100. *Journal of Food Protection*, *73*(8), 1519-1524.

Stepanović, S., Vuković, D., Hola, V., Bonaventura, G. D., Djukić, S., Ćirković, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Apmis*, *115*(8), 891-899.

Tajbakhsh, E., Ahmadi, P., Abedpour-Dehkordi, E., Arbab-Soleimani, N., & Khamesipour, F. (2016). Biofilm formation, antimicrobial susceptibility, serogroups and virulence genes of uropathogenic E. coli isolated from clinical samples in Iran. *Antimicrobial Resistance and Infection Control*, *5*(1), 1-8.

Thiran, E., Di Ciccio, P., Graber, H., Zanardi, E., Ianieri, A., & Hummerjohann, J. (2018). Biofilm formation of Staphylococcus aureus dairy isolates representing different genotypes. *Journal of Dairy Science*, *101*(2), 1000-1012.

Uyanik, T. (2022). Samsun ilindeki hastane kantinlerinde satışa sunulan tüketime hazır sandviçlerde genişlemiş spektrumlu beta-laktamaz üreten Escherichia coli varlığının araştırılması. *Erciyes Üniversitesi Veteriner Fakültesi Dergisi*, *19*(1), 37-42.

Var, I., & Sağlam, S. (2017). Biofilm Structure of foodborne pathogens. *Antimicrobial Research: Novel bioknowledge and educational programs Microbiology Book Series*, *6*, 301-307.

Wang, Y., Yi, L., Wang, Y., Wang, Y., Cai, Y., Zhao, W., & Ding, C. (2016). Isolation, phylogenetic group, drug resistance, biofilm formation, and adherence genes of Escherichia coli from poultry in central China. *Poultry Science*, *95*(12), 2895-2901.

Woo, J., & Ahn, J. (2013). Probiotic-mediated competition, exclusion and displacement in biofilm formation by food-borne pathogens. *Letters in Applied Microbiology*, *56*(4), 307-313.

Zarzecka, U., Zadernowska, A., & Chajęcka-Wierzchowska, W. (2022). Effects of osmotic and high pressure stress on expression of virulence factors among Enterococcus spp. isolated from food of animal origin. *Food Microbiology*, *102*, 103900.

Zhao, X., Zhao, F., Wang, J., & Zhong, N. (2017). Biofilm formation and control strategies of foodborne pathogens: food safety perspectives. *RSC Advances*, *7*(58), 36670-36683.

Zheng, J.-x., Bai, B., Lin, Z.-w., Pu, Z.-y., Yao, W.-m., Chen, Z., Li, D.-y., Deng, X.-b., Deng, Q.-w., & Yu, Z.-j. (2018). Characterization of biofilm formation by Enterococcus faecalis isolates derived from urinary tract infections in China. *Journal of Medical Microbiology*, *67*(1), 60.

Pathological and biochemical investigation of the effects of L-carnitine and gemfibrozil on peroxisome proliferator activated receptors (PPARS) and lipidosis in rabbits on a high-fat diet

ABSTRACT

Obesity and fatty liver is a worldwide health problem in human with detrimental consequences where many investigations are undertaken to overcome this problem. In this study, gemfibrozil and L-carnitine were evaluated in prevention of obesity and lipidosis. The study involved 56 New-Zealand Albino rabbits, divided into 8 equal groups (n=7). The groups were as follow; group I (normal diet), II (normal diet +gemfibrozil), III (normal diet+L-carnitine) and IV (normal diet+gemfibrozil+L-carnitine), V (high fat diet), VI (high fat diet+gemfibrozil), VII (high fat diet+L-carnitine) and VIII (high fat diet+gemfibrozil+L-carnitine). Animals were blood sampled and wieght weekly during the experiment and at the end of the experiment for determination of biochemical parameters (glucose, total lipid). All rabbits were euthanised for histopathological examination and for distrubition of peroxisome proliferator activated receptors (PPARs) in tissues by immunohystochemistry. Gemfibrozil and L-carnitin treatment in rabbits given high fat diet resulted in statistically significant decrease in total lipid when compared to those only received high fat diet. Beta oxidation of high fat diet group was significantly higher than that of groups additionally received gemfibrozil and L-carnitine. Immunohistochemistry revealed an increase in PPAR, PPAR-α and β but not PPAR-γ expression in high fat diet group. On the contrary, L-carnitin administration had no effect on tissue PPAR expression. PPAR-α expression differed between groups received gemfibrozil and high fat diet and those did not. The most marked macroscopy finding was abdominal fat increase in high fat diet group (group V). On the other hand gemfibrozil administration resulted in significant abdominal fat decrease. Furthermore decreased abdominal fat was marked in gemfibrozil and L-carnitine given animals (group VIII) when compared to other groups. In conclusion, gemfibrozil and L-carnitine administration alleviated abdominal and hepatic fattening. Gemfibrozil also caused a significant increase in PPAR-α expression in the liver. It may be of use in avoiding abdominal fat (obesity) due to high fat diet by use of gemfibrozil, a synthetic PPAR-a ligand, and L-carnitine.

Keywords: Gemfibrozil, L-carnitine, Obesity, PPARs, Rabbits

INTRODUCTION

Obesity is a condition that results from the disruption of the balance between the energy intake through feed and consumption. The excess consumption of energy is mainly stored in the form of triglycerides in adipocytes. However, in the development of obesity namely morphological changes such as adipocyte hypertrophy and adipogenesis in adipose tissue, are under the control of numerous mechanisms. Fat deposition in the liver and obesity are inter related as obesity is the most important risk factor for the development of fatty liver (Selek et al, 2011).

How to cite this article
Citil, M., Tunca, R., Uzlu, E., Karapehlivan, M., Adali, Y., Yapar, K., Eroğlu, HA., Erkılıc, EE., Makav, M., Erdogan HM. (2022). Pathological and biochemical investigation of the effects of l-carnitine and gemfibrozil on peroxisome proliferator activated Receptors (PPARS) and lipidosis in rabbits on a high-fat diet. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 346-360. https://doi.org/10.31797/vetbio.1136444

Research Article

Mehmet Citil[1a]
Recai Tunca[2b]
Erdogan Uzlu[3c]
Mahmut Karapehlivan[4d]
Yasemen Adali[5e]
Kursad Yapar[6f]
Huseyin Avni Eroğlu[7g]
Ekin Emre Erkılıc[8h]
Mustafa Makav[9j]
Hidayet Metin Erdogan[10j]

[1]Department of Internal Medicine, Faculty of Veterinary, University of Erciyes, Kayseri, Türkiye
[2]Department of Pathology, Faculty of Veterinary, University of Adnan Menders, Aydin, Türkiye
[3]Department of Internal Medicine, Faculty of Veterinary, University of Balıkesir, Balıkesir, Türkiye
[4]Department of Biochemistry, Faculty of Medicine, University of Kafkas, Kars, Türkiye
[5]Department of Pathology, Faculty of Medicine, Izmir University of Economy, İzmir, Türkiye
[6]Department of Pharmacology and Toxicology, Faculty of Medicine, University of Giresun, Giresun, Türkiye
[7]Department of Physology, Faculty of Medicine, Çanakkale Onsekizmart University, Çanakkale, Türkiye
[8]Department of Internal Medicine, Faculty of Veterinary, University of Kafkas, Kars, Türkiye
[9]Department of Physology, Faculty of Veterinary, University of Kafkas, Kars, Türkiye
[10] Department of Internal Medicine, Faculty of Veterinary, University of Aksaray, Aksaray, Türkiye

ORCİD-
[a]0000-0001-9839-7533
[b]0000-0003-0004-7485
[c]0000-0002-3064-6633
[d]0000-0003-0408-534X
[e]0000-0002-8004-7364
[f]0000-0003-0287-4521
[g]0000-0002-1040-3255
[h]0000-0003-2461-5598
[i]0000-0003-1879-8180
[j]0000-0003-1261-4352

Correspondence
Mehmet CİTİL
mcitil@hotmail.com

Article info

Submission: 28-06-2022
Accepted: 22-12-2022
Online First: 28-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

The severity of liver fat deposition is determined by the balance between fatty acid uptake, endogenous fatty acid synthesis, triglyceride synthesis, oxidation of fatty acids, and triglyceride exports (Stienstra et al., 2007). Activated receptors with peroxisome proliferators (PPARs) are nuclear receptors and have three different isoforms called PPAR-α, -β, and -γ (Wahli, 2002). Peroxisome proliferator activated receptors (PPARs) are nuclear receptors involved in the transcriptional control of genes involved in inflammation, lipid and carbohydrate metabolism, leading to the formation of obesity, hypercholesterolemia, insulin resistance and atherosclerosis (Brown and Plutzky 2007). PPAR-α is excreted from liver, heart and kidneys, PPAR-β is mainly from adipose tissue, skin and brain, and PPAR-γ is from adipose tissue, large intestine, heart, kidney, pancreas and the spleen (Akbiyik et al., 2004; Jeffrey et al., 2000; Tenenbaum et al., 2005; Tunca and Devrim, 2007). In obese animal models, fibrates have been shown to improve insulin sensitivity in addition to beneficial lipid modulation (Ogawa et all.,2000). They are activated by fatty acids and their derivatives. Although PPAR-β is activated by prostaglandin J2, PPAR-α is activated by leukotriene B4 (Xin et al., 1999). This enzyme affects body weight and energy balance as well as regulation of glucose metabolism (Hashimoto et al., 1999). These effects are partly due to the beta oxidation of hepatic fatty acids and lead to the degradation of fatty acids in the liver (Dreyer et al., 1992). PPAR-α fibrate derivatives play an important role in the effects of antilipidemic agents (Akbiyik et al., 2004; Hasimato et al., 1999; Tunca and Devrim, 2007). The PPARγ isoform, which is regarded as the main regulator of glucose homeostasis, is also highly expressed in various cell types and organs including adipocytes, muscle cells, liver and kidney. PPARγ agonists such as thiazolidinediones, pioglitazone, and rosiglitazone, which are currently prescribed as anti-diabetic drugs, act as insulin sensitisers (DeFronzo, 1999).

Fibrates (fenofibrate, gemfibrozil, etc.) are hypolipidemic drugs that stimulate PPARs in the cell (Akbiyik et al., 2004; Hashimoto et al., 1999; Jeffrey et al., 2000). For this reason, they regulate a series of gene expression that is important for lipid and lipoprotein mechanisms. Activated PPAR acts by increasing the expression of genes with PPRE by forming a heterodimer with the retinoid X receptor (Jeffrey et al., 2000). Fibrates also have activity in regulating energy hemostasis. These drugs can suppress obesity by reducing plasma triglycerides and fatty acid concentrations (Kraja et al., 2010; Moutzouri et al., 2010; Schoonjans et al., 1996; Tunca and Devrim, 2007; Yoon et al., 2003). Synthetic agonists of PPARα, such as fibrates, are used clinically to lower plasma triglycerides and increase high-density lipoprotein cholesterol (Tenenbaum et al., 2005). Gemfibrozil is used as a lipid regulator in human health as it lowers cholesterol, triglyceride, very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) levels and increase high-density lipoproteins (HDL) and HDL/cholesterol ratio in the blood. For this reason, it is a main drug of choice against hyperlipidemia, coronary heart failure and myocardial infarction in diabetic patients (Costet et al., 1998; Schoonjans et al., 1996; Tunca and Devrim, 2007;).

L-Carnitine is a biological substance that plays a crucial role in energy production in mitochondria, and is a vital element synthesized in liver and kidneys from amino acids such as lysine and methionine. Oxidation of long chain fatty acids is not possible without L-carnitine. Free fatty acids can pass through the mitochondrial inner membrane by forming an ester with L-carnitine, so that the free fatty acids become ready for β-oxidation (Fritz, 1995; Kopec and Fritz, 1973, Longo et al., 2016) and energy is provided from the burning of fatty acids in mitochondria (Lohninger et al.,

1987). L-carnitine has also been shown to have modulatory and protective effects against metabolic intermediates that cause pathological disorders in organs and tissues in recent studies. These effects are; preparation of active fatty acids necessary for the synthesis of membranes, protection of membrane lipids against peroxidation, modification of membrane structure, transport of long chain fatty acids to mitochondria and oxidation, increasing of ATP production to optimum level, activation and support of immunity system and positive effects on receptor and transport proteins (Binienda and Ali, 2001; Brown, 1999; Kremser et al., 1995; Selek et al., 2011, Longo et al., 2016; Sung et al., 2016; Uhlenbruck, 1996;). Studies in food animals such as cattle and pig, also reported that the administration of exogenous L-carnitine increased digestibility of feed and fat (LaCount et al., 1995), and significantly reduced the amount of fat in the gluteal region resulting in an increase in meat/fat ratio (Owen et al., 1996). L-carnitine supplementation had positive effects in the reduction of serum triglyceride, cholesterol and free fatty acid levels in rats (Mondola et al., 1992), prevention of diabetes in carnivores, treatment of heart diseases such as cardiomyopathy and cardiac insufficiency, obesity and prevention of fatty liver (Keene, 1991).

This study is aimed to investigate pathological (macroscopic, microscopic and histochemical staining methods) and biochemical effects of Gemfibrozil, an antihyperlipoproteinemic fibrate, PPARs and L-carnitine in an experimental model in rabbits on high fed diet.

MATERIAL and METHOD

The study design, management of animals and experimental procedures are given elsewhere (Selek et al, 2011) Briefly a total of 56 New Zealand Albino rabbits, 6 months old, were assigned to 8 equal groups (n = 7). All animals were kept at 12-h light/dark in a ventilated unit with 55% humidity throughout the the experiment. Rabbits were provided normal ad libitum dieat containing 2500 Kcal metabolic energy and 4.5% crude fat (groups I, II, III and IV) and high fat diet containing 3200 Kcal metabolic energy and 15% crude fat (goups V, VI, VII and VIII) and drinking water. Peletted rabbit feed was purchased from Bayramoglu Yem Inc., Erzurum, Türkiye. The animals were medicated daily for nine weeks. Groups were as follow;

Group I: received normal diet only,

Group II; received normal diet+Gemfibrozil (0.05%, 100 mg/kg/day, BW),

Group III: received normal diet+L-carnitine (200 mg/kg/day, BW),

Group IV: received normal diet+Gemfibrozil (0.05%, 100 mg/kg/day, BW)+L-carnitine (200 mg/kg/day, BW)

Group V: received high fat diet only,

Group VI: received high fat diet+Gemfibrozil (0.05%, 100 mg/kg/day, BW),

Group VII: received high fat det+L-carnitin (200 mg/kg/day, BW),

Group VIII: received high fat diet+Gemfibrozil (0.05%, 100 mg/kg/day, BW)+L-carnitin (200 mg/kg/day, BW).

Clinical Examination and Sample Collection

All rabbits were subjected to daily clinical examination following drug administration. The body weights of all rabbits were measured and recorded at a week interval during the study period. Blood samples were collected from vena auricularis into plain tubes at the start of the study (day 0) and every three weeks (week 3, 6 and 9) after a off feed period of 10 h with free access to water. Blood samples were centrifuged for 10 minutes at 3000 rpm and obtained sera was stored at -25ºC until analysis.

Sample Analysis

Glucose was spectrophotometrically determined using a commercial kit (IBL®, Germany) as instructed by manufacturer. Total lipid levels were measured according to the Kunkel phenol method. Peroxisomal β-oxidation analysis of liver tissue was performed spectrophotometrically at 340 nm wavelength according to the method reported by Lazarow (Lazarow, 1981).

Histopathological Examination

At the end of the study, animals were euthanized under general anesthesia and systemic necropsy was performed. Macroscopic changes noted were recorded for each animal. All abdominal organs and abdominal fat tissue (periovarian, perirenal, inguinal, mesenteric and omental adipose tissue) were weighed; tissue samples were taken for histo-pathological examinations. All tissues were fixed in 10% buffered formaldehyde solution. After routine procedures paraffin blocks were prepared and were cut at 4-6 μm thickness and stained with Hematoxylin Eosin (HE) for histopathological examination under light microscope. Histopathological changes were recorded.

For electron microscopic examinations, 1 mm^3 fragments from the liver were washed with 0.1 M cacodylate buffer (Ph 7.4) after 24 h of initial fixation in glutaraldehyde paraformaldehyde solution. It was then subjected to 2 hours fixation with 2% osmic acid. The samples were treated with known methods and blocked with araldite. Semi-thin sections and ultra-thin sections were stained with toluidine blue and uranyl acetate and citrate. Sections were examined on a Zeiss-9S electron microscope. In addition, the expression of PPARs in tissues was determined by immunohistochemical methods.

Immunohistochemical Examination

In this study, avidin-biotin peroxidase method was used as immunohistochemical method. For immunohistochemical staining, 4-6 μm thick sections from paraffin blocks were placed on the slide coated with 3-aminopropyltriethoxysilane (APES, Sigma-Aldrich, St. Louis, Montana, USA). After deparafinization and dehydration, the sections of the citrate buffer (pH 6.0) solution were subjected to microwave treatment at 800 watts and for 10 minutes of treatment with the aim of revealing the antigenic receptors. The sections, spontaneously cooled to room temperature, were incubated for 15 minutes in methanol (3%) solution of hydrogen peroxide to prevent endogenous peroxide activity in the tissues. This was followed by washing three times in PBS (Phosphate Buffer Solution). Primer antibodies used in the study (Table 1) were diluted in PBS. The tissues treated with polyclonal rabbit anti PPAR-α and goat PPAR-β antibodies were incubated with goat anti rabbit and rabbit anti goat immunoglobulin G for 30 minutes, respectively, in biotinylated order. The tissues incubated with monoclonal mouse anti PPAR-γ antibody were incubated with biotinylated rabbit anti mouse immunglobulin G for 30 minutes at room temperature. Negative controls were generated using goat or rabbit serum instead of the primer antibody. Subsequently, all sections were washed three times with PBS and incubated with peroxidase-conjugated streptavidin for an hour. The chromogenic solution of 3.3'-diaminobenzidine tetrahydrochloride (0.5 mg DAB/mL; Dako Corporation, Carpinteria, USA) dissolved in phosphate buffer solution was applied to the tissue sections as a color-evident substrate for 3 minutes. Background painting was done with Mayer Haematoxylin. For this purpose, background dyeing was carried out by staining for 5 seconds and then washing in tap water for 5 minutes. Subsequently, the sections were dehydrated and covered with immunomount. Finally, sections were examined by light microscopy.

Table 1. Specificity, clone, origin, firm and dilutions of primer antibodies used in immunohistochemical staining.

Specificity	Clone	Origin	Firm	Dilution
PPAR-α	Rabbit PAb IgG, H98, sc-9000	Human	SANTA CRUZ	1:100
PPAR-β	Goat PAb IgG, K20, sc-1987	Mouse	SANTA CRUZ	1:100
PPAR-γ	Mouse MAb IgG, E8, sc-7273	Human	SANTA CRUZ	1:100

Analysis of Immunohistochemical Staining Results

PPAR-α, PPAR-β and PPAR-γ immunoreactivity were examined using a grading system based on the number of positive cells in the areas that best reflected the staining character. The analysis for the quantification of immunological staining in the tissue was started based on the high intensity reaction areas. For each sample, a total of 400 magnifications were examined with 10 separate areas, each with a total of 100 grids (10x10) and a total area of 0.025 mm^2. Percentages of positive stained cells in each area were recorded and the average of these 10 sites was taken as the data of that animal. PPAR-α, PPAR-β and PPAR-γ staining intensities seen in the cytoplasm and nuclei of cells were made semiquantitatively for cytoplasm and nuclei separately.

Statistical Analysis

SPSS for Windows 10.0 program was used for the statistical analysis of the data obtained in the study. The data are listed as mean ± standard error on the tables. The comparisons of groups were performed using repeated measure of ANOVA. P-values <0.05 was accepted as significant.

RESULTS

Clinical Findings

Clinical examinations revealed no abnormal signs throughout the experiment.

Biochemical Analysis Findings

Biochemical parameters with regard to liver and oxidative stress parameters are given previously (Selek et al., 2011). Total lipid and glucose levels of the rabbits evaluated in the study are given in Table 2. The results of β-oxidation of fatty acids in the liver are shown in Table 3.

Table 2. Total Lipid and Glucose levels in rabbits fed on Normal and high fat diet (mean ± standard error).

Parameters	Group	Weeks				P
		0.	3.	6.	9.	
Total lipid	GrI	520.4±10.9	522.9±18.7 **B**	530.2±8.3 **BC**	528.7±13.2 **BC**	
	GrII	526.7±8.0	519.3±12.6 **B**	516.3±7.1 **C**	518.8±6.2 **C**	
	GrIII	522.9±8.9	517.8±13.5 **B**	523.3±12.6 **BC**	519.7±8.9 **C**	
	GrIV	521.8.±12.2	515.9±18.0 **B**	519.7±15.8 **BC**	514.7±17.0 **C**	
	GrV	534.1±12.0 **c**	577.1±5.9 **A,b**	607,4±7,3 **A,a**	632.2±7.7 **A,a**	**P<0.001**
	GrVI	538.9±12.6	551.2±12.6 **AB**	557.1±8.4 **B**	560.2±13.1 **B**	
	GrVII	535.4±10.6	548.4±15.2 **AB**	551.1±14.6 **BC**	563.7±12.8 **B**	
	GrVIII	537.2±14.7	547.2±20.2 **AB**	554.2±15.9 **B**	559.4±16.3 **B**	
	P		**P=0.064**	**P<0.001**	**P<0.001**	
Glucose	GrI	118.3±3.3	118.3±2.9	120.9±2.8	120.9±2.8 **BC**	
	GrII	119.4±1.8	122.1±2.8	123.4±2.9	123.1±3.2	

				ABC	
	GrIII	120.3±2.6	124.6±1.9 **ab**	127.7±1.7 **a**	129.4±1.7 **AB,a**	**P<0.05**
	GrIV	118.1±2.9	124.4±3.7	125.7±3.7	128.8±3.5 **AB**	
	GrV	118.6±3.4	118.7±3.2	116.3±2.8	117.4±3.1 **C**	
	GrVI	117.4±3.2	120.9±3.6	122.7±3.9	123.4±3.3 **ABC**	
	GrVII	118.9±3.2	124.1±2.4	126.6±3.7	131.4±3.9 **A**	
	GrVIII	119.4±2.9	123.7±3.2	126.9±3.0	130.6±3.9 **AB**	
P					**P<0.05**	

GrI: Normal diet group; **GrII:** Normal diet + Gemfibrozil group; **GrIII:** Normal diet + L-carnitin group; **GrIV:** Normal diet + L-carnitin + Gemfibrozil group **GrV:** High fat diet group; **GrVI:** High fat diet + Gemfibrozil group; **GrVII:** High fat diet + L-carnitin group; **GrVIII:** High fat diet + L-carnitin + Gemfibrozil group **ABCDE:** Shows statistical significance according to week and intra-group on column base **abcde:** Shows statistical significance according to week and intra-group on line base

Table 3. The levels of peroxisomal β-oxidation in the liver tissue of rabbits fed a normal and high fat diet (µmol/minute/gr liver) (mean ± standard error).

Parameter	**Groups**				
	Group I	Group II	Group III	Group IV	
β- oxidation (µmol/min/gr liver)	0.91±0.24 **b**	0.99±0.11 **b**	1.14±0.13 **b**	1.39±0.32 **a**	**P<0.01**
	Group V	Group VI	Group VII	Group VIII	
	0.89±0.08 b	**1.04±0.08 b**	**1.48±0.27 a**	**1.51±0.33 a**	**P<0.001**

GrI: Normal diet group; **GrII:** Normal diet + Gemfibrozil group; **GrIII:** Normal diet + L-carnitin group; **GrIV:** Normal diet + L-carnitin + Gemfibrozil group **GrV:** High fat diet group; **GrVI:** High fat diet + Gemfibrozil group; **GrVII:** High fat diet + L-carnitin group; **GrVIII:** High fat diet + L-carnitin + Gemfibrozil group **abc:** Shows statistical significance according to week and intra-group on line base

Histopathological Examination Findings

Macroscopic Findings

On gross examination, the most striking finding was a significant increase in abdominal fat tissue observed only in the group treated with high fat diet (Group V). Gemfibrozil administration resulted in a significant decrease in abdominal fat tissue in all groups but the decrease was much more pronounced in gemfibrozil and L-carnitine treated group (Group VIII). The liver was pale and fragile in only animals fed high fat diet (Group V). Gemfibrozil and high fat diet also caused an increase in liver weight (Table 4, 5). The proportion of fat weight to body weight (tissue or organ weight x 100/live weight) is summarized in Table 4.

Histopathological Findings

Steatosis was noted in liver of only rabbits fed high fat diet and the details are given elsewhere (Selek et al 2011). A marked hypertrophy was observed in the adipocytes in omentum, inguinal fat and fat around kidney of rabbits fed with high fat diet. No significant histopathologic changes were observed in the kidneys except for mild glomerular changes in the rabbits fed with high fat diet. In the rabbits treated with gemfibrozil, degenerative and necrotic changes were observed mildly in the proximal tubulus epithelium, but no degenerative changes were observed in any of the rabbits treated with L-carnitine. In group V, mild hyperplasia in the pancreas Langerhans islets on rabbits was noted. In group V and VI vacuolization in the acinar cells of pancreas was

evident whereas no vacuoles found in the acinic cells in the other groups.

Table 4. Weekly animal weight change (gr) and carcass parameters in normal and high fat diet groups (gr) (mean ± standard error).

Parameter	Groups				P
	Group I	**Group II**	**Group III**	**Group IV**	
AW increase (gr)	1042.9±102.0 **a**	442.9±102.0 **c**	757.1±108.8 **ab**	628.6±89.2 **bc**	**P<0.01**
AW increase %	46.1±5.0 **a**	17.9±4.9 b	31.9±5.9 ab	27.7±4.8 b	**P<0.01**
Liver (gr)	103.8±2.9	100.3±5.0	98.9±6.6	104.3±5.9	
Brain (gr)	9.23±0.09 **b**	9.61±0.14 **ab**	9.71±0.17 **a**	9.42±0.14 **ab**	**P<0.05**
Omentum fat (gr)	23.8±3.7	22.7±4.8	20.9±2.3	16.8±3.2	
Upper renal fat (gr)	54.4±7.7	48.7±8.8	41.3±5.3	36.1±5.1	
Total abdominal fat (gr)	78.2±11.0	71.4±13.2	62.2±6.9	52.8±7.9	
Abdominal fat/AW ratio (%)	2.33±0.30	2.32±0.44	1.91±0.21	1.77±0.24	
Abdominal fat/carcas ratio (%)	4.46±0.54	4.45±0.76	3.69±0.38	3.29±0.45	
	Group V	**Group VI**	**Group VII**	**Group VIII**	
AW increase (gr)	771.4±101.7 **ab**	900.0±37.8 **a**	714.3±114.3 **ab**	514.3±96.2 **b**	**P<0.05**
AW increase %	33.8±4.8 **ab**	41.6±2.0 **a**	32.5±5.3 **ab**	23.1±4.9 **b**	**P<0.05**
Liver (gr)	92.9±5.5 **a**	93.7±3.9 **a**	86.8±2.9 **ab**	76.9±3.9 **b**	**P<0.05**
Brain (gr)	9. 57±0.33 **ab**	9.12±0.16 **b**	9.60±0.17 **ab**	9.90±0.12 **a**	**P<0.05**
Omentum fat (gr)	51.7±10.6 **a**	28.7±5.9 **b**	21.6±3.5 **b**	16.9±2.1 **b**	**P<0.01**
Upper renal fat (gr)	134.9±14.9 **a**	88.0±11.6 **b**	65.8±9.9 **bc**	48.1±8.5 **c**	**P<0.001**
Total abdominal fat (gr)	186.6±21.3 **a**	116.8±16.4 **b**	87.4±13.3 **bc**	65.0±10.0 **c**	**P<0.001**
Abdominal fat/AW ratio (%)	6.00±0.60 **a**	3.75±0.44 **b**	2.93±0.39 **bc**	2.27±0.30 **c**	**P<0.001**
Abdominal fat/carcas ratio (%)	**10.36±0.94 a**	**6.83±0.86 b**	**5.29±0.68 bc**	**4.21±0.62 c**	**P<0.001**

GrI: Normal diet group; **GrII:** Normal diet + Gemfibrozil group; **GrIII:** Normal diet + L-carnitin group; **GrIV:** Normal diet + L-carnitin + Gemfibrozil group **abc:** Shows statistical significance according to week and intra-group on line base **GrV:** High fat diet group; **GrVI:** High fat diet + Gemfibrozil group; **GrVII:** High fat diet + L-carnitin group; **GrVIII:** High fat diet + L-carnitin + Gemfibrozil group **abc:** Shows statistical significance according to week and intra-group on line base

Immunohistochemical Examination Findings

PPAR-α expression was significant especially in the liver, kidney and heart. High fat diet and gemfibrozil administration caused a significant increase in PPAR-α expression. On the contrary, L-carnitine administration was found to have no positive or negative effect on PPAR-α expression in tissues.

PPAR-α expression was significant especially in the liver, kidney and heart. The PPAR-α immunoreactive reaction in the liver, especially in hepatocytes, was determined both

cytoplasmic and nuclear. The number of cells stained with PPAR-α primer antibody and the staining intensity were higher in rabbits fed high fat diet+gemfibrozil (Group VI) and high fat diet gemfibrozil + L-carnitine (Group VIII) than rabbits in all groups and high fat diet group were higher compared to rabbits fed normal diet and normal diet+L-carnitine (Figure 1).

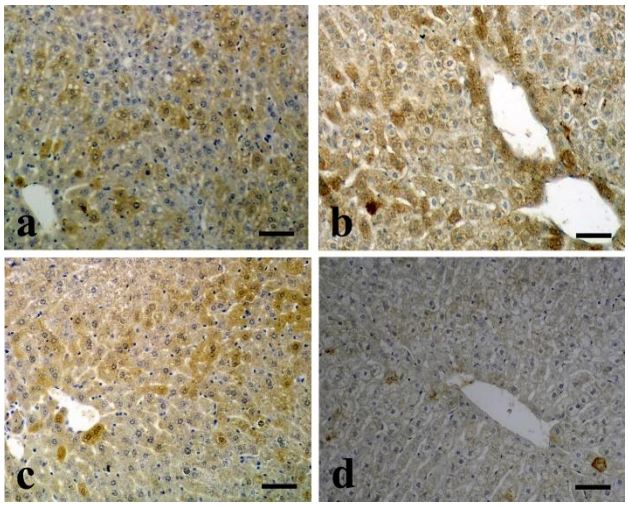


Figure 1. PPAR-α expression in the liver. Avidin Biotin Peroxidase Complex (ABC). High fat diet (a), high fat diet+ gemfibrozil (b), high fat diet+ gemfibrozil+ L-carnitine (d) fed rabbits showed less number of stained cells and staining intensity with PPAR-α in the liver than normal diet (d) fed rabbits. (Bar =50 µm).

There was a difference in PPAR-α expression between gemfibrozil and high fat diet groups and those received normal diet only. However, L-carnitine administration did not affect PPAR-α expression. The PPAR-α positive reaction in liver was more pronounced in periaciner (centrilobular) hepatocytes in rabbits fed normal diet and normal diet+L-carnitine. On the other hand, positive reactions occurred in hepatocytes located in periasiner and midzonal regions and a little less in centriaciner areas in rabbits treated with high fat diet and high fat diet+L-carnitine. Almost all areas of hepatocytes were stained positively in high fat diet+gemfibrozil (Group VI) and high fat diet +gemfibrozil+L-carnitine (Group VIII).

Table 5. Comparison of abdominal fat weight, abdominal fat weight/ animal weight, abdominal fat weight/ carcas ratio between groups with similar nutrition and medication by week in rabbits fed on normal and high fat diet (mean ± standard error).

Groups	Omentum fat (gr)	Upper renal fat (gr)	Total abdominal fat (gr)	Abdominal fat/AW (%)	Abdominal fat/carcas (%)
Group I	23.8±3.7	54.4±7.7	78.2±11.0	2.3±0.3	**4.46±0.55**
Group V	51.7±10.6	134.9±14.9	186.6±21.3	6.0±0.6	**10.36±0.94**
P	**P<0.05**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**
Group II	22.7±4.8	48.7±8.8	71.4±13.2	2.3±0.4	**4.45±0.76**
Group VI	28.7±5.9	88.0±11.6	116.8±16.4	3.8±0.4	**6.83±0.86**
P		**P<0.05**	**P<0.05**	**P<0.05**	**P=0.062**
Grup III	20.9±2.3	41.3±5.3	62.2±6.9	1.9±0.2	**3.69±0.38**
Grup VII	21.6±3.5	65.8±9.9	87.4±13.3	2.9±0.4	**5.29±0.68**
P		**P<0.05**		**P<0.05**	**P=0.064**
Grup IV	16.8±3.2	36.1±5.1	52.8±7.9	1.8±0.2	**3.29±0.45**
Grup VIII	16.9±2.1	48.1±8.5	65.0±10.0	2.3±0.3	**4.21±0.62**
P					

GrI: Normal diet group; **GrII:** Normal diet + Gemfibrozil group; **GrIII:** Normal diet + L-carnitin group; **GrIV:** Normal diet + L-carnitin + Gemfibrozil group **GrV:** High fat diet group; **GrVI:** High fat diet + Gemfibrozil group; **GrVII:** High fat diet + L-carnitin group; **GrVIII:** High fat diet + L-carnitin + Gemfibrozil group

The PPAR-a positive reaction in the kidneys was most prominently found in the proximal tubulus epithelium. In all groups, almost all of the proximal tubular epithelium was stained positively, but the density of staining was more prominent especially in rabbits treated with high fat diet and high fat+gemfibrozil diet than the other groups. Moreover, a positive reaction was found in distal tubular epithelium in these groups. There was no positive reaction in the glomerulus.

PPAR-α expression was also detected in epithelium cells in the stomach and intestines, cortical cells in the adrenal gland, cardiac

muscle and smooth muscle cells, adipocytes and macrophages (Figure 2).

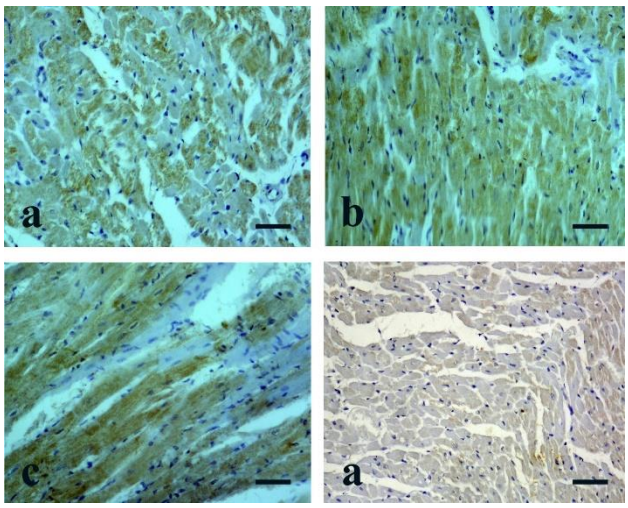


Figure 2. PPAR-α expression in the heart muscle (ABC). High fat diet (a), high fat diet+ gemfibrozil (b), high fat diet+ gemfibrozil+ L-carnitine (d) fed rabbits showed more number of stained cells and staining intensity with PPAR-α in the liver than normal diet (d) fed rabbits. (Bar a, b,c ve d =100 µm, e = 50 µm).

The staining density in the liver was more prominent in the rabbits fed the high fat diet, while no significant difference was found between the groups in terms of number of cells stained positive with PPAR-β primer antibody in tissues (Figure 3). PPAR-β immunoreactivity was present in almost all tissues. The positive reaction in liver tissue was mostly nuclear and cytoplasmic localization, usually granular. However, diffuse staining was also observed in groups that were given high fat diet. Positive immunological reactions in the liver, which were granular in almost all hepatocytes, showed diffuse staining especially in centri-acinar areas in high fat fed animals.

No positive staining was observed in immunohistochemical staining with PPAR-γ primer antibody. Positive reaction to tissue staining with PPAR-γ in mouse tissues was detected to remove doubts about whether the primer antibody is working.

The positive reaction in rabbits treated with normal diet and normal diet+L-carnitine in the kidneys was particularly severe in the tubulus epithelia in the cortical region, and mostly nuclear localization however very dense cytoplasmic staining was observed in the high fat diet animals (Figure 4 a-c). As for the adrenal gland, in almost all groups, PPAR-β expression was observed especially in cortical cells, both cytoplasmic and nuclear localization.

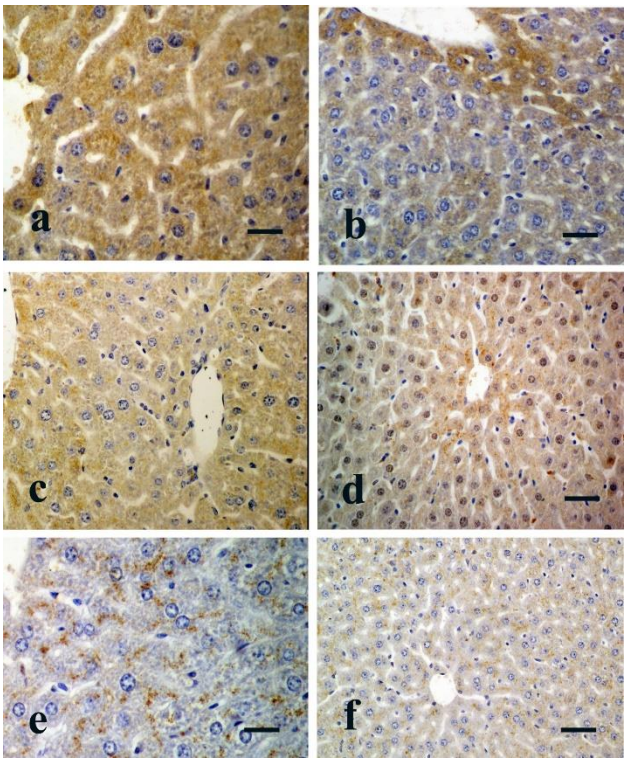


Figure 3. PPAR-β expression in the liver and kidneys. Avidin Biotin Peroxidase Complex (ABC). High fat diet (a,b,d,e,f) and gemfibrozil (b and d) administration resulted in a positive reaction in the liver (a,b,c,d), mostly cytoplasmic and granular. In the kidneys (e and f) both cytoplasmic and nuclear positive reactions are seen. There is no difference between the groups in terms of positive number of stained cells and intensity of staining. (Bar c,d and f = 30 µm, a,b and e = 20 µm).

Positive reaction in heart muscle was in cardiac myocytes and granular style. Cytoplasmic and nuclear staining was also noted in adipocytes in the adipose tissue and stomach and intestinal epithelial cells. In the central nervous system, it was nuclear localization in almost all neurons and sometimes in glia cells (Figure 4 d).

Weak cytoplasmic localized immunoreactivity was observed in beta cells of the pancreas Langerhans islets. In the alpha cells located in the periphery of the islets, intense cytoplasmic and nuclear staining was

observed. In some cases, a mild positive reaction with nuclear localization was observed in exocrine pancreatic acinar cells.

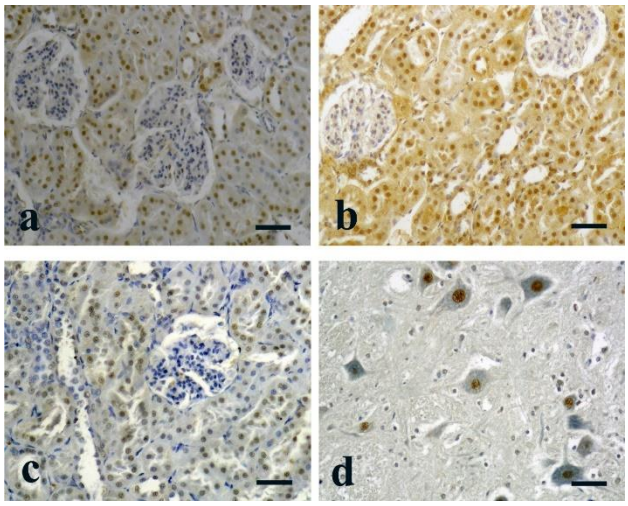


Figure 4. Stomach (a), heart (b), pancreas (c ve d), cerebellum (e) and brain stem (in neurons) (f) cytoplasmic and nuclear PPAR-β expression. (Bar a,b and c = 100 µm, d = 50 µm).

DISCUSSION

In this study, the effects of Gemfibrozil an antihyperlipoproteiemic fibrate, and L-carnitine were investigated on obesity, fattening and distribution of PPARs in tissues histopathologically (macroscopic, microscopic and histochemical staining methods) and biochemically on a model for obesity developed by feeding high fat diet in rabbits. In this study, exogenous administration of gemfibrozil and L-carnitine had various effect on plasma total lipid concentrations and fattening. The relationship between hepatic lipidosis, oxidative stress and Gemfibrozil and L carnitine was discussed elsewhere (Selek et al., 2011)

Gemfibrozil is reported to be used as a lipid regulator in human as it causes elevation in cholesterol, triglycerides, VLDL, LDL levels and cholesterol/HDL cholesterol, and decreases in cholesterol HDL levels (Tunca and Devrim, 2007; Schoonjans et al., 1996; Costet et al., 1998; Stalenhoef et al., 2000; Barter and Rye, 2006). The hypothesis that the effect of gemfibrozil on triglyceride level reduction is due to the lipid-regulating role of fibrates in increasing the activation of PPARs and consequently triglyceride catabolism (Mussoni et al., 2000; Schoonjans et al., 1996; Zambon et al., 2007) has been suggested by researchers.

Mondola et al. (1992) found that L-carnitine addition reduced serum triglycerides, cholesterol and free fatty levels in their studies in rats. It has also been reported that gemfibrozil in combination with other drugs leads to lower levels of serum triglycerides and other lipid profile parameters and is a good lipid regulator in this respect (Wägne et al., 2003). In our study, in Group VIII, in which L-carnitine and gemfibrozil were administered together, a much lower lipid and abdominal fat as compare to Group V-VII was thought to be due to the synergistic effect of these two drugs as this positive synergistic effect between the two drugs was first demonstrated in our study. This synergistic effect supports the findings of the study, in which Ringseis et al. (2008) reported that clofibrate, synthetic PPAR activator, increased intestinal carnitine absorption which in turn had additive effect.

Beta Oxidation

In the present study, beta oxidase was increased by 10% in Group II, 30% in Group III, approximately 40% in Group IV, 20% in Group VI, 40% in Group VII and approximately 50% in Group VIII. Gemfibrozil and L-carnitine addition (Group VIII) caused marked increase in beta-oxidation compared to all other groups.

Increases in β-oxidation rates due to gemfibrozil administration were compatible with the previous studies where gemfibrozil is shown to play an important role in fatty acid catabolism by increasing the gene expression responsible for PPAR-α mitochondrial fatty acid oxidation activated with fibrates, peroxisomal fatty acid oxidation and other fatty oxidation in the cell (Akbiyik et al., 2004; Mandard et al., 2004; Huang et al., 2009). PPAR-α agonists have been widely used for

decreasing plasma triglyceride and low density lipoprotein cholesterol and increasing high density lipoprotein levels (Guerre-Milloet al., 2000). Increasing β-oxidation in the liver and skeletal muscle is one of the reasons that leads to the effects mentioned previously (Minnich et al., 2001). Moreover PPAR-α also regulates liver metabolism by increasing glycolysis and reducing gluconeogenesis in the liver (Atherton et al., 2006) and it is shown that PPAR-α agonists have potent ability to lower glucose concentrations in blood plasma (Kim et al., 2003). In this context, free fatty acids are thought to increase the oxidation of fatty acids in the liver by increasing PPAR-α expression (Akbiyik et al., 2004; Mandard et al., 2004; Huang et al., 2009). These beneficial effects of PPAR-α ligands have been attributed by researchers to the fact that they increase rate-limiting peroxisomal (AOX) and mitochondrial (AD) β-oxidation enzyme activities in hepatocyte mitochondria and peroxisomes and lead to increased oxidation of fatty acids (Akbiyik et al., 2004; Lawrence et al., 2001).

The role of L-carnitine in transporting long chain fatty acids to mitochondria for β-oxidation has long been known (Kopec and Fritz, 1973; Lohninger et al., 1987; McCarty, 2001; Longo et al., 2016). Fritz (1955), for the first time in a mammalian animal study, has shown that L-carnitine increases the oxidation of fats in the liver. We are in the opinion that the findings of β-oxidation increase in our study, especially in Group VIII, are compatible with both other investigators and other findings of our study which led us to think that these two drugs have a positive synergistic effect.

PPAR Expression

High fat diet and gemfibrozil administration caused a significant increase in PPAR-α expression. On the other hand, L-carnitine administration was found to have no effect on PPAR-α expression in tissues, either positively or negatively. There was a difference in PPAR-α expression between gemfibrozil and high fat diet groups and non-high fat diet groups. High fat diet and gemfibrozil administration also caused more PPAR-α expression than gemfibrozil administration alone. This finding supports the data that clofibrate administration was more likely to cause PPAR-α activation than high fat diet administration in rats (Akbiyik et al., 2004) and mice (Tunca and Devrim 2007).

The increase in PPAR-α expression in the liver with the administration of high fat diet is probably been related to the increase in fatty acid concentrations in hepatocytes. Free fatty acids, especially palmitic acid, oleic acid, linolenic acid and arachidonic acid, are known to be natural ligands of PPAR-α (Tunca and Devrin, 2007; Yu et al., 1995). Free fatty acids not only increase the number of these receptors but also activate this receptor, leading to peroxisome proliferation (Akbiyik et al., 2004; Tunca and Devrim 2007). The target genes of PPAR-α are associated with the oxidation of mitochondrial and peroxisomal fatty acids and other lipid metabolites in the cell (Mandard et al., 2004). Thus, free fatty acids increase the PPAR-α expression and increase the oxidation of fatty acids in the liver (Akbiyik et al., 2004; Mandard et al., 2004).

The increase in PPAR-α and β expression in the liver was immunohistochemically increased in the animals fed with the high fat diet, whereas PPAR-γ expression was not affected. While there was no striking difference in the number of cells stained between the groups in terms of the number of cells stained with PPAR- β primer antibody in tissues, the density of staining especially in the liver was more prominent in rabbits fed the high fat diet. Although PPAR-β immunoreactivity was present in almost all tissues, no positive staining was observed in immunohistochemical tissue staining with PPAR-γ primer antibody. As in

PPAR-α, unsaturated fatty acids are natural ligands of PPAR-γ (Lehrke and Lazar, 2005).

Although it has been reported that the target genes of PPAR-γ are responsible for adipocyte differentiation, lipid storage and glucose metabolism, and are important for adipocyte differentiation both in vitro and in vivo (Xu et al., 2003), in this study no positive staining was observed in tissue staining with PPAR-γ primer antibody. The fact that PPAR-α was not affected by high fat diet administration resulted in the ineffectiveness of fatty acids in PPAR-β activation, although PPAR-α had a significant role in the oxidation of fatty acids as opposed to PPAR-γ in the study.

Animal Weight and Organ Weights

High fat diet administration (Group V) caused a significant increase in abdominal fat tissue. A more significant reduction in abdominal fat tissue mass occurred in groups of rabbits given gemfibrozil and L-carnitine separately (Group VI, VII) as well as togather (Group VIII).

It was determined that the liver weights obtained in group VI were higher than the other groups (Group V, VII and VIII). Macroscopically, the most striking finding was an increase in abdominal fat tissue in group only fed high fat diet (Group V). However, gemfibrozil administration resulted in a significant decrease in abdominal fat tissue in all groups. This decrease was much more pronounced in animals treated with gemfibrozil and L-carnitine (Group VIII) than all other groups. Total abdominal fat, abdominal fat/animal weight and abdominal fat/carcass ratios were significantly lower in Group VIII than in Groups V, VI and VII. The animals fed with high fat diet (Group V) had a higher omental fat, upper renal fat, total abdominal fat amount, abdominal fat/ animal weight ratio and abdominal fat/ carcass ratio than the group fed on the normal diet (Group I). This findings are in agreement with those studies where the effect of PPAR-α on obesity was investigated (Tunca and Devrim, 2007). Studies in null mice have reported the lower capacity of PPAR-α to metabolize long-chain fatty acids and consequently, dyslipidemia (Peters et al., 1997) and subsequent occurance of fat deposition (Costet et al., 1998). PPAR-α, activated by synthetic agonists such as unsaturated fatty acids and eicosanoids and synthetic agonists such as fibrates, plays an important role in the catabolism of fatty acids by increasing the expression of the gene responsible for PPAR-α mitochondrial fatty acid oxidation, peroxisomal fatty acid oxidation and other fatty oxidation in the cell (Mandard et al., 2004). Similarly, the lower amount of abdominal fat determined in Group VIII might be due to both the activation of gemfibrozil PPARs and the role of L-carnitine in fat transport to mitochondria (Kopec and Fritz, 1973; Lohninger et al., 1987; McCarty, 2001). Studies in food animals (cattle and pigs) reported increased the digestibility of feed and feed oils (LaCount et al., 1995), decreased the amount of fat in the gluteal region (Smith et al., 1994) and thus markedly increased the meat / fat ratio in the carcass (Owen et al., 1996) following administration of exogenous L-carnitine. Our findings are in line with the previous researchers (Keene, 1991). As PPAR-α ligands such as clofibrate, bezfibrate and gemfibrozil already reduced liver fat (Akbiyik et al., 2004; Toda et al., 2003; Tunca and Devrim, 2007).

CONCLUSION

As a result, gemfibrozil and L-carnitine application in this experimental model had positive effects on abdominal and hepatic fattening and lipid profile improvement. In this model, gemfibrozil administration caused a significant increase in PPAR-α expression in the liver, whereas PPAR-β and PPAR-γ expression were not affected. High fat diet

application increased PPAR-α expression but was ineffective on PPAR-γ and PPAR-β expression. However, L-carnitine administration had no significant enhancing effect on PPARs expression in both normal and high fat diet. It may be suggested that hepatic lipidosis due to high fat diet in rabbits might have been inhibited by gemfibrozil and L-carnitine administration. In addition, abdominal fat (obesity) due to high fat diets could be prevented by gemfibrozil, a synthetic PPAR-a ligand, and L-carnitine, and consequently reduces associated health problems.

ACKNOWLEDGMENT

We sincerely thank The Scientific and Technical Research Council of Turkey as a study team for providing financial support. However, the The Scientific and Technical Research Council of Turkey had no role in the design of this study, data collection, data analysis, data interpretation or the writing of the manuscript.

This study was financially supported The Scientific and Technical Research Council of Turkey (Project Number 106 O 160).

Ethical approval:

The study was approved from the Kafkas University Faculty of Veterinary Animal Experiments Local Ethics Committee
(Protocol number: 20.12.2005/27).

Conflict of interest: All authors have contributed to the design, conduct of the study/data collection, interpretation of data and writing of the manuscript.

REFERENCES

Akbiyik, F., Cinar, K., Demirpence, E., Ozsullu, T., Tunca, R., Haziroglu, R., Yurdaydın, C., Uzunalimoglu, O. & Bozkaya, H. (2004). Ligand-induced expression of peroxisome proliferator-activated receptor α and activation of fatty acid oxidation enzymes in fatty liver. *European Journal of Clinical Investigation*, *34*, 429-435. doi: 10.1111/j.1365-2362.2004.01359.x

Atherton, H.J., Bailey, N.J., Zhang, W., Taylor, J., Major, H., Shockcor, J., Clarke, K. & Griffin, J.L. (2006). A combined 1H-NMR spectroscopy- and mass spectrometry-based metabolomic study of the PPAR-{alpha} null mutant mouse defines profound systemic changes in metabolism linked to the metabolic syndrome. *Physiological Genomics*, *27*, 178-186. doi: 10.1152/physiolgenomics.00060.2006

Barter, P.J. & Rye, K.A. (2006). Cardioprotective properties of fibrates. Which fibrate, Which patients, What mechanism?. *Circulation*, *113*, 1553-1555. doi: 10.1161/CIRCULATIONAHA.105.620450

Binienda, Z.K. & Ali, S.F. (2001). Neuroprotective role of l-carnitine in the 3-nitropropionic acid induced neurotoxicity. *Toxicology Letters*, *25*, 67-73. DOI: 10.1016/s0378-4274(01)00415-5

Brown, J.D. & Plutzky, J. (2007). Peroxisome proliferator-activated receptors as transcriptional nodal points and therapeutic targets. *Circulation*, *115*, 518-533. doi: 10.1161/CIRCULATIONAHA.104.475673

Brown, G.C. (1999). Nitric oxide and mitochondrial respiration. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1411*, 351-369. doi:10.1016/S0005-2728(99)00025-0

Costet, P., Legendre, J., More, J., Edgar, A., Galtier, P. & Pineau, T. (1998). Peroxisome proliferator-activated receptor alpha-isoform deficiency leads to progressive dyslipidemia with sexually dimorphic obesity and steatosis. *Journal of Biological Chemistry*, *273*, 29577-29585. doi: 10.1074/jbc.273.45.29577

DeFronzo, R.A. (1999). Pharmacologic therapy for type 2 diabetes mellitus. *Annals of Internal Medicine*, *131*, 281-303. doi: 10.7326/0003-4819-131-4-199908170-00008

Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G. & Wahli, W. (1992). Control of the peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors. *Cell*, *68*, 879-887. doi: 10.1016/0092-8674(92)90031-7

Fritz, I.B. (1955). The effect of muscle extracts on the oxidation of palmitic acid by liver slices and homogenates. *Acta Physiologica Scandinavica*, *34*, 367-385. doi: 10.1111/j.1748-1716.1955.tb01256.x

Guerre-Millo, M., Gervois, P., Raspé, E., Madsen, L., Poulain, P., Derudas, B., Herbert, J.M., Winegar, D.A., Willson, T.M., Fruchart, J.C., Berge, R.K. & Staels, B. (2000). Peroxisome proliferator-activated receptor α activators improve insulin sensitivity and reduce adiposity. *Journal of Biological Chemistry*, *275*, 16638-16642. doi: 10.1074/jbc.275.22.16638

Hashimoto, T., Fujita, T., Usuda, N., Cook, W., Qi, C., Peters, J.M., Gonzales, F.J., Yeldandi, A.V., Rao, S.M. & Reddy JK (1999). Peroxisomal and mitochondrial fatty acid β-oxidation in mice nullizygous for both PPARα and fatty acyl CoA oxidase: Genotype correlation with fatty liver phenotype. *Journal of Biological Chemistry*, *274*, 19228-19236. doi: 10.1074/jbc.274.27.19228

Huang, T.H., Teoh, A.W., Lin, B.L., Lin, D.S. & Roufogalis, B. (2009). The role of herbal PPAR modulators in the treatment of cardiometabolic syndrome. *Pharmacological Research*, *60*, 195-206. doi: 10.1016/j.phrs.2009.03.020

Jeffrey, M.P., Susanna, S.T.L., Wen, L., Jerrold, M.W., Oksana, G., Carrie, E., Marc, L.R., Lynn,

D.H. & **Frank, J.G. (2000).** Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor-α. *Molecular and Cellular Biology*, *20*, 5119-5128**.** doi: 10.1128/mcb.20.14.5119-5128.2000

Keene, B.W. (1991). L-carnitine supplementation in the therapy of canine dilated cardiomyopathy. *Veterinary Clinics of North America: Small Animal Practice*, *21*, 1005-1009. doi: 10.1016/S0195-5616(91)50108-X

Kim, H., Haluzik, M., Asghar, Z., Yau, D., Joseph, J.W., Fernandez, A.M., Reitman M.L., Yakar, S., Stannard, Bethel., Heron-Milhavet, L., Wheeler, M.B. & LeRoith, D. (2003). Peroxisome proliferator-activated receptor-α agonist treatment in a transgenic model of type 2 diabetes reverses the lipotoxic state and improves glucose homeostasis. *Diabetes*, *52*, 1770-1778. doi: 10.2337/diabetes.52.7.1770

Kopec, B. & Fritz, I.B. (1973). Comparison of properties of carnitine palmitoyltransferase I with those of carnitine palmitoyltransferase II, and preparations of antibodies to carnitine palmitoyltransferases. *Journal of Biological Chemistry*, *248*, 4069-4074. doi: 10.1016/S0021-9258(19)43840-4

Kraja, A.T., Province, M.A., Straka, R.J., Ordovas, J.M., Borecki, I.B. & Arnett, D.K. (2010). Fenofibrate and metabolic syndrome. *Endocrine Metabolic and Immune Disorders Drug Targets*, *10*, 138-148. doi: 10.2174/187153010791213047

Kremser, K., Stangl, H., Pahan, K. & Singh, I. (1995). Nitric oxide regulates peroxisomal enzyme activities. *European Journal of Clinical Chemistry and Clinical Biochemistry*, *33*, 763-774. doi: 10.1515/cclm.1995.33.11.763

LaCount, D.W., Drackley, J.K. & Weigel, D.J. (1995). Responses of dairy cows during early lactation to ruminal or abomasal administration of L-carnitine. *Journal of Dairy Science*, *78*, 1824-1836. doi: 10.3168/jds.S0022-0302(95)76807-2

Lawrence, J.W., Li, Y., Chen, S., DeLuca, J.G., Berger, J.P., Umbenhauer, D.R., Moller, D.E. & Zhou, G. (2001). Differential gene regulation in human versus rodent hepatocytes by peroxisome proliferator-activated receptor (PPAR) alpha. PPAR alpha fails to induce peroxisome proliferation-associated genes in human cells independently of the level of receptor expresson. *Journal of Biological Chemistry*, *276*, 31521-31527.

Lazarow, P.B. (1981). Assay of peroxisomal β-oxidation of fatty acids. *Methods in Enzymology*, *72*, 315-319. doi: 10.1016/S0076-6879(81)72021-4

Lehrke, M. & Lazar, M.A. (2005). The many faces of PPARγ. *Cell*, *123*, 993-999. doi: 10.1016/j.cell.2005.11.026

Lohninger, A., Kaiser, E., Legenstein, E. & Staniek, H. (1987). Carnitine, metabolism and function. Ed. Kaiser E., Lohninger A., Carnitine-Its Role in Lung and Heart Disorders, Karger München, pp: 1-25.

Longo, N., Frigeni, M. & Pasquali, M. (2016). Carnitine transport and fatty acid oxidation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1863*, 2422-2435. doi: 10.1016/j.bbamcr.2016.01.023

Mandard, S., Muller, M. & Kersten S. (2004). Peroxisome proliferator-activated receptor alpha target genes. *Cellular and Molecular Life Sciences*, *61*, 393-416. doi 10.1007/s00018-003-3216-3

McCarty, M. (2001). Hepatothermic therapy of obesity: rationale and invertory of resources. *Medical Hypotheses*, *57*, 324-336.

Minnich, A., Tian, N., Byan, L. & Bilder, G. (2001). A potent PPAR alpha agonist stimulates mitochondrial fatty acid beta-oxidation in liver and skeletal muscle. *American Journal of Physiology Endocrinology and Metabolism*, *280*, E270-E279. doi: 10.1152/ajpendo.2001.280.2.E270

Mondola, P., Santillo, M., De Mercato, R. & Santangelo, F. (1992). The effect of L-carnitine on cholesterol metabolism in rat (*Rattus bubalus*) hepatocyte cells. *International Journal of Biochemistry*, *24*, 1047-1050. doi: 10.1016/0020-711x(92)90372-8

Moutzouri, E., Kei, A., Elisaf, M.S. & Milionis HJ. (2010). Management of dyslipidemias with fibrates, alone and in combination with statins: Role of delayed-release fenofibric acid. *Vascular Health and Risk Management*, *6*, 525-539. doi: 10.2147/VHRM.S5593

Mussoni, L., Mannucci, L., Sirtori, C., Pazzucconi, F., Bonfardeci, G., Cimminiello, C., Notarbartolo, A., Scafidi, V., Bittolo Bon, G., Alessandrini, P., Nenci, G., Parise, P., Colombo, L., Piliego, T. & Tremoli, E. (2000). Effects of gemfibrozil on insulin sensitivity and on haemostatic variables in hypertriglyceridemic patients. *Atherosclerosis*, *148*, 397-406. doi: 10.1016/s0021-9150(99)00283-x

Ogawa, S., Takeuchi, K., Sugimura, K., Fukuda, M., Lee, R., Ito, S. & Sato T. (2000). Bezafibrate reduces blood glucose in type 2 diabetes mellitus. *Metabolism*, *49*, 331-334. doi: 10.1016/s0026-0495(00)90176-8

Owen, K.Q., Nelssen, J.L, Goodband, R.D., Weeden, T.L. & Blum, S.A. (1996). Effect of L-carnitine and soybean oil on growth performance and body composition of early-weaned pigs. *Journal of Animal Science*, *74*, 1612-1619. doi: 10.2527/1996.7471612x

Peters, J.M., Hennuyer, N., Staels, B., Fruchart, J.C., Fievet, C., Gonzalez, F.J. & Auwerx, J. (1997). Alterations in lipoprotein metabolism in peroxisome proliferator-activated receptor alpha-deficient mice. *Journal of Biological Chemistry*, *272*, 27307-27312. doi: 10.1074/jbc.272.43.27307

Ringseis, R., Lüdi, S., Hirche, F. & Eder, K. (2008). Treatment with pharmacological peroxisome proliferator-activated receptor alpha agonist clofibrate increases intestinal carnitine absorption in rats. *Pharmacological Research*, *58*, 58-64. doi: 10.1016/j.phrs.2008.06.006

Schoonjans, K., Staels, B. & Auwerx, J. (1996). The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, *1302*, 93-109. doi: 10.1016/0005-2760(96)00066-5

Selek, Ş., Karapehlivan, M., Citil, M., Tunca, R., Uzlu, E. & Erdoğan, H.M. (2011). Effects of gemfibrozil and L-carnitine on PON1 activity, oxidative/anti-

oxidative parameters and hepatosteatosis in rabbits feed with fat-rich diet. *Kafkas Universitesi Veteriner Fakültesi Dergisi*, 17, 183-190. doi: 10.9775/kvfd.2010.2636

Smith, J.W., Owen, K.Q., Friesen, K.G., Nelssen, J.L., Goodband, R.D. & Tokach, M.D. (1994). The effect of supplemental dietary carnitine, betaine and chromium nicotinate on growth and carcass characteristics in growing-finishing pigs. Kansas State Univ., Swine Day, Agricultural Experiment Station, pp: 154-157.

Stalenhoef, A.F., de Graaf, J., Wittekoek, M.E., Bredie, S.J., Demacker, P.N. & Kastelein, J.J. (2000). The effect of concentrated n-3 fatty acids versus gemfibrozil on plasma lipoproteins, low density lipoprotein heterogeneity and oxidizability in patients with hypertriglyceridemia. *Atherosclerosis*, *153*,129-138. doi: 10.1016/s0021-9150(00)00381-6

Stienstra, R., Duval, C., Müller, M. & Kersten, S. (2007). PPARs, obesity, and inflammation. *PPAR Resesearch*, 2007:95974. doi:10.1155/2007/95974

Sung, D.J., Kim, S., Kim, J.H.S. & So, W.Y. (2016). Role of l-carnitine in sports performance: Focus on ergogenic aid and antioxidant. *Science & Sports*, *31*, 177-188. doi: 10.1016/j.scispo.2016.02.005

Tenenbaum, A., Motro, M. & Fisman, E.Z. (2005). Dual and pan-peroxisome proliferator-activated receptors (PPAR) co-agonism: the bezafibrate lessons. *Cardiovascular Diabetology*, 4:14. doi: 10.1186/1475-2840-4-14

Toda, K., Okoda, T., Miyaura, C. & Saibara, T. (2003). Fenofibrate, a ligant for PPARα, inhibits aromatase cytochrome P450 expression in the ovary of mause. *Journal of Lipid Research*, *44*, 265-270. doi:10.1194/jlr.M200327-JLR200

Tunca, R. & Devrim, A.K. (2007). Deneysel olarak obezite olusturulan farelerde ovarioektomi operasyonu ve gemfibrozilin peroksizom proliferatörleri ile aktive olan reseptörler (PPARs) ve obezite üzerine etkilerinin arastirilmasi. TUBITAK TOVAG, proje no: 104 O 297.

Uhlenbruck, G. (1996). L-Carnitine and the immune system: from the mode of metabolism to the modulation of membranes. *Proc Carnitine Symp Leipzig*, pp: 47-60.

Wägner, A.M., Jorba, O., Bonet, R., Ordóñez-Llanos, J. & Pérez, A. (2003). Efficacy of atorvastatin and gemfibrozil, alone and in low dose combination, in the treatment of diabetic dyslipidemia. *The Journal of Clinical Endocrinology & Metabolism*, *88*, 3212-3217. doi: 10.1210/jc.2003-030153

Wahli, W. (2002). Peroxisome proliferator-activated receptors (PPARs): from metabolic control to epidermal wound healing. *Swiss Medical Weekly*, *132*, 83-91. doi: 10.4414/smw.2002.09939

Xin, X., Yang, S., Kowalski, J. & Gerritsen, M.E. (1999). Peroxisome proliferator-activated receptor gamma ligands are potent inhibitors of angiogenesis in vitro and in vivo. *Journal of Biological Chemistry*, *274*, 9116-9121. doi: 10.1074/jbc.274.13.9116

Xu, H., Barnes, G.T., Yang, Q., Tan, G., Yang, D., Chou, C.J., Sole, J., Nichols, A., Ross, J,S., Tartaglia, L.A. & Chen, H. (2003). Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *Journal of Clinical Investigation*, *112*, 1821-1830. doi: 10.1172/JCI19451

Yoon, M., Jeong, S., Lee, H., Han, M., Kang, J.H., Kim, E.Y., Kim, M. & Oh, G.T. (2003). Fenofibrate improves lipid metabolism and obesity in ovariectomised LDL receptor-nul mice. *Biochemical and Biophysical Research Communications*, *302*, 29-34. doi: 10.1016/s0006-291x(03)00088-3

Yu, K., Bayona, W., Kallen, C.B., Harding, H.P., Ravera, C.P., McMahon, G., Brown, M. & Lazar, M.A. (1995). Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *Journal of Biological Chemistry*, *270*, 23975-23983. doi: 10.1074/jbc.270.41.23975

Zambon, A. & Cusi, K. (2007). The role of fenofibrate in clinical practice. *Diabetes and Vascular Disease Research*, *4*, S15-S20. doi: 10.3132/dvdr.2007.053

Determination of reticular fibers in tissues fixed with sugarcane molasses

Research Article

ABSTRACT

Reticular fiber consists of one or more types of very thin and delicately woven type III collagen strands, which form a highly ordered cellular network and provide a supportive network. Since most of these collagen types are combined with carbohydrates, they react with silver stains and periodic acid-Schiff reagent, but are not shown by ordinary histological stains like those using hematoxylin. In this study, it was aimed to determine the reticular threads by using silver staining technique (Gordon Sweet –GS-) after routine histological tissue follow-up in tissues fixed with sugarcane molasses and formol. The reticular fibers in the tissues fixed with sugarcane molasses were compared with those in the tissues fixed with formol. According to the findings, the staining quality of the liver and spleen tissues fixed with sugarcane molasses showed similar characteristics with the tissues fixed with formol. Very weak staining was observed in stomach, kidney, skin and testicle tissues. The fact that the tissues fixed in the same fixation solution show different results in the Gordon Sweet (GS) staining method shows us that this issue needs to be supported by more detailed studies.

Keywords: Reticular fibers, fixing, sugarcane molasses, formol.

Hatice Yaren Kuloğlu

Department of Histology and Embryology, Faculty of Veterinary Medicine, Aksaray University, Aksaray, Türkiye

ORCİD-
0000-0003-0435-4677

INTRODUCTION

Reticular fibers are a type of connective tissue that is not observed in routine histological staining. They (reticular fibers) are abundant in the organism, but are scattered and mixed with other types of fibers (such as elastic fibers). These fibers are found in very high amounts according to their location in the organism (Alvarenga and Marti, 2014). Their reticular tissue has a high cellular content and is a special type of connective tissue that is predominantly observed in various places. Reticular fibers have a branched and web-like structure called the reticulum. These fibers are actually type III collagen fibers. Compared to type I collagen, which is abundant in the organism, type III fibers are narrower in diameter and have a high carbohydrate content. They consist of collagen molecules, each of which is a trimer of 3 alpha-1 chains. When examined with transmission electron microscopy (TEM), the fibers consist of aggregations of several transverse bands with a distance of 68 nm between two adjacent aggregations (Gelse, 2003; Kumar et al, 2015).

Correspondence

Hatice YAREN KULOĞLU

haticeyyaren@gmail.com

Article info

Submission: 23-08-2022

Accepted: 22-12-2022

Online First: 29-12-2022

Publication: 31-12-2022

e-ISSN: 2548-1150

doi prefix: 10.31797/vetbio

- http://dergipark.org.tr/vetbio

How to cite this article

Yaren Kuloğlu H. (2022). Determination of reticular fibers in tissues fixed with sugarcane molasses. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 361-365. https://doi.org/10.31797/vetbio.1164030

Reticular fibers are found in other types of connective tissue as well as reticular connective tissue, but in very small amounts. In fact, the reticular fibers that develop first in the structure of each tissue containing these fibers serve as an important support for the tissue. They support the early synthesized extracellular matrix during wound healing, scar tissue formation and embryonic development. As the fibers mature, most of them are replaced by stronger type I collagen fibers (Pepin et al, 2000). To become histologically visible under the microscope, reticular fibers need special staining procedures as they are weakly stained with hematoxylin-eosin (H&E). These are: Periodic Acid-Schiff (PAS) reaction, Gomori and Wilder silver nitrate staining methods. Reticular fibers are found in connective tissue in endocrine glands, liver, bone marrow, and lymphoid organs. The structure of these tissues and organs all consist of densely branched septa and internal canals. The reticular fibers move deep into the tissue or organ along the path followed by these septa and canals. Therefore, the task of the skeletal structure formed by the reticular fibers is to support the cells and the small canals that circulate between the related tissues and organs. For example, it supports hepatocytes and sinusoids in the liver.

Histological techniques, which cover the processes such as fixation, tissue tracking and staining, basically aim to capture and visualize the relations between intracellular and extracellular tissue sections and various cells at a certain moment. The first stage of this is to fix the form closest to the living state of the tissue to be examined. It is assumed that the visual product obtained as a result of efforts starting from fixation and continuing with paraffin sections and histochemical or immunohistochemical staining, provides the best possible static image of living tissue. Thus, efforts during fixation and tissue processing must keep the changes following the separation of the tissue from the living organism to a minimum, in terms of structural and chemical integrity. The purpose of the fixation is to coagulate or precipitate proteins, lipids, carbohydrates and other substances in the protoplasm, making them resistant to the reagents they will encounter until the microscopic section is prepared. The fixation process protects the tissue from decomposition by autolysis, bacteria or fungi caused by cellular enzymes, inactivates or kills infectious agents, hardens the tissue, and stabilizes the tissue components. The agents used for fixation are chemicals, which are called fixatives. A good fixative should have lethal, penetrating and hardening qualities in the tissue. Fixation is an essential step in obtaining a quality section. However, fixation has also undesirable effects on tissues. These include changes in protein structure, dissolution of tissue components, shrinkage of tissue, and degradation of nucleic acids.

In this study, it was aimed to determine the reticular fibers in tissues fixed with sugarcane molasses and formol using silver staining technique (Gordon Sweet –GS-). The reticular fibers in the tissues fixed with sugarcane molasses were compared with those in the tissues fixed with formol.

MATERIAL and METHOD

Experimental Procedure

In this study, the experimental animals were obtained from Selcuk University Experimental Medicine Application and Research Center (This study was carried out by Selcuk University Experimental Medicine Application and Research Center with the approval of the ethics committee numbered 2017-14). Laboratory studies were carried out in Aksaray University Central Research Laboratory. Liver, spleen, kidney and testicle tissue samples from 4 rats of both genders were used as material in the study. Tissue samples were divided into two parts and fixed in 30% sugarcane molasses

(Group A) and 10% buffered formal-saline (Group B) for 24 hours at room temperature.

Preparation of Fixative Solutions

Group A- 30% sugarcane molasses – the sugarcane molasses purchased was prepared by diluting with distilled water so that the final sugarcane molasses concentration was 30%.

Group B- 10% buffered formaldehyde - 10% buffered formol solution with pH 7 was prepared with 0.1M phosphate buffer. After 24 hours of fixation, tissue samples were blocked in paraffin after washing, dehydration and polishing with known histological techniques. Gordon Sweet (GS) staining method was applied to 5 µm thick sections taken from the blocks. After staining, the preparations covered with entellan were evaluated under the light microscope.

RESULTS

Sugarcane Molasses Solution (30%)

Liver Well stained reticular fibers were observed around the sinusoids and major blood vessels (Figure 1a).

Spleen: Well stained reticular fibers were observed in the capsule and intercellular space (Figure 2a).

Kidney: Palely stained reticular fibers were observed between the capsule, corpusculum renis and tubules (Figure 3a).

Testicle: Very thin and pale staining was observed around the tubulus seminiferus contours (Figure 4a).

Formol Solution (10%)

Liver: Well stained reticular fibers were observed around the sinusoids (Figure 1b).

Spleen: Well stained reticular fibers were observed in the capsule and intercellular space (Figure 2b).

Kidney: Well stained reticular fibers were observed between the capsule, corpusculum renis and tubules (Figure 3b).

Testicle: Well stained reticular fibers were observed around the tubulus seminiferus contours (Figure 4b).

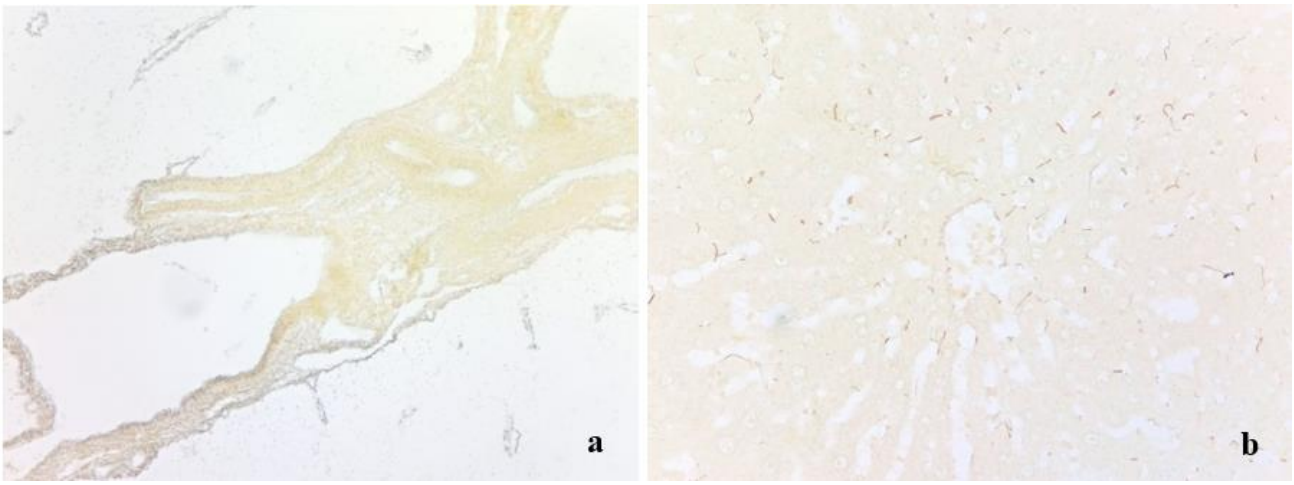


Figure 1. Liver, Gordon Sweet (GS) staining method. a) Tissue fixed with sugarcane molasses solution (30%), b) Tissue fixed with formol solution (10%).

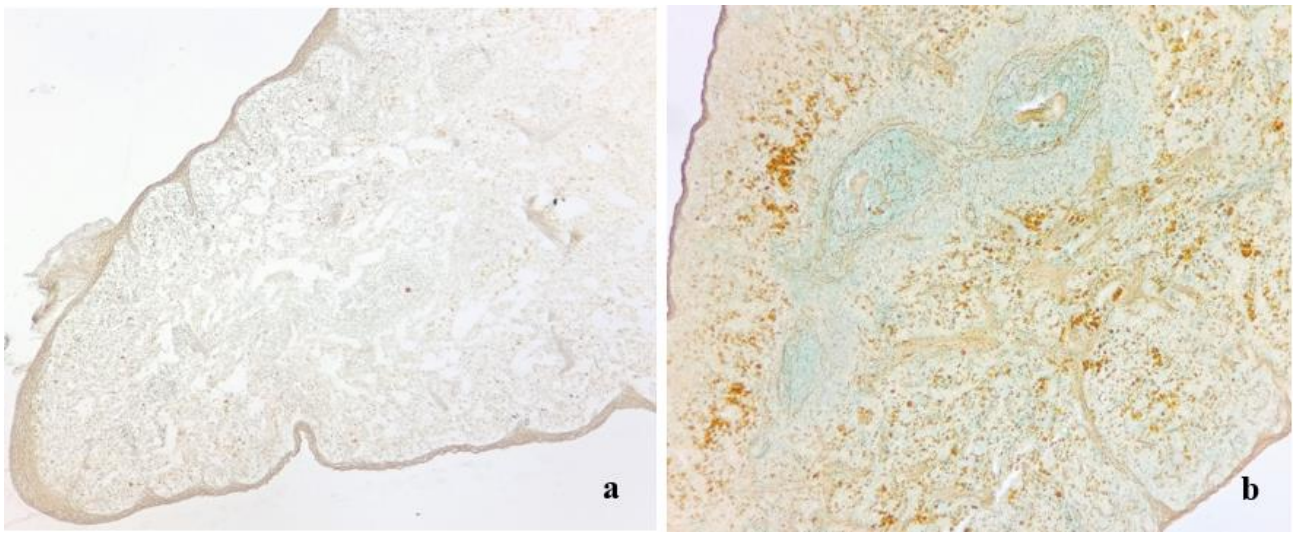


Figure 2. Spleen, Gordon Sweet (GS) staining method. a) Tissue fixed with sugarcane molasses solution (30%), b) Tissue fixed with formol solution (10%).

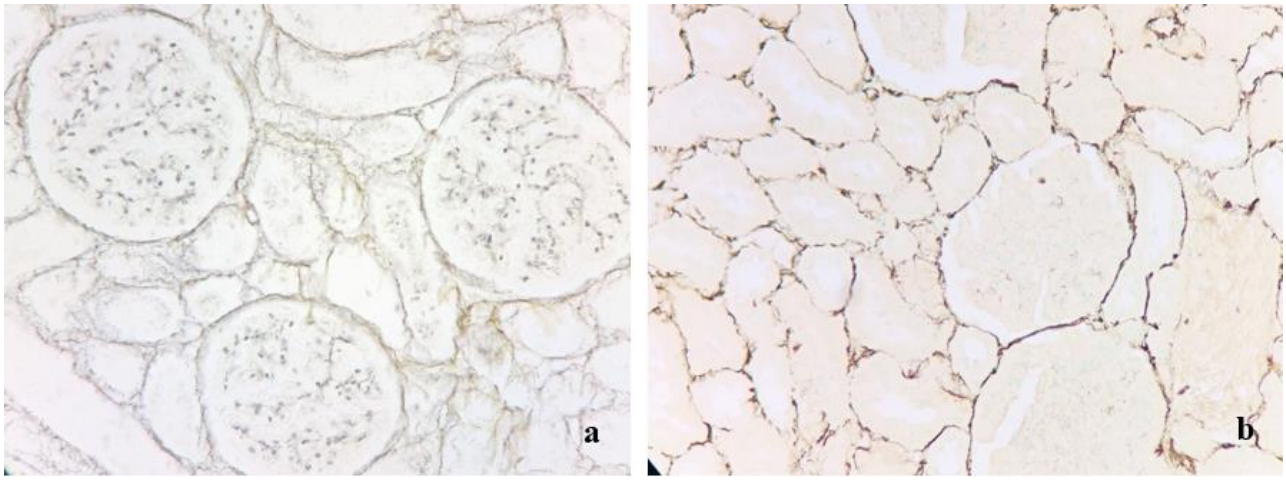


Figure 3. Kidney, Gordon Sweet (GS) staining method. a) Tissue fixed with sugarcane molasses solution (30%), b) Tissue fixed with formol solution (10%).

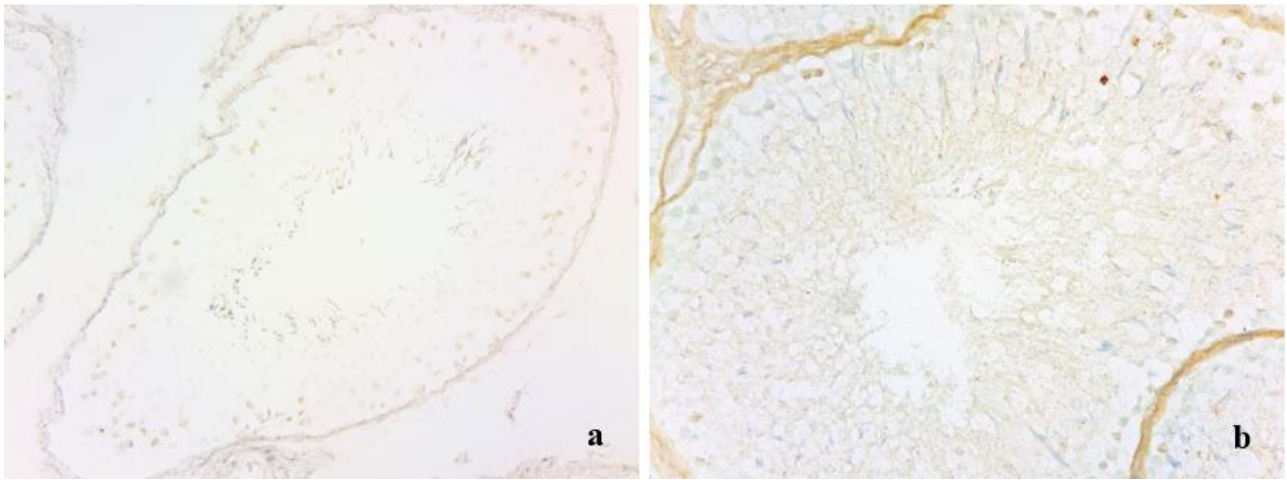


Figure 4. Testicle, Gordon Sweet (GS) staining method. a) Tissue fixed with sugarcane molasses solution (30%), b) Tissue fixed with formol solution (10%).

DISCUSSION

Fixation is an initial and important step in preparing tissue for microscopic examination. The main purpose of fixation is to keep the tissues in the closest form to their living state, to prevent bacterial destruction, to prevent autolysis, and to increase the index of better examination of the tissue. In this study, the staining qualities of reticular fibers in the tissues fixed with sugarcane molasses were compared with those in the tissues fixed with formol by using Gordon Sweet (GS) staining method.

CONCLUSION

According to the findings, the staining quality of the liver and spleen tissues fixed with sugarcane molasses showed similar characteristics with the tissues fixed with formol. Very weak staining was observed in kidney and testicle tissues. The fact that the tissues fixed in the same fixation solution show different results in the Gordon Sweet (GS) staining method shows us that this issue needs to be supported by more detailed studies.

ACKNOWLEDGMENT

This study has been supported by Research Fund of the Aksaray University. Project Number: 2018-021.

Ethical approval:

This study was carried out by Selcuk University Experimental Medicine Application and Research Center with the approval of the ethics committee numbered 2017-14.

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

REFERENCES

Alvarenga, H. G. and Marti, L. (2014). Multifunctional Roles of Reticular Fibroblastic Cells: More Than Meets the Eye?, Journal of Immunology Research, Vol. 10, pp 11-15

Gelse, K. (2003). Collagens-structure, function, and biosynthesis, Advanced Drug Delivery Reviews, Vol. 55, No.12, pp 1531-1546

Kumar, V., Abbas, K. A., Aster, C. J. Robbins and Cotran, Pathologic Basis of Disease, 9th edition, Elsevier Saunders

Mescher Anthony L. Junqueira's Basic Histology: Text and Atlas, 13th edition, McGraw-Hill Education

Pepin, M. et al. (2000). Clinical and Genetic Features of Ehlers–Danlos Syndrome Type IV, the Vascular Type, New England Journal of Medicine, Vol. 342, No. 10, pp 673-680

Ross, M. H. and Pawlina, W. Histology: A text and atlas: With Correlated Cell and Molecular Biology, 6th edition, Lippincott Williams & Wilkins

Williams, P. A. (1947). Creative Reading, The English Journal, Vol. 36, No. 9 (Nov, 1947), pp 454-459

Experimental intravaginal and intrauterine endometritis model: which model is more useful?

ABSTRACT

This study aims to compare the newly created intravaginal endometritis model (IVM) with the intrauterine endometritis model (IUM). *E. coli* infusion was used as intravaginally for IVM and intrauterinally for IUM model. The animals were exeuted on the 7th day. Histopathological and biochemical analyses [malondialdehyde (MDA), glutathione (GSH), Endocan, Endoglin] were performed. A significant inflammation was determined in IVM and IUM compared to the control. A significant decrease in GSH and a significant increase in MDA and Endoglin were determined in IVM and IUM compared to the control. There was a statistically significant increase in the IUM and a numerical increase in the IVM compared to the control. Endometritis was determined by histopathological and biochemical analyses in both IUM and IVM model. It is suggested that intravaginal administration, which is easier to perform, can be used in experimental endometritis model studies.

Keywords: Endometritis, GSH, MDA, Endocan, Endoglin

INTRODUCTION

Endometritis is a widespread gynecological disease characterized by inflammation of endometrial glandular and stromal tissues (Demirel et al., 2019) with mainly resulting in infertility in human and animals. Pelvic inflammatory disease is a clinical infection that results in inflammation of the upper female reproductive tract, including the tube, ovaries, and uterus. Anaerobic gram-negative bacteria generally cause infection. The most common etiological agents are *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Staphylococcus aureus* (Cohen et al., 2002; Ness & Brooks-nelson, 2000; Wiesenfeld & Paavonen, 2010). Endometritis not only causes infertility in farm animals but also causes serious economic losses by affecting milk yield and reproductive performance (Adnane et al., 2017; Demirel et al., 2019; Salah & Yimer, 2017; Sheldon & Dobson, 2004) whereas infertility is the ultimate consequence of the disease in humans (Demirel et al., 2019). Therefore, affecting fertility is important for both human and animal health. Inflammation in the endometrium may cause infertility by preventing the formation of a new cycle. It can also cause symptoms systemically due to its microbial causes (Salah & Yimer, 2017).

How to cite this article
Makav, M., Kuru, M., Beşeren, H., Adalı, Y., Coşkun, MR., Eroğlu HA. (2022). Experimental intravaginal and intrauterine endometritis model: which model is more useful? *Journal of Advances in VetBio Science and Techniques*, *7*(3), 366-375.
https://doi.org/10.31797/vetbio.1147776

Research Article

Mustafa Makav[1a]
Mushap Kuru[2b]
Hatice Beşeren[3c]
Yasemen Adalı[4d]
Mustafa Reha Coşkun[5e]
Hüseyin Avni Eroğlu[6f]

[1]Department of Physiology, Faculty of Veterinary Medicine, Kafkas University, Kars Türkiye
[2]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Kafkas University, Kars Türkiye
[3]Department of Pathology, Health Research and Application Hospital, Kafkas University, Kars Türkiye
[4]Department of Pathology Faculty of Medicine, İzmir University of Economics İzmir, Türkiye
[5]Kafkas University, Faculty of Veterinary Medicine, Department of Microbiology, Kars Türkiye
[6]Department of Physiology, Faculty of Medicine, Çanakkale Onsekiz Mart University, Çanakkale, Türkiye

ORCİD-
[a]0000-0003-1879-8180
[b]0000-0003-4409-251X
[c]0000-0002-4780-540X
[d]0000-0002-8004-7364
[e]0000-0002-1441-3995
[f]0000-0002-1040-3255

Correspondence
Mustafa MAKAV
mustafamakav@gmail.com

Article info
Submission: 24-07-2022
Accepted: 29-12-2022
Online First: 30-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

The bacterial endotoxin-induced uterine infection affects fertility both locally and systemically in the postpartum period. The inflammation causes disruptions in the production of prostaglandin (PG) F2α and PGE2 in the endometrium. However, a change occurs in immune system mediators and cytokine production that may affect the function of the hypothalamus and pituitary (Herath et al., 2009; Sheldon et al., 2009). Detection of bacterial endotoxin lipopolysaccharide in follicular fluid is an indicator that uterine infection adversely affects follicular growth and function (Sheldon et al., 2002). In addition, uterine infection in farm animals causes prolongation of luteal activity and a longer interpregnancy interval (Mohammed et al., 2019).

Physiological reactive oxygen species (ROS) plays an important role in regulating many reproductive processes such as folliculogenesis, oocyte maturation, implantation, embryogenesis, and fetal-placental development. Endometriosis is associated with chronic inflammation and ROS are proinflammatory mediators that modulate cell proliferation (Ngô et al., 2009). As is known, oxidative stress during the formation of endometritis or genital tract inflammation causes pathophysiological disturbance and imbalance in the production of ROS. Therefore, ROS can directly or indirectly damage macromolecules in cases of inflammation (Demirel et al., 2019; Kuru et al., 2018).

Endoglin (CD105) is a transmembrane glycoprotein strongly expressed in several cell types such as macrophages, fibroblasts, and syncytiotrophoblasts, especially vascular endothelial cells (Mitsui et al., 2013). Endoglin dissolved in the plasma is found in the systemic circulation. Circulating endoglin and placental endoglin expression have been reported to be higher in patients with preeclampsia compared to normal pregnancies (Hawinkels et al., 2010). Endoglin expression is known to occur in many types of cancer such as breast, colorectal, and prostate cancers. In addition, increased circulating endoglin levels in colorectal, breast, and other tumors have been reported in studies. It is also known to increase in uterine infections (Mitsui et al., 2013).

Endocan (endothelial cell-specific molecule-1, ESM-1) is a chondroitin/dermatan sulfate proteoglycan specific to vascular endothelial cells that plays a very important role in angiogenesis and inflammation. Serum endocan levels have also been reported to increase in many tumor formations (Laloglu et al., 2017). Serum endocan concentration increases in women with endometriosis and can predict endometriosis with 93% sensitivity and 61% specificity (Güralp et al., 2020).

Experimental studies on endometritis are frequently conducted in recent years. Intrauterine applications are used in the endometritis rat model and this application method may cause some complications due to the need for surgical intervention. This study aims to compare biochemical and histopathological changes in models of intravaginal or intrauterine *Escherichia coli*-induced rat endometritis.

MATERIAL and METHOD

A 15-day period was given to all rats for the adaptation. The cycle periods of all rats were determined by vaginal cytology before starting the study (Cora et al., 2015) and the rats determined to be in the diestrus period were included in the study groups. Serum progesterone concentration of rats found in diestrus on vaginal cytology was also measured using a commercial kit on the AutoAnalyzer (Roche Cobas C501).

Blood was taken from the tail vein of the rats and centrifuged at 3000 RPM for 5 minutes and serum progesterone concentration was measured on the same day from the obtained sera. Rats with a progesterone level higher than

1 ng/mL were used in the study according to the results.

The study was conducted on 21 albino rats. The rats were divided into three groups, each group containing 7 rats, and they were fed as *ad-libitum* with standard normal rat pellet feed and drinking water for 11 days.

Control: Progesterone (P4) (Progestan®, Koçak Farma) was administered subcutaneously to rats at a daily dose of 16 mg/kg between the 0^{th} and 4^{th} days of the study.

Intravaginal endometritis model (IVM): P4 was administered subcutaneously to rats at a daily dose of 16 mg/kg between the 0^{th} and 4^{th} days of the study. *E. coli* was administered intravaginally at 25 µL and 1×10^5 CFU/rat on the 3^{rd} day (Figure 1).

Intrauterine endometritis model (IUM): P4 was administered subcutaneously to rats at a daily dose of 16 mg/kg between the 0^{th} and 4^{th} days of the study. *E. coli* was administered intrauterinally at 25 µL and 1×10^5 CFU/rat on the 3^{rd} day (Figure 1).

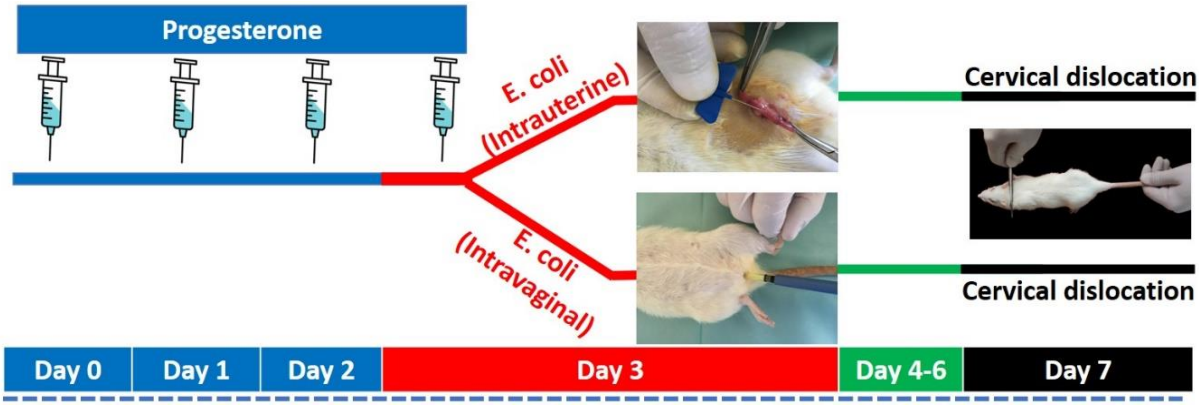


Figure 1. Intravaginal and intrauterine endometritis model protocol

Rats were euthanased at the end of the study (7 days after *E. coli* administration) under anesthesia [ketamine hydrochloride (75 mg/kg) (Ketalar®, Pfizer), and xylazine (15 mg/kg) (Rompun®, Bayer) intramuscular] by cervical dislocation method in accordance with ethical rules. Blood and tissue samples from rats were then taken for biochemical and histopathological analyses. Tissue samples taken for histopathological analysis were placed in 10% formaldehyde. Blood samples were centrifuged at 3000 RPM for 5 minutes and separated. Serum samples were stored at -80°C until the day of the analyses.

Preparation of E. coli strain

Escherichia coli ATCC 25922 strain was used in the study. The concentration of the bacteria was determined according to McFarland standards. McFarland 0.5 contains $1.5x10^8$ bacteria/ml (Garcia, 2010). The McFarland 0.5 density bacterial suspension obtained under sterile conditions was diluted to a density of 10^5 bacteria/25 µL with sterile normal saline.

Intravaginal E. coli Infusion

Rats were anesthesized [ketamine hydrochloride (75 mg/kg) and xylazine (15 mg/kg) intramuscular] and then were positioned on supine position. 100 µL adjustable automatic pipette was used for vaginal application. *E. coli* (25 µL, 1×10^5 CFU/rat) was withdrawn by adjusting the pipette at 25 µL. The pipette tip was advanced into the vagina and 25 µL *of E. coli* was slowly injected in front of the cervix. Care was taken to ensure that the pipette tip did not go into the urinary tract while the pipette was moving inside the vagina (Figure 2).

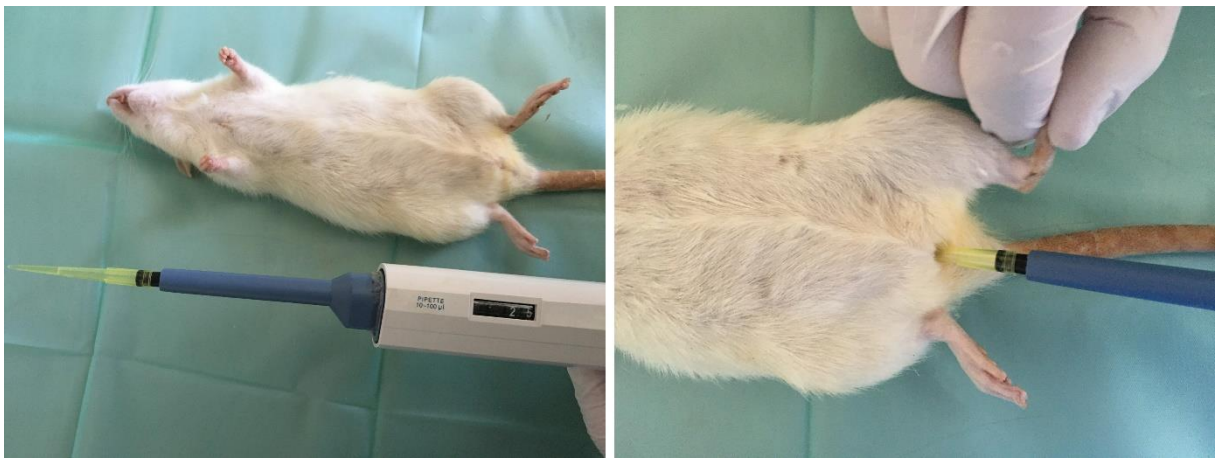


Figure 2. Intravaginal *E. coli* administration

Intrauterine E. coli Infusion

Rats were placed on supine position under ketamine hydrochloride (75 mg/kg) and xylazine (15 mg/kg) intramuscular] anesthesia, the operation area was shaved, and asepsis was achieved. The operation was performed by median incision. The abdominal cavity was reached by cutting the skin, muscle layers, and peritoneum. *E. coli* (25 µL, 1×10^5 CFU/rat) was precisely applied to the left cornu of the uterus by the apparatus attached to the micropipette tip as shown in Figure 3 and made sure that the solution was spread to other cornu. The cornu was returned to the uterine cavity after this procedure. The peritoneum and muscles were stitched with simple continuous sutures and the skin with horizontal U sutures, respectively.

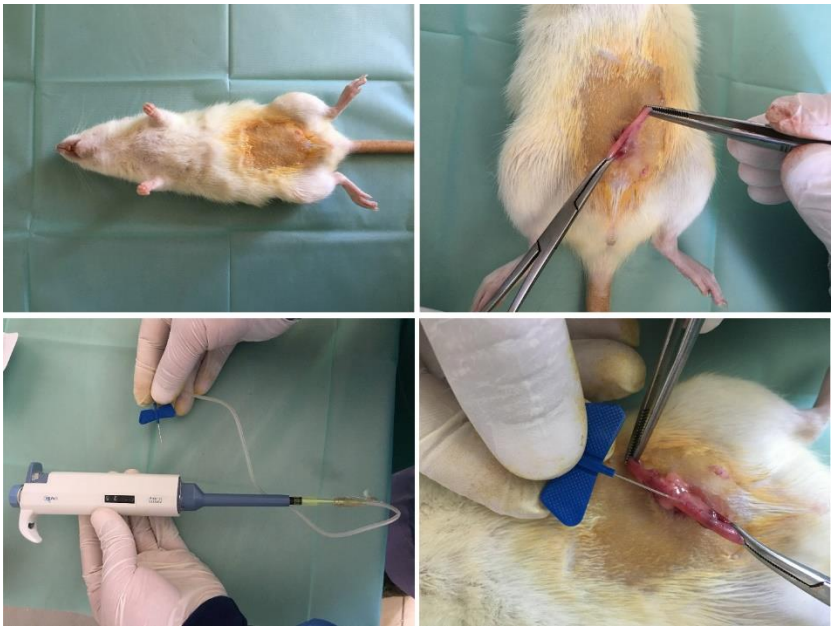


Figure 3: Intrauterine *E. coli* administration

Biochemical Analyses

Antioxidant-oxidant [malondialdehyde (MDA), glutathione (GSH)] and markers of inflammation (Endocan, Endoglin) were determined biochemically. GSH analyses were performed according to the method of Beutler et al. (1963) and MDA analyses were performed according to the method of Yoshoiko et al. (1979). Endocan and endoglin analyses were made using a commercial Enzyme-Linked ImmunoSorbent Assay (ELISA-Elabscience) kit.

Histopathological Analysis

The uterine tissues were kept in 10% buffered formaldehyde solution for 24 hours. The tissues were subjected to tissue processing in an automated device (Leica TP 1020). Sections of 5-µm thickness were taken from the tissues that were manually embedded in paraffin and the sections were stained with hematoxylin & eosin. The stained sections were evaluated under the light microscope (Olympus BX46) blindly for the presence of inflammation. Tissues containing polymorphonuclear leukocytes (PNL) were accepted as active inflammation in the evaluation of two serial cross-sectional areas at 400x magnification. The evaluation of the inflammation is scored by the pathologist subjectively using a two-step scoring system and both glandular and stromal components were considered. In the first step, the inflammation was evaluated by counting the numbers of the inflammatory cells as 0: no inflammation, 1: mild inflammation, 2: moderate inflammation, 3: severe inflammation. In the second step, the inflammation extensity was evaluated by a scoring system 0: no inflammation, 1: mild inflammation, 2: intermediate inflammation, 3: diffuse inflammation. The inflammation score (IS) was calculated by multiplying the scores taken from the first step and the second step (grade X extensity) resulting in a score range 0-9.

Statistical Analysis

The differences in biochemical parameters between the groups were determined through a one-way analysis of variance (ANOVA) method. The results were interpreted using Tukey's HSD test. The significance of the difference between histopathological scored data between the groups was evaluated with the Mann-Whitney U test. GraphPad package program (GraphPad Prism 8/San Diego, CA) was used for statistical analysis. The results were given as mean±standard deviation (SD) and $p<0.05$ was considered statistically significant.

RESULTS

Biochemical results

GSH was affected by *E. coli* infusion in the IVM or the IUM and the serum concentration decreased. There was a statistically significant decrease in GSH concentration in the IVM ($p=0.005$). and IUM ($p=0.03$). compared to the control (IVM-control $p=0.005$, IUM-control $p=0.03$). GSH concentration was not statistically different between IVM and IUM ($p>0.05$) (Figure 4A).

MDA was affected by intravaginal or intrauterine *E. coli* administration. Plasma MDA concentration increased significantly in both the IVM ($p=0.002$) and IUM ($p=0.001$) compared to the control group. MDA concentration was not statistically different between the IVM and IUM ($p>0.05$) (Figure 4B).

Endocan is shown in Figure 4-C and endoglin is shown in Figure 4-D. Both endocan ($p=0.02$) and endoglin ($p=0.03$) were statistically significant in the IUM compared to the control. There was a statistically significant increase in endoglin ($p=0.03$) whereas there was a numerical increase in endocan between the IVM and the control ($p>0.05$).

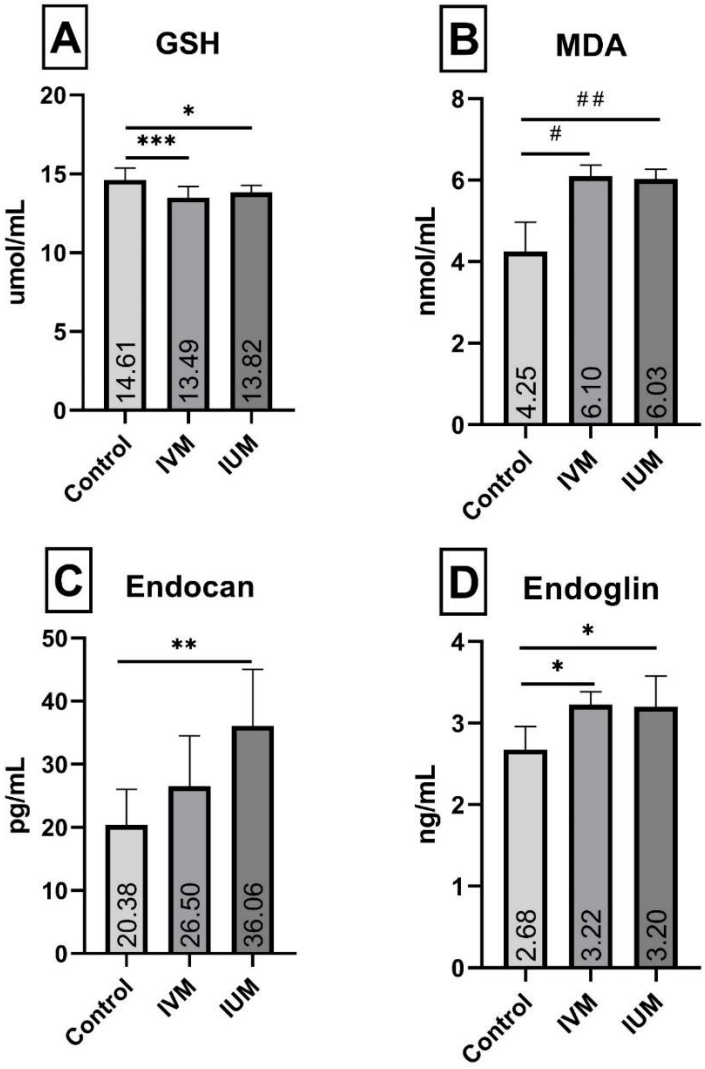


Figure 4. Means + SD of the three groups (A, B, C, D) for biochemical parameters. *p=0,03, **p=0,02, ***p=0,005, $^{\#}$p=0,002, $^{\#\#}$p=0,001

Histopathological results

Table 1 shows the median values of inflammation grade, inflammation extensity, and inflammation scores for the control, IVM, and IUM groups. Figure 5 presents the histological pictures of the groups. Inflammation grade, inflammation extensity, and inflammation score were found to be statistically significantly higher in the IUM compared to the control (p values 0.024, 0.009, and 0.001, respectively). The inflammation grade, inflammation extensity, and the inflammation score were found to be statistically significantly higher in the IVM compared to the control (p values 0.024, 0.024, 0.001, respectively). Finally, no statistically significant difference was found between the IUM and the IVM in terms of inflammation grade, inflammation extensity, and inflammation score (p values 1,000, 1,000, 0.728, respectively).

Table 1. Median values of inflammation grade, inflammation extensity, and inflammation score of control, IVM, and IUM.

Groups	Inflammation grade	Inflammation extensity	Inflammation score
Control	1	1	1
IVM	2	2	3
IUM	2	2	3

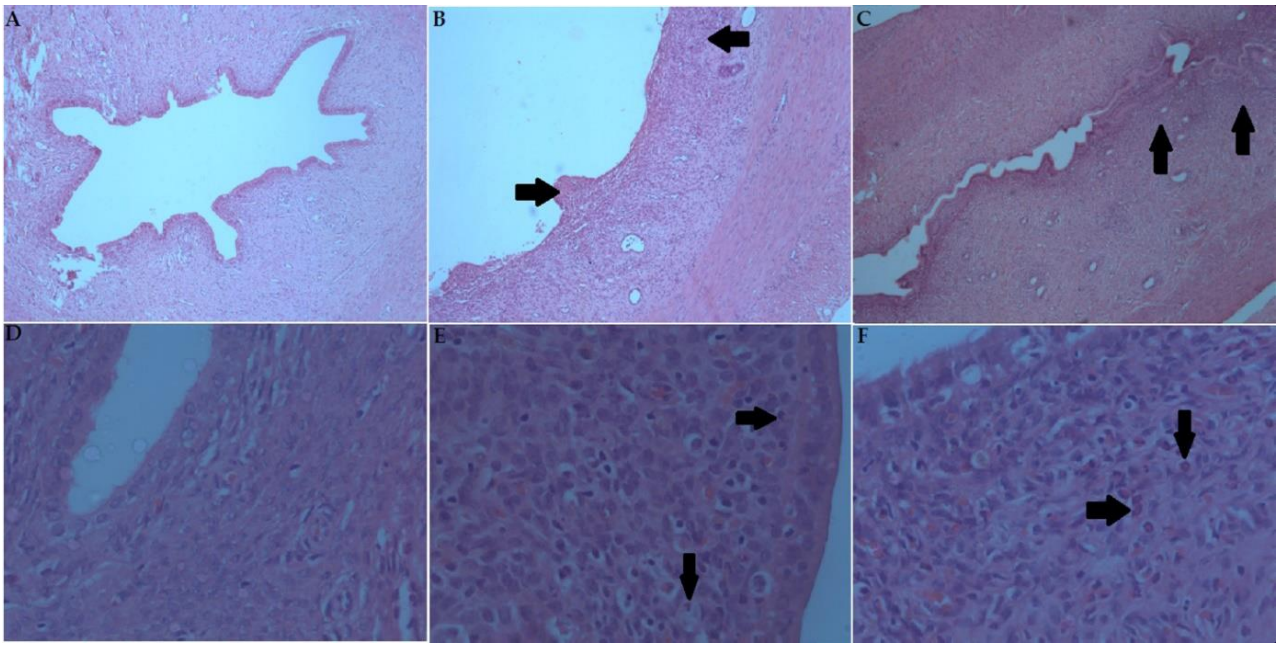


Figure 5. A: Endometrium of control tissue (H&E, 40x), B: Endometrium of intrauterine administration with inflammation, black arrows show inflammation (H&E, 40x), C: Endometrium of intravaginal administration with inflammation, black arrows show inflammation (H&E, 40x), D: Endometrium of control tissue (H&E, 400x), E: Endometrium of intrauterine administration with inflammation, black arrows show inflammatory cells (H&E, 400x), F: Endometrium of intravaginal administration with inflammation, black arrows show inflammatory cells (H&E, 400x).

DISCUSSION

Microorganisms usely detected in the endometrium are common bacteria (Cicinelli et al., 2008, 2009; Kitaya et al., 2017). The host organism can physiologically fight against these infections. Also a wide variety of immunocompetent cells, including macrophages, natural killer cells, and T lymphocyte subsets, infiltrate the endometrium throughout the menstrual cycle under physiological conditions. Endometritis is combated during tissue regeneration. Acute onset of endometritis continues as chronic endometritis unless it is physiologically prevented (Kitaya et al., 2018).

There are many experimental studies with regard to endometritis. Experimental studies with IUM are extensive (He et al., 2008; Nishikawa & Baba, 1985; Tiwari et al., 2018; Xiao et al., 2018). However, the experimental endometritis model with IVM, which is practical and easier in terms of application, is limited (Demirel et al., 2019). Both IVM and IUM were created and compared in our study. IVM is a new protocol and is easier to create than IUM. The IVM reliability was demonstrated by histopathologically evaluating neutrophil infiltration with oxidative stress and inflammation markers as reported previously.

Physiological ROS plays an important role in regulating many reproductive processes such as folliculogenesis, oocyte maturation, implantation, embryogenesis, and fetal-placental development. ROS are proinflammatory mediators that modulate cell proliferation in chronic cases of endometritis (Ngô et al., 2009). ROS may directly or indirectly damage macromolecules by activating signaling pathways (Roberts et al., 2009). ROS increase causes GSH and NADPH depletion and thus, a decrease in plasma level (Franco et al., 2007). GSH level in the IVM and IUM showed a significant decrease compared to the control in our study. The decrease in GSH shows an increase in ROS activity. ROS increase is also known to increase in inflammation and infectious conditions (Franco et al., 2007). This condition supports IVM by showing similar oxidative/antioxidative results

compared to IUM. In addition, an increase in GSH is known in the case of toxicity and inflammation of the uterus and ovary (Kaygusuzoglu et al., 2018). One of the ROS species is hydrogen peroxide (H_2O_2). H_2O_2 is a more stable compound than ROS. Therefore, it plays an important role in cell signaling, especially in response to stress. Hydroxyl radicals produced by H_2O_2 at high levels attack polyunsaturated fatty acids in the membranes. Thus, it causes lipid peroxide formation, which leads to widespread damage and eventually membrane fragmentation. Lipid peroxides decompose to form MDA, often used as an analytical marker of lipid peroxidation. An increase in MDA level is formed due to lipid peroxidation (Mazaheri-Tirani & Madadkar Haghjou, 2019). Our study found a statistically significant increase in both IVM and IUM compared to the control. This increase may be due to lipid peroxidation. The increase in both methods suggests that there is no difference between the two methods and supports IVM as a reliable model. Another study reported an increase in MDA levels in uterine and ovarian injuries (Kaygusuzoglu et al., 2018) as was also the case in our study.

Endocan-specific (endothelial cell-specific) molecule 1 (ESM-1) is a proteoglycan secreted by vascular endothelial cells (Sarrazin et al., 2006). It plays an important role in regulating the endothelial cell due to inflammation and in the functioning of lymphocytes (Afsar et al., 2014). It can be used as a biomarker for inflammation in many diseases (Balta et al., 2015; Sarrazin et al., 2006). The physiopathology of endometritis is characterized by inflammation of endometrial glandular and stromal tissues (Cohen et al., 2002). Our study has shown an increase in inflammation-related endometritis levels in our models. Therefore, it is predicted that there may be a relationship between endometritis and endocan concentration and it may be a biomarker of genital tract infections.

Endoglin is a proliferation-related, hypoxia-induced protein, mostly expressed in angiogenic endothelial cells (Baydın & Akbulut, 2013). Endoglin is expressed at low or even absent in normal endothelial cells whereas it is expressed at high rates in vascular endothelial cells in active local angiogenesis, inflamed tissues, and tumors inside and tumors around during embryogenesis (Ten Dijke et al., 2008). Our study found a statistically high level of inflammation in both IVM and IUM compared to the control. In particular, the fact that IVM was not different from IUM as shown by the results of inflammation due to *E. coli*.

Endometritis is a disease that causes inflammation in uterine tissue due to bacterial infections (Ness & Brooks-nelson, 2000). Demirel et al. (2019) histopathologically showed inflammation in the uterus in the experimentally developed endometritis model. Our study has also determined inflammation in both IVM and IUM. These results proved IVM as usable model.

CONCLUSION

IVM was determined by both biochemical analyses and histopathological analyses. It has been demonstrated biochemically and histopathologically that there is no difference between IVM and IUM. Complications that may occur during the application of IUM may adversely affect the studies to be conducted. The application of the operative method is a difficult method that requires both a surgeon and is time-consuming. In conclusion, both easy procedures in induction and uncomplicated intravaginal administration make IVM more suitable method for an experimental protocol.

ACKNOWLEDGMENT

This study was financially supported by the Scientific Research Projects Coordinatorship of Kafkas University under the project number 2022-TS-13.

Ethical approval:

The experimental procedures were approved by the Kafkas University Animal Experiments Local Ethics Committee before starting the study (KAU-HADYEK 2020-071).

Conflict of interest: The authors declare that they have no competing interests

REFERENCES

Adnane, M., Kaidi, R., Hanzen, C., & England, G. C. W. (2017). Risk factors of clinical and subclinical endometritis in cattle: A review. *Turkish Journal of Veterinary & Animal Sciences*, *41*, 1–11.

Afsar, B., Takir, M., Kostek, O., Covic, A., & Kanbay, M. (2014). Endocan: A new molecule playing a role in the development of hypertension and chronic kidney disease? *The Journal of Clinical Hypertension*, *16*(12), 914–916. https://doi.org/10.1111/jch.12440

Balta, S., Mikhailidis, D. P., Demirkol, S., Ozturk, C., Celik, T., & Iyisoy, A. (2015). Endocan: A novel inflammatory indicator in cardiovascular disease? *Atherosclerosis*, *243*(1), 339–343. https://doi.org/10.1016/j.atherosclerosis.2015.09.030

Baydın, P. Ö., & Akbulut, H. (2013). Designing gene therapy vectors targeting tumor cell endothelium. *TURKISH JOURNAL OF ONCOLOGY*, *28*(4), 179–186.

Beutler, E., Duron, O., & Kelly, B. (1963). Improved method for the determination of blood glutathione. *J. Lab. Clin. Med.*, *61*, 882–888.

Cicinelli, E., De Ziegler, D., Nicoletti, R., Colafiglio, G., Saliani, N., Resta, L., Rizzi, D., & De Vito, D. (2008). Chronic endometritis: Correlation among hysteroscopic, histologic, and bacteriologic findings in a prospective trial with 2190 consecutive office hysteroscopies. *Fertility and Sterility*, *89*(3), 677–684. https://doi.org/10.1016/j.fertnstert.2007.03.074

Cicinelli, E., Ziegler, D. D., Nicoletti, R., Tinelli, R., Saliani, N., Resta, L., Bellavia, M., & Vito, D. D. (2009). Poor reliability of vaginal and endocervical cultures for evaluating microbiology of endometrial cavity in women with chronic endometritis. *Gynecologic and Obstetric Investigation*, *68*(2), 108–115. https://doi.org/10.1159/000223819

Cohen, C. R., Manhart, L. E., Bukusi, E. A., Astete, S., Brunham, R. C., Holmes, K. K., Sinei, S. K., Bwayo, J. J., & Totten, P. A. (2002). Association between mycoplasma genitalium and acute endometritis. *The Lancet*, *359*(9308), 765–766. https://doi.org/10.1016/S0140-6736(02)07848-0

Cora, M. C., Kooistra, L., & Travlos, G. (2015). Vaginal cytology of the laboratory rat and mouse: Review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicologic Pathology*, *43*(6), 776–793. https://doi.org/10.1177/0192623315570339

Demirel, M. A., Han, S., Tokmak, A., Gokay, N. E., Uludag, M. O., Ustun, T. Y., & Cicek, A. F. (2019). Therapeutic effects of resveratrol in Escherichia coli-induced rat endometritis model. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *392*(12), 1577–1589.

Franco, R., Schoneveld, O., Pappa, A., & Panayiotidis, M. (2007). The central role of glutathione in the pathophysiology of human disease. *Archives of Physiology and Biochemistry*, *113*, 234–258. https://doi.org/10.1080/13813450701661198

Garcia, L. (2010). Preparation of routine media and reagents used in antimicrobial susceptibility testing. In *Clinical Microbiology Procedures Handbook* (3rd Edition, p. p 181-201). ASM Press.

Güralp, O., Acikgöz, S., Tüten, N., Ekmekci, H., Schild-Suhren, M., Malik, E., & Tüten, A. (2020). Evaluation of serum endocan levels in endometriosis: A case-control study: Evaluation of serum endocan levels in endometriosis: A case-control study. *La Clinica Terapeutica*, *171*(6).

Hawinkels, L. J. A. C., Kuiper, P., Wiercinska, E., Verspaget, H. W., Liu, Z., Pardali, E., Sier, C. F. M., & Dijke, P. ten. (2010). Matrix metalloproteinase-14 (MT1-MMP)–mediated endoglin shedding inhibits tumor angiogenesis. *Cancer Research*, *70*(10), 4141–4150. https://doi.org/10.1158/0008-5472.CAN-09-4466

He, C., Wu, R., Li, L., Wang, X., Wang, J., Wang, S., Liu, W., & Yang, K. (2008). Establishment of a rat model of endometritis and its treatment with a Chinese-Western medicine emulsion. *Acta Laboratorium Animalis Scientia Sinica*, *3*.

Herath, S., Lilly, S. T., Fischer, D. P., Williams, E. J., Dobson, H., Bryant, C. E., & Sheldon, I. M. (2009). Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F2α to prostaglandin E2 in bovine endometrium. *Endocrinology*, *150*(4), 1912–1920.

Kaygusuzoglu, E., Caglayan, C., Kandemir, F. M., Yıldırım, S., Kucukler, S., Kılınc, M. A., & Saglam, Y. S. (2018). Zingerone ameliorates cisplatin-induced ovarian and uterine toxicity via suppression of sex hormone imbalances, oxidative stress, inflammation and apoptosis in female wistar rats. *Biomedicine & Pharmacotherapy*, *102*, 517–530. https://doi.org/10.1016/j.biopha.2018.03.119

Kitaya, K., Matsubayashi, H., Takaya, Y., Nishiyama, R., Yamaguchi, K., Takeuchi, T., & Ishikawa, T. (2017). Live birth rate following oral antibiotic treatment for chronic endometritis in infertile women with repeated implantation failure. *American Journal of Reproductive Immunology*, *78*(5), e12719. https://doi.org/10.1111/aji.12719

Kitaya, K., Takeuchi, T., Mizuta, S., Matsubayashi, H., & Ishikawa, T. (2018). Endometritis: New time, new concepts. *Fertility and Sterility*, *110*(3), 344–350. https://doi.org/10.1016/j.fertnstert.2018.04.012

Kuru, M., Öğün, M., Kulaksız, R., Kükürt, A., & Oral, H. (2018). Comparison of oxidative/nitrosative stress, leptin and progesterone concentrations in pregnant and non-pregnant abaza goats synchronized with controlled internal drug release application.

Kafkas Üniversitesi Veteriner Fakültesi Dergisi, *24*(6), 887–892.

Laloglu, E., Kumtepe, Y., Aksoy, H., & Yilmaz, E. P. T. (2017). Serum endocan levels in endometrial and ovarian cancers. *Journal of Clinical Laboratory Analysis*, *31*(5), e22079. https://doi.org/10.1002/jcla.22079

Mazaheri-Tirani, M., & Madadkar Haghjou, M. (2019). Reactive oxygen species (ROS), Total antioxidant capacity (AOC) and Malondialdehyde (MDA) make a triangle in evaluation of Zinc stress extension. *Journal of Animal and Plant Sciences*, *29*.

Mitsui, H., Shibata, K., Mano, Y., Suzuki, S., Umezu, T., Mizuno, M., Yamamoto, E., Kajiyama, H., Kotani, T., Senga, T., & Kikkawa, F. (2013). The expression and characterization of endoglin in uterine leiomyosarcoma. *Clinical & Experimental Metastasis*, *30*(6), 731–740. https://doi.org/10.1007/s10585-013-9574-9

Mohammed, Z. A., Mann, G. E., & Robinson, R. S. (2019). Impact of endometritis on post-partum ovarian cyclicity in dairy cows. *The Veterinary Journal*, *248*, 8–13. https://doi.org/10.1016/j.tvjl.2019.03.008

Ness, R., & Brooks-nelson, D. (2000). 29—Pelvic Inflammatory Disease. In M. B. Goldman & M. C. Hatch (Eds.), *Women and Health* (pp. 369–380). Academic Press. https://doi.org/10.1016/B978-012288145-9/50033-4

Ngô, C., Chéreau, C., Nicco, C., Weill, B., Chapron, C., & Batteux, F. (2009). Reactive oxygen species controls endometriosis progression. *The American Journal of Pathology*, *175*(1), 225–234. https://doi.org/10.2353/ajpath.2009.080804

Nishikawa, Y., & Baba, T. (1985). Effects of ovarian hormones on manifestation of purulent endometritis in rat uteruses infected with Escherichia coli. *Infection and Immunity*, *47*(1), 311–317.

R**oberts, R. A., Laskin, D. L., Smith, C. V., Robertson, F. M., Allen, E. M. G., Doorn, J. A., & Slikker, W. (2009).** Nitrative and oxidative stress in toxicology and disease. *Toxicological Sciences*, *112*(1), 4–16. https://doi.org/10.1093/toxsci/kfp179

Salah, N., & Yimer, N. (2017). Cytological endometritis and its agreement with ultrasound examination in postpartum beef cows. *Veterinary World*, *10*(6), 605–609. https://doi.org/10.14202/vetworld.2017.605-609

Sarrazin, S., Adam, E., Lyon, M., Depontieu, F., Motte, V., Landolfi, C., Lortat-Jacob, H., Bechard, D., Lassalle, P., & Delehedde, M. (2006). Endocan or endothelial cell specific molecule-1 (ESM-1): A potential novel endothelial cell marker and a new target for cancer therapy. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, *1765*(1), 25–37. https://doi.org/10.1016/j.bbcan.2005.08.004

Sheldon, I. M., Cronin, J., Goetze, L., Donofrio, G., & Schuberth, H.-J. (2009). Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. *Biology of Reproduction*, *81*(6), 1025–1032.

Sheldon, I. M., & Dobson, H. (2004). Postpartum uterine health in cattle. *Animal Reproduction Science*, *82–83*, 295–306. https://doi.org/10.1016/j.anireprosci.2004.04.006

Sheldon, I., Noakes, D., Rycroft, A., Pfeiffer, D., & Dobson, H. (2002). Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. *Reproduction (Cambridge, England)*, *123*, 837–845. https://doi.org/10.1530/rep.0.1230837

Ten Dijke, P., Goumans, M.-J., & Pardali, E. (2008). Endoglin in angiogenesis and vascular diseases. *Angiogenesis*, *11*(1), 79–89.

Tiwari, A., Singh, P., Jaitley, P., Sharma, S., Prakash, A., Mandil, R., Choudhury, S., Gangwar, N. K., & Garg, S. K. (2018). Eucalyptus robusta leaves methanolic extract suppresses inflammatory mediators by specifically targeting TLR4/TLR9, MPO, COX2, iNOS and inflammatory cytokines in experimentally-induced endometritis in rats. *Journal of Ethnopharmacology*, *213*, 149–158. https://doi.org/10.1016/j.jep.2017.10.035

Wiesenfeld, H., & Paavonen, J. (2010). 6—Pelvic Inflammatory Disease. In S. A. Morse, R. C. Ballard, K. K. Holmes, & A. A. Moreland (Eds.), *Atlas of Sexually Transmitted Diseases and AIDS (Fourth Edition)* (pp. 94–110). W.B. Saunders. https://doi.org/10.1016/B978-0-7020-4060-3.00006-5

Xiao, H.-B., Sui, G.-G., Lu, X.-Y., & Sun, Z.-L. (2018). Elevated levels of ADMA are associated with lower DDAH2 and higher PRMT1 in LPS-Induced endometritis rats. *Inflammation*, *41*(1), 299–306. https://doi.org/10.1007/s10753-017-0687-1

Yoshioka, T., Kawada, K., Shimada, T., & Mori, M. (1979). Lipid peroxidation in maternal and cord blood and protective mechanisms. *Am J Obstet Gynecol*, *135*, 972–976.

The influence of blood, seminal plasma testosterone, growth hormone and cortisol levels on the sperm quality in merino rams

ABSTRACT

The research was conducted on 10 Merino rams to determine the effect of testosterone, growth hormone and cortisol levels on the sperm quality. Beginning in January, blood samples were taken from the jugular veins of rams every month, centrifuged for 20 min at 3000 rpm and obtained plasma. On the days the blood samples were taken, the semen samples which were collected with an artificial vagina, were divided between two eppendorf tubes, one of which was used for the determination of the semen characteristics, while the other was centrifuged to obtain seminal plasma. A significant relationship was detected between blood and seminal plasma levels of testosterone ($P<0.01$). No growth hormone (GH) on a measurable level was detected in the seminal plasma, and it was determined that plasma GH levels, just as testosterone levels began to increase with the approach of the reproductive season, and peaked at the start of the season. Plasma and seminal plasma cortisol levels, on the other hand, while increasing before the season, decreased significantly at the start of the season, and a remarkable correlation between plasma and seminal plasma levels was ascertained ($P<0.01$). An important negative relation which was observed between plasma GH levels and seminal plasma cortisol levels in Merino rams. It was found out that the hormones that were examined did not have a significant influence on sperm qualities, but there was an important negative relation only between the plasma GH levels and sperm volumes in the Merino ($P<0.01$). In conclusion, it may be stated that in rams, especially plasma testosterone and cortisol levels determine the plasma levels; both plasma and seminal plasma testosterone levels increase to a significant degree in the reproductive season. It can be said that there is no significant relationship between semen quality and plasma and seminal plasma hormone levels.

Keywords: Cortisol, Growth Hormone, Sperm, Testosterone.

INTRODUCTION

In all living beings, sexual activity and reproduction are healthy maintained under the effect of hormones and several other factors. Among the domestic livestock, sexual activity in sheep in particular follows a seasonal course, and after the months of August and September when the days are shorter, the hormone profiles of animals change and they enter the sexual cycle. As the days shorten, melatonin production in sheep stimulates the biosynthesis and release of gonadotropins from the anterior lobe of the pituitary gland, gonadotropins activate the ovaries and the sexual cycle begins (Boland et al., 1985; Hamidi et al., 2012). Despite the fact that rams do not display a precise seasonal cycle, producing semen throughout the year, it has been hypothesized that they experience changes in hormone profiles, testicular weight and diameter, and semen characteristics, and this has been proven with subsequent studies (Abecia et al., 2012; Belkadi et al., 2017).

How to cite this article
Ergin Eğritağ, H., Özgökçen, N., Harman, H., Güngör, Ş., Öztürk, C., Serpek B. (2022). The influence of blood, seminal plasma testosterone, growth hormone and cortisol levels on the sperm quality in merino rams. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 376-384. https://doi.org/10.31797/vetbio.1195892

Research Article

Hale Ergin Eğritağ[1a]
Nagehan Özgökçen[2b]
Halil Harman[3c]
Şükrü Güngör[4d]
Caner Öztürk[5e]
Behiç Serpek[6f]

[1]Department of Biochemistry, Faculty of Veterinary Medicine,Mehmet Akif Ersoy University, Burdur,Türkiye
[2]Department of Biochemistry, Faculty of Veterinary Medicine, Selcuk University, Konya,Türkiye
[3]Bahri Dağdaş International Agricultural Research Institute, Konya,Türkiye
[4]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur,Türkiye
[5]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Aksaray University, Aksaray,Türkiye
[6]Department of Biochemistry, Faculty of Veterinary Medicine, Selcuk University, Konya,Türkiye

ORCİD-
[a]0000-0003-4240-4698
[b]0000-0002-3930-9860
[c]0000-0002-3761-5602
[d]0000-0003-3460-522X
[e]0000-0003-0566-0684
[f]0000-0001-6817-1525

Correspondence
Halil HARMAN
halilharman70@hotmail.com

Article info
Submission: 28-10-2022
Accepted: 29-12-2022
Online First: 30-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

An important point in animals is how the relationship between plasma levels of hormones such as testosterone, growth hormone, cortisol, and seminal plasma levels, affects qualities of sperma. For example, Graves and Eiler (1979), found that exogenous cortisol administration in bulls increased cortisol concentrations in both the plasma and seminal plasma significantly, and that increasing the cortisol concentrations caused an increase in total sperm counts. The number of studies of rams addressing this issue is limited. In the present study, the plasma and seminal plasma concentrations of growth hormone, testosterone and cortisol were analyzed in rams every month for a period of one year, and annual variations in hormone concentrations, relationships between hormone concentrations and the effects of hormones on semen characteristics were determined.

MATERIAL and METHOD

Selection of animals

The study was carried out with healthy 10 Merino rams. All rams were barned in the Research Farm of Selcuk University, Faculty of Veterinary Medicine throughout the study period, and fed ad libitum.

Collection of blood and semen samples

Beginning in January, blood samples were taken from the jugular veins of rams every month, centrifuged for 20 min at 3000 rpm, and kept in the freezer at - 20 °C until the analyses were carried out. On the days the blood samples were taken, the semen samples which were collected with an artificial vagina, were divided between two eppendorf tubes, one of which was used for the determination of the semen characteristics, while the other was centrifuged to obtain seminal plasma, and kept in the freezer at -20 °C until the analyses were carried out.

Hormone analyses

Testosterone, cortisol and growth hormone concentrations were analyzed in the plasma and seminal plasma. The growth hormone analyses were carried out using the sheep growth hormone enzyme immunoassay (EIA) method developed by the Endocrinology Laboratory of Selçuk University Faculty of Veterinary Medicine, Department of Biochemistry (Serpek and Haliloğlu, 2000) while cortisol and testosterone concentrations were analyzed using DRG Cortisol EIA (EIA – 1887) and DRG Testosterone EIA (EIA – 1559) kits developed by DRG Instruments GmbH, Germany Division of DRG International, Inc.

Determination of semen characteristics

In the semen samples that were taken, semen volume, sperm motility, mass motility in the seminal plasma, semen concentration, host values, sperm counts, percentage of normal spermatozoa, percentage of spermatozoa with head defects and the percentage of spermatozoa with other defects were determined.

Mass Movement examination was carried out with 4× microscope lens in 1 drop fresh sperma and spermatozoon groups scored 0 - 5 intervals. The Mass Movement was evaluated done as follows; 0 (-): No movement. 1 point (+): There is very little mass movement. Ripple motion is not observed. 2 points (++): There is a low degree of mass movement and superficial ripple movement. 3 points (+++): Vivid ripple movement with a moderate mass movement. There is some blackening in places. 4 points (++++): Black areas are formed with a strong mass movement, strong wave movements andswirls are visible. 5 points (+++++): Dense black areas are formed with a very strong mass movement. Very strong wave movements and eddies are seen.

For motility examination, sperma was diluated with tris base extender solution to 100 $\times 10^6$ and 1 drop sample was dripped on slide and invastigated in 5 different zones with 20×

lens phase contrast microscope and rates were calculated in percentage value.

Sperm density was carried out with hemocytometric method by using thoma slide and spermatozoon was counted in 1 / 200 diluated sperm in 10 big square with 40× lens microscope. Hayem solution [sodium sulfate (5 grams) sodium citrate (1 gram) mercury chloride (0.5 grams) bidistilled water (200 ml)] was used to dilute the samples taken from native semen. Finally density was calculated with formula that given belove. [Number of spermatozoon / (big square volume × number of square × dilution rate)] × 1000.

Abnormal sperma exemination was carried out with liquid fixation method by using Hancock solution and 300 spermatozoon were observed in 1 drop sample obtained from 1 / 200 diluated sperm with 100× lens microscope and disorder rates were calculated in percentage value.

For HOST (hypo osmotic swelling test) thawed sperm and HOST solution mixture that incubated for 1 hr at 37 °C was examinated 100× lens microscope and counted 300 spermatozoons. Curl tailed ones were evaluated as live and strong.

Statistical analysis

The statistical analysis of the data was performed using the SPSS 21. Because of the abnormal distribution of data Mann – Whitney U Test was performed to determine the statistical significance. Correlation analyses was performed with Spearmen's method.

RESULTS

Hormone concentrations in blood plasma

The plasma testosterone, cortisol and growth hormone concentrations of rams, and variations in the concentrations by month, are presented in Table 1. The plasma testosterone concentrations, which were low in the spring months, started to increase gradually from August, and concentrations remained high until December. The plasma cortisol concentrations were low in the winter months, but increased in the spring, and it was observed that concentrations decreased significantly as the reproductive season began (Table 1). The mean plasma growth hormone concentrations, which were 7-8 ng/ml in January, started to decrease significantly in the Merino samples in June but bounced back significantly to 12-13 ng/ml in the in July. (Table 1).

Table 1. Plasma hormone levels in merino rams, ng/ml (n=10)

M	1	2	3	4	5	6	7	8	9	10	11	12
T	2.92 ± 0.89	3.19 ± 1.3	2.58 ± 1.42	3.99 ± 0.85	3.22 ± 0.95	2.51 ± 0.82	1.76 ± 0.51	6.35 ± 2.21	9.03 ± 2.34	9.09 ± 2.03	10.36 ± 1.96	6.67 ± 1.97
C	9.04 ± 1.97	16.72 ± 2.05	13.83 ± 3.9	36.27 ± 6.37	16.54 ± 4.25	21.4 ± 4.81	17.88 ± 4.99	26.54 ± 10.8	9.41 ± 2.6	8.13 ± 2.46	14.66 ± 2.52	13.2 ± 5.08
GH	8.01 ± 0.86	7.03 ± 0.94	8.23 ± 1.82	8.92 ± 0.9	6.52 ± 0.54	4.86 ± 0.45	13.43 ± 0.8	13.23 ± 0.81	15.33 ± 1.17	8.67 ± 0.74	5.66 ± 0.97	6.57 ± 0.39

(M: Months, T: Testosterone, C: Cortisol, GH: Growth Hormone)

Hormone concentrations in the seminal plasma

No growth hormone was detected at any measurable concentration in the seminal plasma. In Merino rams, the testosterone concentration in the seminal plasma, which was 8.01 ± 0.86 ng/ml in January, showed fluctuations, but the concentrations were maintained until June, after which they started to increase, reaching a peak in October (15.33 ± 1.17 ng/ml) (Table 2).

Table 2. Seminal plasma hormone levels in merino rams, ng/ml

M	1	2	3	4	5	6	7	8	9	10	11	12
T	8.01 ± 0.86	7.03 ± 0.94	8.23 ± 1.82	8.92 ± 0.9	6.52 ± 0.54	4.86 ± 0.45	13.43 ± 0.8	13.23 ± 0.81	15.33 ± 1.17	8.67 ± 0.74	5.66 ± 0.97	6.57 ± 0.39
C	9.11 ± 2.69	7.76 ± 2.01	7.37 ± 2.62	21.3 ± 9.18	8.91 ± 2.55	15.9 ± 5.49	9.49 ± 3.61	10.8 ± 5.09	1.78 ± 0.61	2.62 ± 0.58	5.27 ± 1.32	3.54 ± 1.16
n	10	10	10	10	9	10	9	7	8	7	5	6

(M: Months, T: Testosterone, C: Cortisol, n: Number of animals)

Despite the fact that the cortisol concentrations in the seminal plasma only showed a statistically significant bounce in April and June. The seminal plasma cortisol levels decreased significantly in September. (Table 2).

Semen characteristics

Among the semen characteristics, semen volume showed high monthly fluctuations in the 1-year period in Merino rams, while the lowest volumes were observed in July and the highest volumes were observed in December. The volumes noted in February, May, June and December were significantly higher ($P < 0.05$) than in the other months. (Table 3).

Table 3. Some semen charasteristics of merino rams.

M	1	2	3	4	5	6	7	8	9	10	11	12
SA (ml)	1.31 ± 0.16	1.72 ± 0.2	1.35 ± 0.15	1.26 ± 0.14	1.66 ± 0.11	1.64 ± 0.11	1.06 ± 0.18	1.2 ± 0.42	1.28 ± 0.13	1.31 ± 0.16	1.4 ± 0.26	1.92 ± 0.25
MM	2.0 ± 0.2	2.1 ± 0.2	2.3 ± 0.3	2.6 ± 0.3	2.8 ± 0.2	2.2 ± 0.3	2.6 ± 0.3	2.0 ± 0.5	2.3 ± 0.3	2.1 ± 0.3	2.6 ± 0.4	2.17 ± 0.4
SD ($\times 10^6$)	1334 ± 223.2	1860 ± 335.5	1505 ± 190.2	2280 ± 283	1850 ± 161.8	1995 ± 230.9	2189 ± 61.1	1600 ± 305.2	2425 ± 302.7	1644 ± 160.4	2030 ± 133.6	2600 ± 216.4
SM (%)	76.5 ± 2.36	77 ± 2.38	76.1 ± 3.05	81.0 ± 2.08	80.0 ± 2.35	75.5 ± 2.41	80.0 ± 1.67	78.3 ± 2.79	77.5 ± 2.99	73.9 ± 2.64	81.0 ± 5.51	79.17 ± 2.66
HV (%)	77.5 ± 1.64	77.5 ± 2.01	78.0 ± 2.26	81.0 ± 2.08	73.3 ± 3.73	67.0 ± 2.94	80.0 ± 1.67	80.0 ± 2.37	78.1 ± 2.83	76.2 ± 2.45	82.0 ± 1.23	79.17 ± 3.0
NSR (%)	77.2 ± 2.19	77.2 ± 1.14	76.0 ± 1.46	76.5 ± 2.25	76.4 ± 2.49	76.1 ± 1.89	78.9 ± 1.87	76.3 ± 2.81	74.88 ± 2.75	76.88 ± 1.68	76.6 ± 2.21	76.3 ± 2.40
HDR (%)	6.20 ± 2.62	7.10 ± 1.66	7.30 ± 1.42	5.20 ± 2.04	6.89 ± 3.26	5.80 ± 2.62	4.67 ± 2.55	8.33 ± 1.97	6.0 ± 2.27	6.88 ± 1.55	7.20 ± 1.64	6.67 ± 0.52
ODR (%)	16.60 ± 2.10	15.70 ± 1.53	16.70 ± 1.09	18.70 ± 1.68	16.67 ± 2.54	17.1 ± 2.04	16.89 ± 1.07	17.0 ± 2.61	19.38 ± 2.21	16.25 ± 1.17	16.2 ± 2.04	17.0 ± 2.27
n	10	10	10	10	9	10	9	7	8	7	5	6

(M: Months, SA: Semen Amount, MM: Mass Movement, SD: Sperma Density, SM: Sperma Motility, HV: Host Value, NSR: Normal Spermatozoon Rate, HDR: Head Disorder Rate, ODR: Other Disorders Rate, n: Number of animals)

The highest mass motility in sperma was observed in April and May (2.60 ± 0.30 and 2.80 ± 0.20), and for one year the values fluctuated between 2.00 ± 0.20 and 2.80 ± 0.20. No differences were detected in terms of the reproductive season or the seasons of the year (Table 3).

Sperm concentrations showed very high fluctuations in Merino rams throughout the year. Concentrations were low in the first

months of the year, but started to increase from April, with the highest concentrations observed in September and December, being significantly higher than the concentrations in January, March, August and October (P < 0.05) (Table 3). The highest sperm motility percentages were observed in April, May, July and November in Merino rams (81 ± 2.08, 80 ± 2,35, 80 ± 1,67 and 81 ± 5.51%, respectively) while the lowest percentage was observed in October (73.88 ± 2.64%) (Table 3). Host values showed a variation similar to sperm motility, with the lowest percentage observed in June (67.00 ± 2.94%) and the highest percentages in April, July, August and November (81 ± 2.08, 80 ± 1.67, 80 ± 2.37 and 82 ± 1.23% respectively), representing a statistically significant difference (P < 0.05) (Table 3). The mean percentage of the normal spermatozoa varied between 74.88 ± 2.75 and 78.89 ± 1.87 in Merino rams, although no significant difference was found between the monthly percentages over the year (Table 3). The mean percentage of spermatozoa with head defects varied between 4.67 ± 2.55 (July) and 8.33 ± 1.97 (August) in Merino rams, with no significant difference noted between the monthly percentages (Table 3). The ratio of spermatozoa with other defects varied between 15.70 ± 1.63 and 19.38 ± 2.21 throughout the year in Merino rams, with no statistically significant difference noted between the monthly percentages (Table 3).

Relationships between analyzed parameters

In Merino rams, a statistically significant negative correlation was found between the plasma concentrations of growth hormone and cortisol (P < 0.01) and, it was found that the same negative correlation continued with seminal plasma cortisol levels. (P<0,05). It has been determined that plasma testosterone levels and plasma cortisol levels significantly affect seminal plasma testosterone and cortisol levels and there is a significant positive relationship between them (P< 0.01). In the examination of the relationship between semen qualities and plasma and seminal plasma hormone levels, a statistically significant difference was determined only between plasma GH levels and semen volume (P<0.05). It was also found that plasma testosterone and cortisol concentrations significantly affected seminal plasma testosterone and cortisol concentrations, with a significant positive correlation (P<0.01). When the semen characteristics and relationships between the plasma and seminal plasma hormone concentrations were analyzed, a statistically significant difference was noted only between plasma growth hormone concentrations and semen volume (P<0.05) (Table 4).

Table 4. Relationships between parameters in merino rams

		Pl.Cor	Pl.GH	SpTes	SpCor	Vol	MM	Mot	Dens.	Host	Normal	Head D.	Other D.
Pl. Tes	r	0.174	0.039	0.325**	0.007	-0.067	-0.1	-0.047	0.085	0.001	-0.01	0.054	0.002
Pl. Cor	r		-.213*	-0.141	0.630**	-0.038	0.04	0.095	0.15	0.064	-0.174	0.07	0.171
Pl. GH	r			0.119	-0.253*	-0.325**	0.07	0.013	0.122	0.124	-0.052	-0.087	0.148
Sp.Tes	r				-0.113	0.021	0	0.029	0.182	-0.03	-0.038	0.035	0.025
Sp.Cor	r					-0,045	0.06	0,16	0.03	0.009	-0.088	0.07	0.053
Vol	r						0.16	0.146	0.004	-0.046	0.128	-0.087	-0.142
MM	r							0.530**	0.291**	0.397**	0.047	0.015	0.001
Mot	r								0.359**	0.316**	0.105	-0.006	-0.075
Dens	r									0.403**	-0.012	-0.068	0.006
Host	r										0.007	0.109	0.025
Normal	r											-0.512**	-0.875**
Head D.	r												0.184
Other D.	r												

(Plasma: Pl, Cor: Cortisol, Tes: Testosterone, GH, Growth Hormone Vol: Volume, MM: Mass Movement, Mot: Motility, Dens: Density, D: Disorder, r: Correlation)

DISCUSSION

In January, being the first month of the study, plasma testosterone concentrations were similar to Zamiri et al. (2010), who indicated a decrease in testosterone concentrations during winter months. As reported by researchers (Zamiri and Khodaei, 2005; Hamidi et al., 2012), testosterone concentrations show fluctuations in rams between the pre-reproductive and reproductive season. Palacios et al. (2016) reported that high plasma cortisol concentrations in sexual activity, when rams kept inside, separate from ewes. Ansari et al. (2017) observed that serum cortisol concentration in antelopes, higher in summer than winter in line with present study findings. The high concentrations of plasma testosterone levels, which were detected until October and November, returned to the concentrations noted in January due to the separation of the rams and ewes (Table 1).

Plasma growth hormone concentrations, which were 8.01 ± 0.86 ng/ml in Merino rams in January, decreased gradually during the spring months, similar to the findings reported in literature with a statistically significant decrease occurring in May (Table 1) and the concentrations started to increase in July with the increase in temperatures, as well as the increase in plasma testosterone concentrations associated with the stress of the beginning of the reproductive season (Bex et al., 1978; Bartlett et al., 1990; King et al., 2005; Kalra et al., 2008). The growth hormone concentrations were statistically significant until September when compared to other months, which was a finding supported by Hamidi et al. (2012) whose findings indicated that growth hormone and testosterone concentrations increased during the reproductive season. In the present study, testosterone concentrations in seminal plasma showed a seasonal variation similar to the figure observed by Casao et al. (2013) and Javed et al. (2000) findings.

Graves and Eiler (1979) reported that plasma and seminal plasma basal cortisol concentrations in bulls (3-4 µ/dl) increased 238 - fold in the plasma and 32 - fold in the seminal plasma 20 min after the administration of 500 mg exogenous cortisol. Ansari et al. (2017) investigated the effect of the summer and winter seasons on epididymal sperm quality in antelopes, and found that heat stress increased serum cortisol concentrations, but no significant variation in T3 and T4 concentrations. Maurya et al. (1990) found that the incorporation of low levels of concentrates (100 g/day) into the forage diets of Malpura rams with low body condition scores (2.5) between March and June caused a decrease in live weights, as well as in seminal plasma cortisol concentrations ($P<0.05$) when compared to rams with medium and high body condition scores, and while semen volume and sperm motility were affected negatively, sperm counts did not change. In the study, the variations in the seminal plasma cortisol concentrations of Merino rams by month were similar to the variations in the plasma cortisol concentrations, and this finding is in line with the findings of Graves and Eiler (1979), who suggested that exogenous cortisol administration in bulls increased cortisol concentrations in both the plasma and seminal plasma, suggesting that blood cortisol passes to the seminal plasma (Table 2).

İleri et al. (1998) reported that the average semen volume, which can vary according to age, season, nutrition, the technical ability of the person taking the sample, the management of and method used for taking the samples, the sampling frequency, the temperament of the ram and the body condition is around 1 ml (0.7 - 3.0 ml). According to the data from the study, the semen volumes taken of the Merino rams were within the normal range, but showed fluctuations, with higher volumes seen in the 2nd and 12th months than in the other months (Table 3). In an analysis of the relationship

between semen volume and hormone concentrations in the plasma and seminal plasma, a significant negative correlation was found only between the plasma growth hormone concentrations ($P<0.05$) (Table 4). Semen with a mass motility score (varying between 0 and 5 in adult rams (Rege et al., 2000; David et al., 2015) higher than 4 can be used for artificial insemination (Alvarez et al., 2012; David et al., 2015), Rege et al. (2000) reported that concentrations in 9 - month old Menz and Horro rams in Ethiopia were 1.92 ± 0.27. In the present study, concentrations were seen to vary between 2.00 ± 0.20 and 2.80 ± 0.20 over one year, with no significant variation noted between months (Table 3). The detected concentrations were lower than those reported by David et al. (2015) and Alvarez et al. (2012), but in line with the figures reported by Rege et al. (2000). This difference was considered to be related to the breed or to regional climatic variances. When the relationships between semen characteristics were analyzed (Table 4), a significant correlation was found between mass motility and sperm motility, semen concentration and host values ($P<0.01$), as could be expected. İleri et al. (1998) reported sperm concentrations of $1 – 4 \times 10^9$/ml in rams, and these concentrations were reported to vary according to the age of the bull (Maurya et al., 2010) and rams, and were affected also by nutritional status, season and melatonin, cortisol, growth hormone and testosterone concentrations in the blood (Bartlett et al., 1990; King et al., 2005; Kalra et al., 2008; Hamidi et al., 2012; Ansari et al., 2017). The lowest concentrations in Merino rams were found in January (1334 ± 223.2) and the highest concentrations in December (2600 ± 216.4 × 10^6/ml), with concentrations were found to be within the normal range throughout the year (Table 3). In Merino rams, sperm motility varied between 75.5% (June) and 81% (May and November) throughout the year, and it was observed that the monthly variations were not significant, with concentrations falling within the normal range determined for healthy animals (Benmoula et al., 2017) (Table 3). Camara et al. (2017) reported host values of 44.6 ± 3.4% shortly after diluting the semen samples of Santa Ines rams. Ömür and Çoyan (2014) found host values of 52 ± 2.6% in semen samples taken from Merino rams during the reproductive season and subjected to freezing and thawing. Akalın et al. (2015) identified host values of 57.0 ± 1.2% after freezing and thawing semen samples from Konya Merino rams. In the present study, the semen samples were not subjected to any processes, and so the host values were lowest in June (67 ± 2.94%), which is outside the reproductive season, and highest in November (82 ± 1.23%), which is within the reproductive season. The percentages were found to be significantly higher during the reproductive season when compared to the months outside the reproductive season (Table 3). Mickelsen et al. (1981) found that the ratio of normal spermatozoa in the semen samples of Suffolk and Lincoln rams, taken during the reproductive season using an electro - ejaculator was 92,8% for both breeds in October, and 77.0% for Suffolk rams and 84.7% for Lincoln rams in November, while Moore (1985) found that the ratio of normal spermatozoa in the semen samples of Romney rams, taken using an artificial vagina, was 79%, and 82% in samples taken using an electro - ejaculator In the present study, the ratio of normal spermatozoa was lowest in September (74.88 ± 2.75%) and highest in July (78.89 ± 1.87%), but the percentages followed a horizontal course. Although these ratios were lower than those reported by Mickelsen et al. (2000) and Moore (1985) in samples taken using an electro - ejaculator, our findings were in line with the ratios in the samples taken using an artificial vagina, as in our study (Table 3).

CONCLUSION

As a result, it could be suggested that testosterone concentrations in blood plasma of Merino rams fed ad libitum for one year increase significantly at the beginning of reproductive season, and are maintained until the end of the year; that testosterone concentrations in the seminal plasma of Merino rams followed a course similar to the testosterone concentrations in the plasma, with a statistically significant correlation ($P < 0.01$) between them; that plasma cortisol concentrations, which were low at the beginning of the year, increased gradually from May, when the weather started to get warmer, due to heat stress, and with the end of heat stress the concentrations started to decrease rapidly and were maintained until the end of the year; that a significant correlation between plasma and seminal plasma concentrations can be seen in Merino rams ($P < 0.01$); that plasma growth hormone concentrations follow a similar course to the reproductive cycle; that the concentrations, which increased significantly as the reproductive season approaches, start to decrease with the beginning of the fall; that no significant correlation exists between hormone concentrations, that there is no significant correlation between plasma and seminal plasma concentrations of hormones and semen characteristics; and that a significant correlation exists among sperm mass motility, semen concentration and host values, as well as a significant correlation between head defects and other defects, as expected. It is considered that the detection of blood testosterone and growth hormone concentrations during the reproductive season could be useful in determining the performance of rams.

ACKNOWLEDGMENT

This study was supported by the Selçuk University Scientific Projects Coordinator (Project Number: 09401085).

Ethical approval:

The necessary board of ethics approval was obtained from the Board of Ethics of the Selçuk University Faculty of Veterinary Medicine (No: 2009/50).

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

REFERENCES

Abecia, J.A., Forcada, F., Gonzalez-Bulnes, A., (2012). Hormonal control of reproduction in small ruminants. *Anim. Reprod. Sci. 130*, 173-179.

Akalın, P.P., Başpınar, N., Çoyan, K., Bucak, M.N., Ataman, M.B., Ömür, A., Bilgili, A., Güngör, Ş., Sarıözkan, S., Öztürk, C., Bodu, M., Acibaeva, B., (2015). Metiyonin ve trehaloz içeren sulandırıcıların koç spermasının dondurma-çözdürme sonrası sperm akrozom bütünlüğü ve biyokimyasal parametreleri üzerine etkileri. *Bahri Dağdas Hay. Ars. Derg.*, 3, 7-15.

Alvarez, M., Tamayo- Canul, J., Anel, E., Boixo, J.C., Mata-Campuzano, M., Martinez-Pastor, F., Anel, L., Paz., P., (2012). Sperm concentration at freezing affects post-thaw quality and fertility on ram semen. *Theriogenology, 77*, 1111-1118.

Ansari, S.P., Singh, H.S., Patel, P., Mishra, A., (2017). Biochemical and hormonal profile of epididymis of buck during winter and summer season. *Indian J. Anim. Res. 51*, 449-452.

Bartlett, J.M., Charlton, H.M., Robinson, I.C., Nieschlag, E., (1990). Pubertal development and testicular function in the male growth hormone-deficient rat. *J. Endocrinol*, *126*, 193-201.

Belkadi S, Safsaf B, Heleili N, Tlidjane, M., Belkacem, L., Oucheriahet, Y., (2017). Seasonal influence on sperm parameters, scrotal measurements, and serum testosterone in ouled djellal breed rams in Algeria. *Vet. World. 10*, 1486-1492.

Benmoula, A., Badi, A., El Fadili, M., EL Khalil, K., Allai, L., (2017). Effect of season on scrotal circumference, semen characteristics, seminal plasma composition and spermatozoa motility during liquid storage in INRA180 rams. *Anim. Reprod. Sci., 180*, 17-22.

Bex, F., Bartke, A., Goldman, B.D., Dalterio, S., (1978). Prolactin, growth hormone, luteinizing hormone receptors, and seasonal changes in testicular activity in the golden hamster. *Endocrinology, 103*, 2069-2080.

Boland, M.P., Al Kamari, A.A., Crosby, T.F., Haynes, N.B., Howles, Kelleher D.L., Gordonet, I., (1985). The influence of breed, season and photoperiod on semen characteristics, testicular size, libido and plasma hormone concentrations in rams. *Anim. Reprod. Sci., 9*, 241-252.

Câmara, D.R., Mello-Pinto, M.M.C., Pinto, L.C., Brasil, O.O., Nunes, J.F., Guerra, M.M.P., (2011). Effects of reduced glutathione and catalase on the kinematics and membrane functionality of sperm

during liquid storage of ram semen. *Small Rumin. Res.*, *100*, 44-49.

Casao, A., Perez, R., Abecia, A., Forcada, F., Muiño-Blanco, T., Cebrián-Pérez, J. Á., (2013). The effect of exogenous melatonin during the non-reproductive season on the seminal plasma hormonal profile and the antioxidant defence system of Rasa Aragonesa rams. *Anim. Reprod. Sci*., *138*, 168-174.

David, I., Kohnke, P., Lagriffoul, G., Praud, O., Plouarboue, F., (2015). Mass sperm motility is associated with fertility in sheep. *Anim. Reprod. Sci.*, *161*, 75-81.

Graves, C.N., Elier, H., (**1979).** Cortisol content of semen and the effect of exogenous cortisol on the concentration of cortisol and minerals (Ca, Mg, K and Na) in semen and blood plasma of bulls. *Biol. Reprod. 21*, 1225-1229.

Hamidi, A., Mamomei, M., Khalil, M., Tabatabaei, S., Hedayat-allah, R., (2012). Correlation between blood growth hormone profile and reproduction performance in Arabic rams. *Comp. Clin. Path. 21*, 819-823.

İleri, İ.K., Ak, K., Pabuççuoğlu, S., Birler, S., (1998). Evcil hayvanlarda reprodüksiyon ve suni tohumlama. İÜ Vet Fak Yayınları, İstanbul Türkiye.

Javed, M.T., Khan, A., Ali, M., (2000). Influence of season on seminal plasma testosterone and oestrogen in healthy and abnormal buffalo bulls and their relationship with other semen parameters. *Vet. Arh.*, *70*, 141-149.

Kalra, S., Kalra, B., Sharma, A., (2008). Growth hormone improves semen volume, sperm count and motility in men with idiopathic normogonadotropic infertility. *Endocrine Abstracts*, *16*, 613.

King, B.J., Da Silva, V.V., Harper, J.D., Scott, C.J., Sillence, M.N., (2005). Equine growth hormone enhances motility and extends longevity of ram spermatozoa in vitro. *Reprod. Fertil. Dev.*, *17*, 85.

Maurya, V.P., Seljan, V., Kumar, D., Naqvi, S.M.K., (2010). Effect of induced body condition score differences on sexual behavior, scrotal measurements, semen attributes and endocrine responses in Malpura rams under hot semi-arid environment. *J. Anim. Physiol. Anim. Nutr*., *94*, 308-317.

Mickelsen, D.W., Paisley, L.G., Dahmen, J.J., (1981). The effects of season on the scrotal circumference and sperm motility and morphology in rams. *Theriogenology*, *16*, 45-51.

Moore, R.W., (1985). A comparison of electro-ejaculation with the artifical vagina for ram semen collection. *N. Z. Vet. J*., *33*, 22-23.

Ömür, A.D., Çoyan, K., (2014). Sezon içi ve sezon dışında koç spermasının dondurulmasında antioksidanların etkisi. *Erciyes Üniv. Vet. Fak. Derg*., *11, 37-42.*

Palacios, C., Alvarez, S., Martín-Gil, J., Abecia, A., (2016). Plasma hormonal levels of rams are affected by sexual activity and confinement in a semen collection centre. *ARC J. Anim. Vet. Sci*. *2*, 15-21.

Rege, J.E., Toe, F., Mukasa-Mugerwa, E., Tembely, S., Anindo, D., Baker, R. L., Kassi, L., (2000). Reproductive characteristics of Ethiopian highland sheep: II. Genetic parameters of semen characteristics and their relationships with testicular measurements in ram lambs. *Small Rumin. Res*., *3*, 173-187.

Serpek, B., Haliloğlu, S., (2000). Development of an enzyme immunoassay forthe determination of ovine growth hormone in plasma. *Turk J. Vet. Anim. Sci. 24*, 163-168.

Zamiri, M.J., Khodaei, H.R., (2005). Seasonal thyroidal activity and reproductive characteristics of Iranian fat-tailed rams. *Anim. Reprod. Sci. 88*, 245-255.

Zamiri, M.J., Khalili, B., Jafaroghli, M., Farshad, A., (2010). Seasonal variation in seminal parameters, testicular size, and plasma testosterone concentration in Iranian Moghani rams. *Small Rumin. Res. 94*, 132-136.

Investigation of antifungal activity mechanisms of alpha-pinene, eugenol, and limonene

Conference Paper

Hatice Büşra Lüleci[1a]
Bengü Ergüden[1b]

[1]Department of Bioengineering, Faculty of Engineering, Gebze Technical University Kocaeli, Türkiye

ORCİD-
[a]0000-0002-7115-7211
[b]0000-0002-8621-3474

Correspondence
Bengü ERGÜDEN
b.sezen@gtu.edu.tr

Article info
Submission: 10-09-2022
Accepted: 29-12-2022
Online First: 30-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

ABSTRACT

Plant essential oils are preferred in cosmetics, medicine, food, and beverage industries for various purposes. α-Pinene is found mainly in eucalyptus oils, eugenol is the active ingredient in clove oil, and limonene is the major component in the oil of citrus fruit peels. In this study, we aimed to determine the antifungal activity of α-pinene, eugenol, and limonene against *Saccharomyces cerevisiae* yeast cells. Besides, we focused on revealing the target side of the compounds on the yeast cells. Firstly, the antifungal activity of compounds was tested via minimum inhibitory concentration (MIC) measurement. After that, we performed a sorbitol effect assay to understand whether it acts on the cell wall or not. With sorbitol, the MIC values were not changed. It means that they are not effective on the yeast cell wall. Then, we measured the extracellular conductivity increase upon treatment with the compounds to understand the effect on the cell membrane. Eugenol and limonene were not changed the extracellular conductivity, and there was no ion leakage from the cell membrane. On the other hand, α-pinene damaged the yeast cell membrane causing a sudden increase in conductivity due to ion leakage. An ergosterol effect assay with α-pinene was performed to detect cell membrane disruption via ergosterol or not. With ergosterol, the MIC value was not changed. α-Pinene must have another target than the ergosterol in the yeast cell membrane. Finally, revealing the mode of action of compounds against yeast cells will provide new insights into their usage in various fields.

Keywords: Antifungal activity, Eugenol, Limonene, Mode of action, α-Pinene, *Saccharomyces cerevisiae*.

INTRODUCTION

Natural compounds are vital sources in medicine for discovering drugs (Harvey et al., 2015)and for the food industry to improve food safety against microbial growth (Burt et al., 2013; Harvey et al., 2015). Several plant essential oils have been studied, and various studies have proven their biological activities, such as anti-inflammatory (Koudou et al., 2005), antiviral (Loizzo et al., 2008), cytotoxic (Zarai et al., 2011), and antimicrobial properties (Alviano & Alviano, 2009). Essential oils including pine, rosemary, lavender, and turpentine can contain pinenes and bicyclic terpenes. They have various chemical structures and biological properties. α-Pinene and β-pinene are two constitutional isomers (Silva et al., 2012). α-Pinene, ((1RS,5RS)-2,6,6-trimethylbi-cyclo [3.1.1] hept-2-ene) with a sum formula of C10H16 is an essential secondary metabolite in many conifer-derived essential oils (Allenspach et al., 2020), and it is an important biologically active natural monoterpene (Wei et al., 2020).

How to cite this article
Lüleci, HB., Ergüden B. (2022). Investigation of antifungal activity mechanisms of alpha-pinene, eugenol, and limonene. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 385-390. https://doi.org/10.31797/vetbio.1173455

Limonene is another cyclic monoterpene, the major component of citrus fruit peels. As a phenolic compound, eugenol is an important essential oil involved in clove oil (Cai et al., 2019). All three biologically active compounds have high industrial and commercial values (Allenspach & Steuer, 2021), and they are widely used in cosmetics, medicines, food industry and fine chemicals (Alonso et al., 2015; Wei et al., 2020) because of their antimicrobial activity against bacterial and fungal cells (Cahyari et al., 2018; Cai et al., 2019; Silva et al., 2012).

There have been many studies on the antibacterial (Allenspach & Steuer, 2021; Bevilacqua et al., 2010; Yang et al., 2015), antifungal (Bevilacqua et al., 2010; Cai et al., 2019; Nóbrega et al., 2021), and antiparasitic (da Franca Rodrigues et al., 2015) activities of α-pinene, limonene and eugenol in recent years. For antifungal activity, Candida species have been mostly used (Nóbrega et al., 2021). However, to our knowledge, studies concerning the antifungal activity of these compounds against *S. cerevisiae* are limited. *S. cerevisiae* is a significant spoilage yeast type for food and beverage industries (Stratford, 2006), and it is also frequently used in studies associated with diseases (Shen et al., 2004). Thus, determining the antifungal activity of the corresponding compounds against *S. cerevisiae* is crucial for various fields.

Natural compounds, especially essential oils, have a various modes of action to inhibit the yeast cells (Konuk & Ergüden, 2017). The cell wall, cell membrane, intracellular protein, mitochondria, DNA, and RNA are prominent and significant target sites of the natural compounds (Burt et al., 2013; Hyldgaard et al., 2012). In this study, we aimed to improve the understanding of the antifungal activity mechanism of the α-pinene, limonene and eugenol against yeast cells for usage in antifungal drugs or food/beverage industries.

MATERIAL and METHOD

Chemicals and yeast strain

D-Glucose, peptone, yeast extract powder, agar powder (HIMEDIA), dimethyl sulfoxide (DMSO), α-pinene, limonene, eugenol, sorbitol, and ergosterol (Sigma-Aldrich) were used in all the experiments. An ultrapure purification system produced distilled water (Thermo Scientific, Smart2Pure Water Purification System). YPH499 (MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1; (Sikorski & Hieter, 1989) was used as the *S. cerevisiae* strain in all the experiments. Cells were grown in a yeast extract peptone glucose (YPD) medium at 28 °C and then precultured in a fresh YPD medium.

Minimum inhibitory concentration (MIC) measurement

The *S. cerevisiae* yeast cells were cultured overnight at 28 °C in a YPD medium and were diluted in the YPD to give a final density of 1×10^6 CFU/mL. Different concentrations of α-pinene, limonene and eugenol were prepared with DMSO, and put in a 24-well microtiter plate. After that, the *S. cerevisiae* cells were added to each well separately for three compounds. The yeast cells suspension without α-pinene, limonene and eugenol were used as a control groups. 24-well microtiter plates were incubated for 48 h at 28 °C. After the incubation, MIC values were detected to be the lowest concentration of compounds completely inhibiting the visual growth of the yeast cells.

Extracellular conductivity measurement

Extracellular conductivity measurement was performed like Konuk et al. (Konuk & Ergüden, 2017). All experiments were carried out in parallel with dH_2O and yeast cells as a negative control and dH_2O, yeast cells, and α-pinene, limonene and eugenol as the experimental groups. 20 mM, and 40 mM for α-pinene; 1 mM, 2 mM, and 4 mM for limonene / eugenol

were added to the cell suspensions at zero point, separately. Extracellular conductivity was recorded every 10 min in the first 60 min with an AD 31 Waterproof EC/TDS tester.

Sorbitol and ergosterol effect assay

Sorbitol and ergosterol effect assays were performed similarly to MIC. After adding α-pinene, limonene, and eugenol, 8 mM sorbitol was added to each well for the sorbitol effect assay (Pereira et al., 2015). Ergosterol effect assay was performed for α-pinene. 200 µg/mL ergosterol was added to each well after adding α-pinene for the ergosterol effect assay (Pereira et al., 2015). Sorbitol and ergosterol effect assays were performed separately, on different plates. 24-well microtiter plates were incubated for 48 h at 28 °C. The lowest concentration of compounds that completely inhibits visual growth of the yeast cells was detected visually for sorbitol and ergosterol effect assays.

Statistical analyses

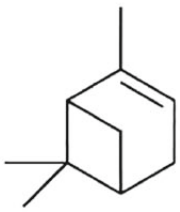
MIC, extracellular conductivity, sorbitol, and ergosterol effect assays were repeated at least three times to decrease the experimental errors. Averages and standard deviations of all groups were calculated by using Microsoft Excel. To understand whether treatment with α-pinene caused the statistically significant change, student's t-tests were performed to compare the plotted regression line slopes (Andrade & Estévez-Pérez, 2014)

RESULTS and DISCUSSION

MIC shows the antifungal activity of α-pinene, limonene and eugenol but not via yeast cell wall

The antifungal activity of the three biologically active compounds against *S. cerevisiae* cells was detected via MIC measurement. Here, we focused on the antifungal activity of the compounds and their modes of action against *S. cerevisiae* cells, because similar studies with *S. cerevisiae* are limited. Moreover, *S. cerevisiae* is a significant spoilage yeast for food, and beverage industries (Stratford, 2006), and medical areas (Shen et al., 2004). The MIC values of α-pinene, limonene and eugenol were demonstrated in Table 1. In addition to detecting antifungal activity, understanding the mode of action of these compounds is an indispensable necessity for medical or various industrial areas (Etebu & Arikekpar, 2016; Schäfer & Wenzel, 2020). After determining antifungal activity, the mechanism by which compounds exert an antifungal effect was determined. The cell wall of yeasts may be considered the first target for antifungal agents because of its structure involving chitin, glucan, and mannan (Nazzaro et al., 2017). Therefore, a sorbitol effect assay was performed to test the effectiveness of the compounds on the cell wall. The cell wall protects the yeast cells from environmental stresses (Pereira et al., 2015). With sorbitol, the MIC value of α-pinene, limonene and eugenol were not changed (Table 1). It means that the antifungal activity of the compounds does not interfere with the yeast cell wall. This result is in accord with the previously published work by Miron et. al. who investigated the damage of eugenol and α-pinene on the cell wall of another fungal cell type and observed no effect of sorbitol on MIC values (Miron et al., 2014).

Table 1. MICs (mM)value for α-pinene, limonene, and eugenol after MIC measurement, sorbitol and ergosterol effect assay



Compounds	MIC measurement (mM)	Sorbitol effect assay (mM)	Ergosterol effect assay (mM)
α-pinene	10 - 20	10 - 20	10 - 20
limonene	1 - 2	1 - 2	-
eugenol	1 - 2	1 - 2	-

α-Pinene triggered a higher amount of ion leakage but not via ergosterol biosynthesis

α- pinene is a well-known representative of the monoterpenes group and is found in various essential oils. It has a wide range of biological activities, including antibacterial effects against *E.coli* and *S. aureus*; antifungal effects against *C. albicans* (Salehi et al., 2019). In this study, we tried to elucidate the antifungal activity mechanism of the compound against the *S. cerevisiae* cells. The extracellular conductivity of the yeast cells treated with 20 mM and 40 mM α-pinene for 0–60 min are demonstrated in Fig. 1. Ion release was found to be statistically significantly different ($p < 0.05$) between control group (no compound), and 40 mM α-pinene (Fig. 1.). Ion release from the cell membrane with 40 mM α-pinene demonstrated that it disrupts cell membrane integrity with the corresponding concentration against *S. cerevisiae* cells.

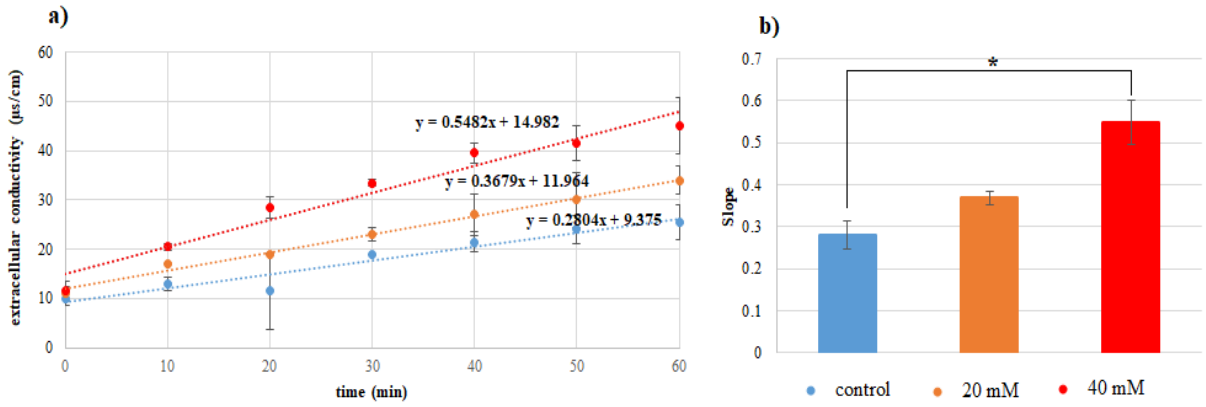


Figure 1. Extracellular conductivity measurement. a) Effect of α-pinene on the extracellular conductivity of *S. cerevisiae* yeast cells. b) The bars indicated standard deviation of the results from the mean values. Asterisk (*) indicates that values are statistically significantly different from each other ($p < 0.05$).

On the other hand, 1,2, and 4 mM limonene or eugenol did not cause efficient ion leakage from the yeast cell membrane. They must have another target side than the cell membrane in the yeast cell. Based on the conductivity measurement, 40 mM α-pinene caused efficient ion leakage from the yeast cell membrane. Ion leakage from the cytoplasm is associated with membrane disruption and cell death (Ergüden & Ünver, 2022).

After detecting ion leakage, we tested whether the membrane deformation was due to ergosterol or not. Ergosterol is a sterol included in the cell membrane of yeast cells, and it is an essential regulator of membrane fluidity. Since ergosterol is not present in animal and human cell membrane structure, it is the target site for many antifungal agents. Hence, disruption of ergosterol biosynthesis inhibits fungal growth (Minnebruggen et al., 2010). Nevertheless, MIC value of α-pinene after ergosterol effect assay did not change (Table 1). The findings indicate that α-pinene does not act by a mechanism that seems to involve inhibition of the ergosterol biosynthesis. In other words, the antifungal activity of α-pinene is not due to the disruption of ergosterol biosynthesis. It must have another target than the ergosterol in the yeast cell membrane.

CONCLUSION

The antifungal properties of α-pinene, limonene and eugenol against *S. cerevisiae* yeast cells were revealed, and the compounds' mode of action was studied. It has been proven that α-pinene, a biologically active natural monoterpene, has antifungal activity on the yeast cells. We have also shown that α-pinene disrupts cell membrane integrity and causes ion leakage resulting in cell death. These observations might be helpful for further

investigation for their medical applications or various industrial usage of monoterpenes.

ACKNOWLEDGMENT

This study was presented in ICABB 2022.

Ethical approval:

Ethics committee is not required

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

REFERENCES

Allenspach, M., & Steuer, C. (2021). α-Pinene: A never-ending story. *Phytochemistry*, *190*, 112857.

Allenspach, M., Valder, C., Flamm, D., Grisoni, F., & Steuer, C. (2020). Verification of chromatographic profile of primary essential oil of Pinus sylvestris L. combined with chemometric analysis. *Molecules*, *25*(13), 2973.

Alonso, A., Belda, I., Santos, A., Navascués, E., & Marquina, D. (2015). Advances in the control of the spoilage caused by Zygosaccharomyces species on sweet wines and concentrated grape musts. *Food Control*, *51*, 129–134.

Alviano, D. S., & Alviano, C. S. (2009). Plant extracts: search for new alternatives to treat microbial diseases. *Current Pharmaceutical Biotechnology*, *10*(1), 106–121.

Andrade, J. M., & Estévez-Pérez, M. G. (2014). Statistical comparison of the slopes of two regression lines: A tutorial. *Analytica Chimica Acta*, *838*, 1–12.

Bevilacqua, A., Corbo, M. R., & Sinigaglia, M. (2010). In vitro evaluation of the antimicrobial activity of eugenol, limonene, and citrus extract against bacteria and yeasts, representative of the spoiling microflora of fruit juices. *Journal of Food Protection*, *73*(5), 888–894.

Burt, S. A., Ahad, D., Kinjet, M., & Santos, R. R. (2013). Cinnamaldehyde, carvacrol and selected organic acids affect expression of immune related genes in IPEC-J2 cells exposed to Salmonella enterica serotype Typhimurium or Escherichia coli K88. *Planta Medica*, *79*(13), PJ7.

Cahyari, K., Syamsiah, S., Hidayat, M., & Sarto, S. (2018). Inhibitory kinetics study of limonene and eugenol towards mixed culture of dark fermentative biohydrogen production. *AIP Conference Proceedings*, *2026*(1), 20038.

Cai, R., Hu, M., Zhang, Y., Niu, C., Yue, T., Yuan, Y., & Wang, Z. (2019). Antifungal activity and mechanism of citral, limonene and eugenol against Zygosaccharomyces rouxii. *Lwt*, *106*, 50–56.

da Franca Rodrigues, K. A., Amorim, L. V., Dias, C. N., Moraes, D. F. C., Carneiro, S. M. P., & de Amorim Carvalho, F. A. (2015). Syzygium cumini (L.) Skeels essential oil and its major constituent α-pinene exhibit anti-Leishmania activity through immunomodulation in vitro. *Journal of Ethnopharmacology*, *160*, 32–40.

Ergüden, B., & Ünver, Y. (2022). Phenolic chalcones lead to ion leakage from Gram-positive bacteria prior to cell death. *Archives of Microbiology*, *204*(1), 1–6.

Etebu, E., & Arikekpar, I. (2016). Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. *Int J Appl Microbiol Biotechnol Res*, *4*(2016), 90–101.

Harvey, A. L., Edrada-Ebel, R., & Quinn, R. J. (2015). The re-emergence of natural products for drug discovery in the genomics era. *Nature Reviews Drug Discovery*, *14*(2), 111–129.

Hyldgaard, M., Mygind, T., & Meyer, R. L. (2012). Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Frontiers in Microbiology*, *3*, 12.

Konuk, H. B., & Ergüden, B. (2017). Antifungal activity of various essential oils against Saccharomyces cerevisiae depends on disruption of cell membrane integrity. *Biocell*, *41*(1), 13.

Koudou, J., Abena, A. A., Ngaissona, P., & Bessière, J. M. (2005). Chemical composition and pharmacological activity of essential oil of Canarium schweinfurthii. *Fitoterapia*, *76*(7–8), 700–703.

Loizzo, M. R., Saab, A., Tundis, R., Statti, G. A., Lampronti, I., Menichini, F., Gambari, R., Cinatl, J., & Doerr, H. W. (2008). Phytochemical analysis and in vitro evaluation of the biological activity against herpes simplex virus type 1 (HSV-1) of Cedrus libani A. Rich. *Phytomedicine*, *15*(1–2), 79–83.

Minnebruggen, G. Van, François, I., Cammue, B. P. A., Thevissen, K., Vroome, V., Borgers, M., & Shroot, B. (2010). A general overview on past, present and future antimycotics. *The Open Mycology Journal*, *4*(1).

Miron, D., Battisti, F., Silva, F. K., Lana, A. D., Pippi, B., Casanova, B., Gnoatto, S., Fuentefria, A., Mayorga, P., & Schapoval, E. E. S. (2014). Antifungal activity and mechanism of action of monoterpenes against dermatophytes and yeasts. *Revista Brasileira de Farmacognosia*, *24*, 660–667.

Nazzaro, F., Fratianni, F., Coppola, R., & De Feo, V. (2017). Essential oils and antifungal activity. *Pharmaceuticals*, *10*(4), 86.

Nóbrega, J. R., Silva, D. de F., Andrade Júnior, F. P. de, Sousa, P. M. S., Figueiredo, P. T. R. de, Cordeiro, L. V., & Lima, E. de O. (2021). Antifungal action of $α$-pinene against Candida spp. isolated from patients with otomycosis and effects of its association with boric acid. *Natural Product Research*, *35*(24), 6190–6193.

Pereira, F. de O., Mendes, J. M., Lima, I. O., Mota, K. S. de L., Oliveira, W. A. de, & Lima, E. de O. (2015). Antifungal activity of geraniol and citronellol, two monoterpenes alcohols, against Trichophyton rubrum involves inhibition of ergosterol biosynthesis. *Pharmaceutical Biology*, *53*(2), 228–234.

Salehi, B., Upadhyay, S., Erdogan Orhan, I., Kumar Jugran, A., LD Jayaweera, S., A. Dias, D., ... & Sharifi-Rad, J. (2019). Therapeutic potential of α-

and β-pinene: A miracle gift of nature. *Biomolecules*, *9*(11), 738

Schäfer, A.-B., & Wenzel, M. (2020). A how-to guide for mode of action analysis of antimicrobial peptides. *Frontiers in Cellular and Infection Microbiology*, *10*, 540898.

Shen, Y.-C., Chou, C.-J., Wang, Y.-H., Chen, C.-F., Chou, Y.-C., & Lu, M.-K. (2004). Anti-inflammatory activity of the extracts from mycelia of Antrodia camphorata cultured with water-soluble fractions from five different Cinnamomum species. *FEMS Microbiology Letters*, *231*(1), 137–143.

Sikorski, R. S., & Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics*, *122*(1), 19–27.

Silva, A. C. R. da, Lopes, P. M., Azevedo, M. M. B. de, Costa, D. C. M., Alviano, C. S., & Alviano, D. S. (2012). Biological activities of a-pinene and $β$-pinene enantiomers. *Molecules*, *17*(6), 6305–6316.

Stratford, M. (2006). Food and beverage spoilage yeasts. In *Yeasts in food and beverages* (pp. 335–379). Springer.

Wei, L.-J., Zhong, Y.-T., Nie, M.-Y., Liu, S.-C., & Hua, Q. (2020). Biosynthesis of $α$-pinene by genetically engineered Yarrowia lipolytica from low-cost renewable feedstocks. *Journal of Agricultural and Food Chemistry*, *69*(1), 275–285.

Yang, C., Hu, D.-H., & Feng, Y. (2015). Antibacterial activity and mode of action of the Artemisia capillaris essential oil and its constituents against respiratory tract infection-causing pathogens. *Molecular Medicine Reports*, *11*(4), 2852–2860.

Zarai, Z., Kadri, A., Ben Chobba, I., Ben Mansour, R., Bekir, A., Mejdoub, H., & Gharsallah, N. (2011). The in-vitro evaluation of antibacterial, antifungal and cytotoxic properties of Marrubium vulgare L. essential oil grown in Tunisia. *Lipids in Health and Disease*, *10*(1), 1–8.

Changes in cardiac markers in a calf died of foot and mouth disease

ABSTRACT

This report disclosed changes in cardiac troponin expression during myocardial degeneration in a calf with foot and mouth disease (FMD). The case presented was a 7-day-old calf milk fed by its mother which diagnosed as having FMD (O-type) by Ministry of Agriculture and Forestry Şap (Foot and Mouth Disease) Institute. The calf suffered from myocarditis due to suckling FMD infected dam. Blood sample was taken from the jugular vein of calf. Serum cTn-T, cTn-I, CK-MB, LDH, AST and ALT levels were measured using commercial kits. cTn-T, cTn-I, CK-MB, LDH, AST and ALT levels were determined as 1.00 ng/mL, 26.68 ng/mL, 262 U/L, 4503 U/L, 56.6 U/L, 55.3 U/L, respectively and these values were higher than those of healthy calf. It is thought that the use of cardiac troponins (cTn-I, cTn-T) for the diagnosis of the disease will give accurate and rapid results.

Keywords: Calf, CK-MB, cTn-I, cTn-T, FMD.

INTRODUCTION

Foot and Mouth disease (FMD) an acute, contagious viral disease of ruminants causes serious economic losses (Kitching et al., 2007; AlSaad, 2020). The course of the disease depends on factors such as the entry site, strain, and type of host (Barnett and Cox, 1999; Hughes et al., 2002). The incubation period varies between 2-14 days (Alexandersen et al., 2003). Infected animals spread the virus through secrete and excrete (Çiftçi and İnanç, 2017). FMD causes sudden death due to myocardial degenerations in young and sensitive calves (Gülbahar et al., 2007). While the morbidity rate of the disease can reach 100%, the mortality rate is 1-2% in adults and 20% in young animals (Lubroth, 2002; Çiftçi and İnanç, 2017; Aly et al., 2020).

In recent years, cardiac troponins (cTn) have widely been used in veterinary cardiology, as they have a high sensitivity and specificity for myocardial damage (El-Khuffash and Molloy, 2008; Tunca et al., 2009; Undhad et al., 2012; AlSaad, 2020). Troponin consists of three subunits: troponin I (cTn-I), troponin T (cTn-T), and troponin C. In humans, cTn-I has been considered more sensitive and specific than cTn-T (Rajappa and Sharma, 2005). It has been reported that cardiac troponin level is more sensitive than creatinine kinase myocardial band (CK-MB) (Ooi et al. 2000). Moreover, creatine kinase (CK) and CK-MB levels are at their normal values during the onset of myocardial damage, while cTn-I levels increase significantly. This leads to wide use of troponins for diagnostic purposes (Bayraktar, 2014).

How to cite this article
Ayvazoğlu, C., Gökçe, E., Erdoğan HM. (2022). Changes in cardiac markers in a calf died of foot and mouth disease. *Journal of Advances in VetBio Science and Techniques, 7*(3), 391-394. https://doi.org/10.31797/vetbio.1107357

Case Report

Cemalettin Ayvazoğlu[1a]
Erhan Gökçe[2b]
Hidayet Metin Erdoğan[3c]

[1]Nihat Delibalta Göle Vocational High School, Ardahan University, 75100, Ardahan, Türkiye
[2]Department of Internal Medicine, Faculty of Veterinary Medicine, Kafkas University, 36100, Kars, Türkiye
[3]Department of Internal Medicine, Faculty of Veterinary Medicine, Aksaray University, 68000, Aksaray, Türkiye

ORCİD-
[a]0000-0003-2064-0657
[b]0000-0003-2674-1010
[c]0000-0003-1261-4352

Correspondence
Cemalettin AYVAZOĞLU
cemayvazoglu@hotmail.com

Article info
Submission: 22-04-2022
Accepted: 07-12-2022
Online First: 25-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

In this case report, changes in cTn-I, cTn-T, CK-MB, lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values of a calf died due to myocarditis resulting from FMD were investigated and the results were evaluated.

CASE HISTORY

Foot and Mouth disease cases in cattle were recorded and immediately quarantina was implemented in Akyaka district of Kars province by Kars Agriculture and Forestry Directorate on 31.01.2022. A 7-day-old calf was referred with clinical signs of lethargy, dispnea. The history revealed that the calf was suckling its mother with clinical signs of FMD and sample taken from udder by the Provincial Directorate of Agriculture and Forestry disclosed FMD (O variant) positive by PCR analysis at the Ministry of Agriculture and Forestry Foot and Mouth Disease Institute. The rectal temperature of the calf was 40.1°C, the pulse rate was 119 beats/min, and the respiratory rate was 42 breaths/min. Arrhythmia was also detected on cardiac auscultation. There were no vesicular or erosive lesions of FMD. These findings were in accordance to those related myocardial form of FMD (Dawood and Alsaad, 2018). Following physical examination, 8 mL blood sample without anticoagulant was taken from jugular vein in order to determine serum cTn-I, cTn-T, CK-MB, LDH, AST and ALT concentrations. The calf died on the the following day of sampling. A blood sample was taken from another healthy calf under the same care and feeding conditions in order to make comparisons. Samples were stored at room temperature for one hour and centrifuged at 3000 rpm for 15 minutes, then harvested serum was stored at –20 °C until analysis. CTn-I, cTn-T and CK-MB were measured using commercial kits (BT LAB, China for cTn-T and ELK Biotechnology, P.R.C for cTn-I, CK-MB) on ELISA reader (ELISA reader®-DAS for cTn-I and Elecsys® 2010-Roche for cTn-T, CK-MB). LDH, AST and ALT activities were measured on a fully automatic autoanalyzer device (Roche, Cobas C501). The data obtained from the case was made available with the permission of the owner through signed informed consent form.

Table 1. Serum cTn-I, cTn-T, CK-MB LDH, AST and ALT levels in healthy and diseased calf.

Parameters	Healthy	Patient
cTn-I (ng/mL)	0.16	26.7
cTn-T (ng/mL)	0.018	1.00
CK-MB (U/L)	17.88	262.0
LDH (U/L)	341.60	895
AST (U/L)	27.13	56.6
ALT (U/L)	18.90	8.1

Abbreviations: cTn-T=Cardiac Troponin T, cTn-I=Cardiac Troponin I, CK-MB: Creatine phosphokinase, LDH: Lactate Dehydrogenase, AST: Aspartate aminotransferase, ALT: Aspartate transaminase.

The cTn-I, cTn-T, CK-MB, LDH, AST and ALT values of healthy calves and calves that died of myocardial form of Foot and Mouth disease are presented in Table 1. Accordingly, cTn-I, CTn-T, CK-MB, LDH and AST values were higher than healthy calves except for ALT which was higher in healthy calf.

DISCUSSION

Foot and Mouth disease, is of great importance due to high economic losses incurred by disease. The most important clinical sign of Foot and Mouth disease is vesicle formation in mouth and foot (Windsor et al., 2011). However, in the myocarditis form, death usually occur before vesicle formation (Lubroth, 2002). For this reason, early diagnosis as well as prevention is of great importance (Sobrino et al., 1986; Çiftçi and İnanç, 2017).

In a study conducted on beef cattle with FMD, cTn-I analysis was reported to be sensitive and specific to detect myocardial degeneration and a threshold value of ≥ 3.618 ng/mL for cTn-I was recommended to determine myocardial degeneration and also reported higher cTn-I and LDH levels in calves

with FMD when compared with healthy calves (Akın et al., 2015).

In another study, cTn-T, cTn-I, LDH levels in healthy calves were reported as 0.344 ng/mL, 0.03 ng/mL, 959.02 U/L, respectively, and increased in premature calves (Aydoğdu et al., 2016). Similarly the cTn-I, LDH, AST and ALT levels of calves with foot and mouth were reported significantly higher (Tunca et al., 2008; Cha et al., 2017; Salim et al., 2019; Aly et al, 2020). In this case report, the cTn-T, cTn-I, CK-MB, LDH, AST and ALT levels of the calf with myocarditis were 1.00 ng/mL, 26.68 ng/mL, 262.0 U/L, 4503 U/L, 56.6 U/L, 55.3 U/L, respectively. These values were higher than those of healthy calves.

As a result; Foot and Mouth disease is still a major problem due to myocardial form that causes sudden death without clinical symptoms in young animals, despite massive vaccination campaigns. Therefore, early diagnosis of the disease is important. It is thought that the use of cardiac troponins (cTn-I, cTn-T) for the diagnosis of disease-related myocardial degeneration will give accurate and rapid results.

ACKNOWLEDGMENT

Ethical approval:

Republic of Türkiye Ministry of Agriculture and Forestry Şap Enstitüsü, Date: 17.03.2022 No: 93369491-300-278.

Conflict of interest: No conflict of interest between the authors.

REFERENCES

Akın, H., Güneş, V., & Bekdik, İ. K. (2015). Practice Cardiac Troponin I Kit Expressions and Concentrations of Cardiac Troponin I in Beef Cattle with Foot and Mouth Disease. *Erciyes Üniversitesi Veteriner Fakültesi Dergisi, 12*(3), 163-170.

Alexandersen, S., Quan, M., Murphy, C., Knight, J., & Zhang, Z. (2003). Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. *Journal of Comparative Pathology*, *129*(4), 268-282.

AlSaad, K. M., Al-Autaish, H. N., & Ahmed, J. A. (2020). Evaluation of cardiac enzymes and acute phase response as biomarkers for rapid diagnosis of myocarditis in calves with FMD. *Iraqi Journal of Veterinary Sciences*, *34*(1), 31-37.

Aly, M., Nayel, M., Salama, A., Ghazy, E., & Elshahawy, I. (2020). Cardiac troponin I as a cardiac biomarker has prognostic and predictive value for poor survival in Egyptian buffalo calves with foot-and-mouth disease. *Veterinary World*, *13*(5), 890-895.

Aydogdu, U., Yildiz, R., Guzelbektes, H., Coskun, A., & Sen, I. (2016). Cardiac biomarkers in premature calves with respiratory distress syndrome. *Acta Veterinaria Hungarica*, *64*(1), 38-46.

Barnett, P. V., & Cox, S. J. (1999). The role of small ruminants in the epidemiology and transmission of foot-and-mouth disease. *The Veterinary Journal*, *158*(1), 6-13.

Bayraktar, B. (2014). Investigation of the effectiveness of cardiac troponins in the determination of myocardial damage in lambs and kids. (Publication No. 376893) [PhD Thesis. Adnan Menderes University]. PQDT Open. http://adudspace.adu.edu.tr:8080/jspui/bitstream/11607/367/1/Bahar%20BAYRAKTAR%20_tez.pdf

Cha, C. N., Park, E. K., Yoo, C. Y., Kim, S., Yun, Y. W., & Lee, H. J. (2017). Blood parameter changes in Korean traditional calves and pigs after foot-and-mouth disease vaccination. *Korean Journal of Veterinary Research*, *57*(1), 43-45.

Çiftçi, M. K., & İnanç, M. (2017). Şap Hastalığının Miyokardiyal Formundan Ölen İki Kuzuda Kalp ve Dilde Gözlenen Patolojik Bulgular. *Bahri Dağdaş Hayvancılık Araştırma Dergisi, 6*(2), 15-22.

Dawood, A. A., & Alsaad, K. M. (2018). Clinical and diagnostic studies of myocarditis result from FMD in lambs. *Journal of Agriculture and Veterinary Science*, *11*(7), 01-10.

El-Khuffash, A. F., & Molloy, E. J. (2008). Serum troponin in neonatal intensive care. *Neonatology*, *94*(1), 1-7.

Gulbahar, M. Y., Davis, W. C., Guvenc, T., Yarim, M., Parlak, U., & Kabak, Y. B. (2007). Myocarditis associated with foot-and-mouth disease virus type O in lambs. *Veterinary Pathology*, *44*(5), 589-599.

Hughes, G. J., Mioulet, V., Haydon, D. T., Kitching, R. P., Donaldson, A. I., & Woolhouse, M. E. (2002). Serial passage of foot-and-mouth disease virus in sheep reveals declining levels of viraemia over time. *Journal of General Virology*, *83*(8), 1907-1914.

Kitching, P., Hammond, J., Jeggo, M., Charleston, B., Paton, D., Rodriguez, L., & Heckert, R. (2007). Global FMD control is it an option?. *Vaccine*, *25*(30), 5660-5664.

Lubroth, J. (2002). Foot-and-mouth disease: a review for the practitioner. *Veterinary Clinics: Food Animal Practice*, *18*(3), 475-499.

Ooi, D. S., Isotalo, P. A., & Veinot, J. P. (2000). Correlation of antemortem serum creatine kinase, creatine kinase-MB, troponin I, and troponin T with cardiac pathology. *Clinical Chemistry*, *46*(3), 338-344.

Rajappa, M., & Sharma, A. (2005). Biomarkers of cardiac injury: an update. *Angiology*, *56*(6), 677-691.

Salim, S. A. S., Talb, O. Q., Saad, M. S., & Yousif, A. S. (2019). Clinico-pathological and biochemical aspects of foot and mouth disease in calves. *Advances in Animal and Veterinary Sciences*, *7*(10), 835-843.

Sobrino, F., Palma, E. L., Beck, E., Davila, M., De La Torre, J. C., Negro, P., Villanueva, N., Ortín, J., & Domingo, E. (1986). Fixation of mutations in the viral genome during an outbreak of foot-and-mouth disease: heterogeneity and rate variations. *Gene*, *50*(1-3), 149-159.

Tunca, R., Erdoğan, H. M., Sözmen, M., Çitil, M., Devrim, A. K., Erginsoy, S., & Uzlu, E. (2009). Evaluation of cardiac troponin I and inducible nitric oxide synthase expressions in lambs with white muscle disease. *Turkish Journal of Veterinary and Animal Sciences*, *33*(1), 53-59.

Tunca, R., Sozmen, M., Erdogan, H., Citil, M., Uzlu, E., Ozen, H., & Gokçe, E. (2008). Determination of cardiac troponin I in the blood and heart of calves with foot-and-mouth disease. *Journal of Veterinary Diagnostic Investigation*, *20*(5), 598-605.

Undhad, V. V., Fefar, D. T., Jivani, B. M., Gupta, H., Ghodasara, D. J., Joshi, B. P., & Prajapati, K. S. (2012). Cardiac troponin: an emerging cardiac biomarker in animal health. *Vet World*, *5*(8), 508-511.

Windsor, M. A., Carr, B. V., Bankowski, B., Gibson, D., Reid, E., Hamblin, P., Gubbins, S., Juleff, N., & Charleston, B. (2011). Cattle remain immunocompetent during the acute phase of foot-and-mouth disease virus infection. *Veterinary Research*, *42*(1), 1-11.