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RESEARCH ARTICLE

Evaluation of Teaching Methods and Technical Opportunities for the Distance Education Process of Veterinary Faculty Students in Türkiye During the COVID-19 Pandemic

Gökhan ASLIM[1*], Mustafa Agâh TEKİNDAL[2], Ali YİĞİT[3], Ayşe MENTEŞ GÜRLER[4], Şule SANAL[5]

[1]*Department of History of Veterinary Medicine and Deontology, Faculty of Veterinary Medicine, Selçuk University, Konya, Türkiye*
[2]*Department of Biostatictics, Faculty of Medicine, İzmir Kâtip Çelebi University, İzmir, Türkiye*
[3]*Department of History of Veterinary Medicine and Deontology, Faculty of Veterinary Medicine, Dokuz Eylül University, İzmir, Türkiye*
[4]*Department of History of Veterinary Medicine and Deontology, Faculty of Veterinary Medicine, Harran University, Şanlıurfa, Türkiye*
[5]*Department of History of Veterinary Medicine and Deontology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Türkiye*

ABSTRACT

Covid-19 has caused the death of approximately 7 million people and caused severe problems in all areas of life, especially in education. Upon the detection of the first case in Turkey, education was suspended in all educational institutions on March 16, 2020, and it was decided to continue education in a digital environment through distance and open education for the 2019-2020 spring semester. This study aimed to evaluate the opinions and thoughts of veterinary faculty students about the methods and technical possibilities of distance education courses. A questionnaire was administered to 1599 students via "Google Docs" between December 10, 2020, and January 11, 2021, to those who were willing to participate in the survey among 2nd, 3rd, 4th, and 5th-year students who were studying in veterinary faculties in Turkey and had face-to-face and distance education experience. The statistical package program was used for the SPPS 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Descriptive frequencies and percentages were given for categorical variables. In the study, it was determined that 67.2% of the participants used their computers. However, lack of professional development (75.4%), technical problems (73.3%), access to the internet (56.8%), etc. were identified as the difficulties of online courses. Although students found the course duration sufficient, they thought the courses should be supported with PDF, Word, and PowerPoint documents (78.2%). In addition to the security concerns of distance education, students also stated that they did not prefer distance education because veterinary medicine education is a practical field and because of technical problems. Students' concerns, such as professional development and professional-social interaction due to being away from school, can be addressed with support training after the pandemic.

Keywords: Covid-19, distance education, online education, pandemic, veterinary students

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Türkiye'de Veteriner Fakültesi Öğrencilerinin COVID -19 Pandemisinde Uzaktan Eğitim Sürecine Yönelik Derslerin İşlenme Yöntemi ve Teknik Olanakların Değerlendirilmesi

ÖZ

Covid-19 yaklaşık yedi milyon insanın ölümüne sebep olmuş, başta eğitim olmak üzere hayatın her alanında ciddi sorunlara neden olmuştur. Türkiye'de ilk vakanın tespit edilmesi üzerine 16 Mart 2020 tarihinde tüm eğitim kurumlarında eğitime ara verilmiş, 2019-2020 bahar dönemi için uzaktan ve açık öğretim yoluyla dijital ortamda eğitime devam edilmesine karar verilmiştir. Çalışmada, veteriner fakültesi öğrencilerinin uzaktan eğitim ile verilen derslerin yöntemleri ve teknik olanakları hakkındaki görüş ve düşüncelerini değerlendirmesi amaçlandı. Türkiye'de veteriner fakültelerinde öğrenim görmekte olan, yüzyüze ve uzaktan eğitim deneyimi yaşamış 2, 3, 4 ve 5. sınıf öğrencilerinden ankete katılmak isteyenlere 10 Aralık 2020- 11 Ocak 2021 tarihleri arasında "*Google docs*" aracılığıyla 1.599 öğrenciye anket uygulaması yapıldı. Verilerin değerlendirilmesinde SPPS 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) istatistik paket programı kullanıldı. Çalışmada kategorik değişkenler için tanımlayıcı olan frekans ve yüzdelik dilimleri verildi. Çalışmada kendi bilgisayarını kullananların oranının %67.2 olduğu tespit edildi. Bununla birlikte, çevrimiçi derslerin zorlukları olarak mesleki gelişim eksikliği (%75.4); teknik sorunlar (%73.3); internete erişim (%56.8) vb. belirlendi. Öğrenciler ders süresini yeterli bulmakla birlikte, derslerin PDF, Word ve PowerPoint dokümanlarıyla desteklenmesi gerektiğini düşünmektedir (%78.2). Uzaktan eğitimin güvenlik kaygılarının yanı sıra öğrenciler, veteriner hekimliği eğitiminin uygulamaya yönelik bir alan olması ve teknik sorunlar nedeniyle tercih etmediklerini de belirtmektedir. Öğrencilerin okuldan uzak kalmaya bağlı mesleki gelişim ve mesleki-sosyal etkileşim gibi kaygılarının pandemi sonrası yapılacak destek eğitimleri ile giderilebileceği önerilebilir.

Anahtar Kelimeler: Covid-19, online eğitim, pandemi, uzaktan eğitim, veteriner fakültesi öğrencileri

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ORCID ID; *GA: 0000-0001-5976-8186, MAT: 0000-0002-4060-7048, AY: 0000-0002-1180-3517, AMG: 0000-0001-8402-7291, ŞS: 0000-0002-5703-5752*
*Corresponding author e-mail: gokhan.aslim@selcuk.edu.tr

INTRODUCTION

A Pandemic is an epidemic occurring worldwide or over a vast area, crossing international boundaries and usually affecting many people (Last 2001). Epidemics such as influenza, HIV, plague, malaria, cholera, and SARS have occurred in the past (Shoals 2019). COVID-19, which emerged in 2019, has also been defined as a pandemic by the World Health Organization (WHO) as of March 11, 2020. The first COVID-19 case in Türkiye was reported on March 11, 2020. Since it started, the total number of reported cases worldwide has reached 185 million, and nearly 7 million people have died because of this disease (WHO 2021). The mode of transmission of COVID-19, like general influenza, is by the respiratory route. Since the risk of catching it increases in indoor places, it has become necessary to suspend education in educational institutions to prevent its spread. Social distancing (individual and social isolation) has been put into practice in Türkiye to prevent the transmission of COVID-19. In order to avoid interruptions in the field of education, the "distance education" method has been employed by using the available opportunities of digital world (MH 2019). Distance education is a form of education made from a particular center using various communication tools without the face-to-face presence of the student and teacher (TLS 2020).

During the pandemic, in Türkiye, The Council of Higher Education (CHE) first decided on March 13 to suspend education at universities for three weeks as of March 16, 2020 (CHE 2020a), and then on March 23 due to the uncertainty of the process, decided to continue education in the digital environment through distance and open education for the 2019-2020 spring academic semester (CHE 2020b). During this time, it was found that the majority of Turkish universities have the technology infrastructure to allow distant learning, and CHE would quickly assist those who do not. (Dikmen and Bahçeci 2020).

Following period, the publication of the "New Normalization Process Guide in the Global Epidemic" by CHE was announced on July 30, 2020. It is stated that "the guide provides a general framework according to possible scenarios and gives relevant university committees authority to apply for different programs according to the regional and local course of the epidemic." The guide includes framework decisions and suggestions under "Distance Education Practices, Applied Trainings, Assessment and Evaluation Practices, Foreign Students, Meetings, Congresses and Exchange Programs." This guide has allowed different practices to be implemented in universities according to the regional and local course of the epidemic during the epidemic's progression. The "New Normalization Process in the Global Epidemic 2020" guide published by CHE states that distance education performed due to the COVID-19 global epidemic is the emergency distance education" application of the new normal, and it also emphasizes that they were different from the distance education implemented by preplanning (CHE 2020c).

The online learning method used in distance education is described as a tool that can make the teaching-learning process more student-centered, innovative, and flexible. Online learning refers to educational experiences that take place in either synchronous or asynchronous settings on various internet-connected devices, such as laptops and mobile phones. These settings allow for anywhere (independent) learning and interaction between students, teachers, and other students (Singh and Thurman 2019). Serçemeli and Kurnaz (2020) stated that students did not face any problems in terms of self-efficacy regarding the use of the distance education system; they approached it positively because of re-watchable video recordings, flexible education opportunities, and time-saving.

This study was prepared to evaluate the opinions and thoughts of students about the methods and technical possibilities of distance education courses that started with the COVID-19 pandemic in veterinary medicine education.

MATERIAL and METHODS

In the study, a survey was created based on the studies on "distance education during the pandemic period" (Serçemeli and Kurnaz 2020; Kürtüncü and Kurt 2020). According to 2019 data, there are 10.946 students studying in veterinary faculties in Turkey and the questionnaire applied to the other grades (2nd, 3rd, 4th and 5th) except for the first year students. The questionnaire evaluated seven demographic questions and eight questions about the method of teaching the courses. The survey form will be applied to the students who want to participate in the study, and the name and surname of the participating students will not be requested in the survey forms. It was sufficient to apply the questionnaire form to approximately 700 people with a 95% confidence interval, and a total of 1599 students, 518 from 2nd grade, 379 from 3rd grade, 342 from 4th grade and 360 from 5th grade, participated in the study (Table 1). The questionnaire was administered to students who volunteered to participate in the study. The survey application was delivered to students via "Google" (docs.google) between December 10, 2020 and January 11, 2021, and data were obtained. "SPPS 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.)" statistical package software was used to evaluate the data. In the study, the frequencies and percentages of the data obtained from participants'

tool choice to follow online courses, their preferences about the method of the theoretical and practical courses, the problems they encountered, the course duration preferences, and the tools that the courses should be supported by are given in the tables. The demographic distribution of the data was presented in tabular form based on the classes of the participants.

RESULTS

The distribution of these students according to region, gender, grade point average, and place of residence is shown in Table 1. 55.6% of the participants stated that they lived in the city center. Gender distribution was in favor of male students (F: 45.7%/M: 54.3%) and the highest participation was from faculties in the Eastern Anatolia Region (20.2%).

Table 1. Distribution of demographic data based on the class of the participants

Demographic Criteria	Class							
	2nd		3rd		4th		5th	
	Number	%	Number	%	Number	%	Number	%
Region								
Mediterrenian	89	5.6	72	4.5	89	5.6	60	3.8
Eastern Anatolia	125	7.8	67	4.2	39	2.4	92	5.8
Aegean	12	0.8	25	1.6	14	0.9	20	1.3
Southeastern Anatolia	69	4.3	47	2.9	79	4.9	29	1.8
Central Anatolia	107	6.7	67	4.2	77	4.8	38	2.4
Black Sea	81	5.1	47	2.9	29	1.8	46	2.9
Marmara	35	2.2	54	3.4	15	0.9	75	4.7
Total	518	32.4	379	23.7	342	21.4	360	22.5
Gender								
Female	272	17.0	188	11.8	130	8.1	141	8.8
Male	246	15.4	191	11.9	212	13.3	219	13,7
Total	518	32.4	379	23.7	342	21.4	360	22.5
Point average								
Less than 2.00	8	0.5	6	0.4	8	0.5	5	0.3
2.00-2.50	53	3.3	52	3.3	68	4.3	94	5.9
2.51-3.00	174	10.9	186	11.6	163	10.2	182	11.4
3.01-3.50	198	12.4	112	7.0	82	5.1	70	4.4
3.51 and above	85	5.3	23	1.4	21	1.3	9	0.6
Total	518	32.4	379	23.7	342	21.4	360	22.5
Place of residence								
Provincial center	277	17.3	193	12.1	195	12.2	224	14.0
District	132	8.3	108	6.8	85	5.3	87	5.4
Town	9	0.6	5	0.3	6	0.4	3	0.2
Neighborhood (Village)	100	6.3	73	4.6	56	3.5	46	2.9
Total	518	32.4	379	23.7	342	21.4	360	22.5

More than one answer was received for the "Which of the following tools do you use during online/distance education?" (Table 2) question and a total of 2214 responses were received. It was determined that the rate of those using their computer is 67.2%; those using their smartphone is 51.5%; those using someone else's computer is 14.4%, and those using someone else's smartphone is 1.8%.

Table 2. Participants' means for following online courses*

Lesson tracking tool	Number	%
My own computer	1075	67.2
My own tablet	48	3.0
My own phone	824	51.5
Someone else's computer	230	14.4
Someone else's tablet	8	0.5
Someone else's phone	29	1.8
Other	3	0.2

* More than one option is marked

While 52.9% of students preferred online or distance learning for theoretical courses, 21.3% of students preferred online or distance learning for practical courses. While 17.8% of the students thought that theoretical courses should be given face-to-face, 60.7% of the students said that practical courses should be given face-to-face (Table 3).

Table 3. Preferences of the participants regarding the way of teaching theoretical and practical courses

Type of course	Theoric		Practical	
	Number	%	Number	%
Online/remote	846	52.9	340	21.3
Face to face	284	17.8	970	60.7
Hybrid	496	29.3	259	16.2
Other	-	-	30	1.9

For the question "What are the difficulties of following courses online/remotely in the veterinary department?", it was determined that the lack of professional development (75.4%) was the most frequently mentioned problem, followed by technical problems (73.3%), difficulties in accessing the internet (56.8%), being away from school (53.0%) and social environments (43.6%) (Table 4).

Table 4. Problems faced by the participants in online courses*

The difficulty experienced	Number	%
Difficulties accessing the Internet	909	56.8
Facing technical problems (computer, tablet, phone malfunction, system problems, etc.)	1172	73.3
Being away from social situations	697	43.6
Getting away from the school environment	847	53.0
Not being able to ask questions about incomprehensible matters	560	35.0
Lack of professional development	1206	75.4
Excessive intensity (Video, homework, etc.)	9	0.6
Other	22	1.4
I don't have any problems	22	1.4

* More than one option is marked

Regarding "the duration of a lesson hour," 36.0% of the students answered that it should be in the range of 21-30 minutes; 35.3% of them said that it should be between the range of 31-40 minutes; 12.9% of them answered that it should be between the range of 41-50 minutes (Table 5).

Table 5. Opinions of the participants about the duration of a lesson

Lesson duration	Number	%
5-10 min	37	2.3
11-20 min	136	8.5
21-30 min	576	36.0
31-40 min	564	35.3
41-50 min	206	12.9
51-60 min	80	5.0

To the question of, "Which of the following methods would you like digital education course to be supported with?", 78.2% of the participants answered PDF Word and PowerPoint documents, 77.2% with the lecturers' notes/papers, 66.1% with online video recordings (Table 6).

Table 6. Opinions of the participants about which tools should be used in distance education courses*

Method to be supported	**Number**	**%**
Online / Live broadcast	783	49.0
Textbook	761	47.6
PDF, Word, Power point document	1250	78.2
Offline video recordings	1057	66.1
With the lecturers' notes/papers	1235	77.2
Other	19	1.2

* More than one option is marked

DISCUSSION

With the emergence of the COVID-19 pandemic, distance education was started in veterinary departments too. However, with the maintenance of the process, problems related to practical courses, especially for the year 2020, began to be expressed. It can be said that this study, which was carried out to determine how students come up with problems such as teaching methods and technical opportunities of the courses, is essential by including all university departments in Türkiye, by having a high number of participants and by including the opinions of people who had experienced both face-to-face and distance education.

Although it has been asserted that universities worldwide are increasingly running to distance education, it is reported that there are problems in terms of technological competence and security (Ali 2020). Studies on distance education reveal that there are various options regarding the means of class participation. Smartphones, laptops, tablets, and personal computers are the most commonly used electronic devices to attend online classes during the COVID-19 pandemic (Abushammala et al. 2021). A similar study by Karatepe et al. (2020) of teacher candidates states that 68.7% of students participating in the study follow lessons with a computer and 59.5% of them with a mobile phone. In a study in which students from the Department of Physiotherapy and Rehabilitation participated, it is stated that 61.5% of the students follow lessons by computer and 35.5% by mobile phone (Altuntaş Yılmaz 2020). The results of the study conducted by Serçemeli and Kurnaz (2020) showed that 75.5% of students used a smartphone, while 42.4% of students used a computer. Those who used someone else's computer or smartphone made up 12.8% and 3.9% of the sample, respectively. In the study conducted with a total of 1,392 veterinary students and researchers from 92 different countries, it was determined that the most used device was a smartphone (51.0%), followed by a laptop (32.8%) and tablet (9.6%) (Mahdy 2020). Another study determined that 61.5% of students accessed education using a computer, 35.5% using a phone, and 3% using a tablet[15] (Altuntaş Yılmaz 2020). In the study, in which the participants were allowed to choose more than one device, like in other studies, it was found that veterinary department students primarily used their computers (67.2%) and their smartphones (51.5%) in lessons (Table 2). According to Mahdy (2020), research must be done to give students access to computers, the internet, and tablets. Nearly all students should also own a computer, and these devices must always be available to support successful distance learning, which is expected to be implemented in 40% of informal education (CHE 2020c), regardless of the pandemic period. In addition, determining which devices students use in their online learning will help to ensure the accessibility of the prepared educational content by many students.

World Organization for Animal Health (OIE) has specified the characteristics that 'Day 1 graduates' veterinarians should have in order to ensure the quality of national veterinary medicine services of member countries and has developed a "Model Core Veterinary Curriculum" (OIE 2013). This curriculum was prepared for Veterinary Education Establishments of OIE member states where most courses are applied. Similarly, in the evaluation processes of EAEVE (European Association of Establishments for Veterinary Education), for which 13 university departments from Türkiye have applied for membership, it is observed that an applied learning-based approach is prioritized at every stage of veterinary medicine education (EAEVE 2021). It has been stated that in units with applied education, students need more face-to-face education. Performing the applications after the academic lessons is crucial for the student's professional practice skills (Keskin and Özer Kaya 2020). Mahdy (2020) reports that fulfilling students' veterinary competencies with only the online education system is not easy. In a survey of veterinary students in Egypt, 53% of the students stated that online anatomy learning cannot replace face-to-face teaching

(Mahdy and Sayed 2021). Some pre-clinical courses, such as Anatomy and Histology, require direct macroscopic and microscopic demonstration of different organs and structures of different animals to students (Brassett et al. 2020). Some courses, such as Medicine, Surgery and Theriogenology (Gynecology, Obstetrics and Andrology) require direct handling of clinically ill animals to learn the management of critically ill animal species (Dedeilia et al. 2020). Students will need a practical demonstration of practice (Brassett et al. 2020; Dedeilia et al. 2020). The study determined that veterinary department students also preferred face-to-face (60.7%, n:970) for applied courses in parallel with the abovementioned studies (Table 3). It can be said that this situation aligns with the recommendations of OIE and EAEVE regarding their efficiency and veterinary faculty students' professional development.

The student's binary named "Distance learning strategies: What do we know about the effectiveness?" organized by UNESCO (2021) due to the COVID-19 pandemic stated that technological preparations should come at the beginning of the preparations. Internet-related issues such as availability, connectivity, speed, infrastructure, and cost in developing countries hurt students' online learning (Mahdy and Sayed 2021). In the studies on the subject (Al-Balas et al. 2020; Keskin and Özer Kaya 2020; Kurtüncü and Kurt 2020; Serçemeli and Kurnaz 2020), internet or technical problems were the main ones among the difficulties. In this study, "What are the difficulties of following the courses taught in the veterinary department online/remotely without coming to school?" was asked, and it was determined that the most critical problem among the experienced difficulties was the lack of professional development (75.4%), followed by technical problems (73.3%), difficulties in accessing the Internet (56.8%), being away from school (53.0%), and social environments (43.6%) (Table 4). These results show that veterinary department students, unlike other university students, prioritize professional development and are more willing to obtain face-to-face education due to their anxiety in this regard, as seen in Table 3. The problems encountered, such as Internet access and technical issues, match those of other studies. Although the rate of participants living in city centers where internet access is more possible than in rural areas was 55.6% (Table 1), the rate of internet access difficulty was determined as 56.8%. Students need Internet access to participate in online courses, creating a significant global inequality in veterinary medical education programs (Edwards, 2004). It should be noted that poor connectivity in rural areas can result in lost educational opportunities for students living in these areas (Mohammed et al. 2019). In order to maintain distance education smoothly and successfully, infrastructures should be developed, and students should be supported in terms of internet access in city centers and rural areas.

The 45-minute lesson period that were applied before the pandemic was changed to 30 minutes in primary, middle, high school education (MNE 2020). Higher Education Institutions maintained this period as 40-45 minutes. If online courses take a long time, students feel sleepy and tired and lose their motivation to participate in online learning (Mahdy 2020; Wilcha, 2020). A study conducted in the field of social sciences reported that the highest rate of students (36.2%) preferred the lecture duration to be between 15 and 20 minutes (Serçemeli and Kurnaz 2020). Afşar and Büyükdoğan (2020) determined in their study that 90.4% of 432 students claim 25 minutes lessons or more. It was determined in the study that 36.0% of the students stated that it should be in the range of 21-30 minutes, 35.3% in the range of 31-40 minutes, and 12.9% between 41-50 minutes (Table 5). Even though departments have different preferences regarding course length, it is crucial to keep these durations within reasonable bounds to maintain followability. The ideal course duration should be determined in line with the student's opinions and applied in distance education processes in this manner.

A study conducted with students from the Education Department studying in the Central Anatolia Region stated that 22% of the students prefer oral presentations, and 18.7% prefer scanned lecture notes (Karatepe et al. 2020). In the study of Serçemeli and Kurnaz (2020), 79.4% of the students expressed that lectures should be supported with lecturer's notes, 34.6% with online video recordings, and 33.5% with documents such as PDF, word, and PowerPoint. Frequent access to asynchronous learning materials improves veterinary students' performance (Schoenfeld-Tacher and Dorman 2021). Can (2020) also stated that students prefer written materials and less participation in online courses and that the reason for this is the problems experienced in accessing the Internet. Mahdy (2020) reported that participants were provided online study materials mainly through PDF lecture notes, e-books, YouTube videos, university platforms, educational websites, and applications. In the study, students were asked, "Which of the following methods would you like distance education courses to be supported with?" and 78.2% of the participants answered with documents such as PDF, word, PowerPoint; 77.2% with the lecturers' notes; 66.1% with online video recordings (Table 6). Thus, students prefer sources such as the instructor's notes, PDF, word, and PowerPoint because they are always accessible and not likely to be affected by internet access problems.

CONCLUSION

As a result, given the importance of practical/applied veterinary education, students from veterinary departments significantly prefer face-to-face education. This preference may concern that their

professional development will be improved with face-to-face practical/applied learning. Accordingly, mainly applied learning lessons should be done face-to-face as much as possible while taking all necessary precautions and giving students the right to choose. Studies should be carried out to eliminate the concerns of the students by fulfilling the missing training after the pandemic. Veterinary students' thoughts and opinions can guide this issue by identifying problems in distance education in general.

Ethics Committee Approval: The permission for the study was received on December 04, 2020, from the "Non-Interventional Research Ethics Board of the Department of Health Sciences of Kafkas University."

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RESEARCH ARTICLE

Investigation of Ochratoxin A Level in Feed Used in Bovine Feeding in Ardahan Province

Ertan DOĞAN[1]

[1]*Department of Laborant and Veterinary Health, Vocational School of Nihat Delibalta Göle, Ardahan University, Ardahan, Türkiye*

ABSTRACT

This study's objective is to determine the quantity of Ochratoxin A (OTA) in the feed given to cattle in the Ardahan region. The research was carried out on 30 randomly selected bovine farms in the Ardahan region. Cattle farms were visited in February, March, and April 2023. Thirty samples (10 meadow grass, 10 hay and 10 crushed barley) were collected each month, one sample from each farm visited. In all, 90 feed samples were examined for OTA within the three-month period. The amount of OTA in feed samples was determined by using an ELISA kit. The study did not detect OTA in the meadow grass, hay and crushed barley collected in February. In hay samples, 0.19 μg/kg was detected in March, 0.21 μg/kg in April, and in crushed barley 0.20 μg/kg in March and 0.21 μg/kg OTA in April. In this study, the highest amount of OTA was determined in meadow grass (0.28 μg/kg) collected in April. The difference between the average OTA amounts detected in meadow grass in March and April was statistically significant ($p<0.05$). It was seen that the amount of OTA detected in the meadow grass, hay and crushed barley was below the maximum limit (5.0 μg/kg) set by the Turkish Food Codex Contaminants Regulation. In this study, it was concluded that meadow grass, hay, and crushed barley given to cattle in February, March, and April in Ardahan region contain ochratoxin A at a level that does not pose a risk to animal and human health.

Keywords: Ardahan, Cattle, Feed, Mycotoxin, Ochratoxin A.

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Ardahan Yöresinde Sığır Beslenmesinde Kullanılan Yemlerde Okratoksin A Düzeyinin Araştırılması

ÖZ

Bu araştırmanın amacı Ardahan yöresinde sığırlara verilen yemlerde Okratoksin A (OTA) miktarını belirlemektir. Araştırma Ardahan yöresinden rastgele seçilen 30 sığır işletmesinde yürütüldü. Sığır işletmeleri 2023 yılı Şubat, Mart ve Nisan aylarında ziyaret edildi. Her ziyaret edilen işletmeden bir numune olmak üzere her ay 30 numune (10 adet çayır otu, 10 adet saman ve 10 adet arpa kırması) toplandı. Üç aylık dönemde toplam 90 yem örnekleri OTA yönünden incelendi. Yem örneklerinde OTA miktarının belirlenmesinde ELİSA kiti kullanıldı. Araştırmada Şubat ayında toplanan çayır otu, saman ve arpa kırmasında OTA tespit edilmedi. Saman örneklerinde Mart ayında 0,19 μg/kg, Nisan ayında 0,21 μg/kg, arpa kırmasında ise Mart ayında 0,20 μg/kg, Nisan ayında 0,21 μg/kg OTA tespit edildi. Bu araştırmada en yüksek OTA miktarı Nisan ayında toplanan çayır otunda (0,28 μg/kg) belirlendi. Mart ve Nisan ayında çayır otunda tespit edilen ortalama OTA miktarları arasındaki fark istatistiksel olarak anlamlı ($p<0,05$) bulundu. Çayır otu, saman ve arpa kırmasında tespit edilen OTA miktarı Türk Gıda Kodeksi Bulaşanlar Yönetmeliğinin belirlediği maksimum limitin (5,0 μg/kg) altında olduğu görüldü. Bu araştırmada Ardahan yöresinde Şubat, Mart ve Nisan ayında sığırlara verilen çayır otu, saman ve arpa kırmasının hayvan ve insan sağlığı için risk oluşturmayacak düzeyde okratoksin A içerdiği sonucuna varıldı.

Anahtar kelimeler: Ardahan, Mikotoksin, Okratoksin A, Sığır, Yem.

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ORCID ID; *ED: 0000-0003-0751-0559*

*Corresponding author e-mail: ertandogan@ardahan.edu.tr

INTRODUCTION

Various toxins are synthesized by some species of fungi in foods and feedstuffs. These toxins are called mycotoxins (Tao et al. 2018). Ochratoxins, a type of mycotoxin, are secondary metabolites synthesized by some fungi of the genus Penicillium and Aspergillus (Battacone et al. 2010). *Penicillium verrucosum, Penicillium nordicum and Aspergillus ochraceus*are the most important species synthesizing ochratoxin.According to their chemical structure, ochratoxins are divided into three groups: ochratoxin A, ochratoxin B and ochratoxin C. The most toxic of these is ochratoxin A (OTA) (Wang et al. 2022). OTA is a white-colored, odorless, heat-resistant, water-soluble, crystallized solid molecule. Its chemical structure is formed by dihydroisocoumarin, which binds to beta-phenylalanine via 7-carboxyl (Tao et al. 2018).

When food containing OTA is consumed by humans and animals, severe health problems occur. The liver and kidneys are especially sensitive to this toxin. Furthermore, mutagenic, immunotoxic, teratogenic, genotoxic, embryotoxic, and carcinogenic effects have been reported (Turkoglu and Keyvan 2019; El-Sayed et al. 2022; Miguel Alfonso et al. 2022; Wang et al. 2022). OTA causes chronic kidney damage (Balkan Endemic Nephropathy) in humans. It is also reported to induce liver and kidney tumors. For this reason, it is included in the 2B carcinogen group by the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) (Bernardini et al. 2014; Stoev 2021; El-Sayed et al. 2022).

The susceptibility of animal species to OTA is associated with the structure of the digestive tract. In single-stomach animals, nearly all the toxin is absorbed, whereas in ruminants, microbial decomposition occurs in the rumen. It undergoes peptide hydrolysis microbial enzymes. Following this hydrolysis, phenylalaninand dihydroisocoumarin are released. The degradation products are also known as ochratoxine α. Ochratoxin α is less poisonous than OTA (Boudra et al. 2007; Marin et al. 2013; Miguel Alfonso et al. 2022). The toxins absorbed through the gastrointestinal tract bind strongly to serum proteins (particularly albumin). This relationship determines its half-life in the bloodstream and varies depending on the animal species (Battacone et al. 2010). OTA and ochratoxin α are mostly excreted in urine and feces (Boudra et al. 2007).

The types of fungi in the contaminated agricultural products vary depending on the geographic area. *Penicillium verrucosum* generally reproduces in temperate areas, while *Aspergillus ochraceus* synthesizes ochratoxin by reproducing in warm climates (Battacone et al. 2010). The toxin inhibits protein synthesis in cells. It also leads to mitochondrial lesions and DNA damage, lipid peroxidation and oxidative stress. As a result of the toxic effect of the toxin on DNA, mutagenic, genotoxic, and carcinogenic effects occur (Marin et al. 2013; Miguel Alfonso et al. 2022). OTA was detected in 76% of tumorous kidneys that underwent nephrectomy in humans (Malir et al. 2021).

OTA can be found in numerous agricultural products. Cereals, wine, beer, coffee, and grapes are some of them. OTA is synthesized when suitable conditions (such as humidity, temperature, and oxygen) are created in contaminated nutrients. It passes into the meat and milk of animals fed with contaminated feedstuffs. The OTA content in animal products presents a risk to human health (Battacone et al. 2010; Miguel Alfonso et al. 2022; Wang et al. 2022). Moreover, they are very heat resistant and do not decompose with normal milk processes (such as pasteurization and UHT). It is necessary to apply at least 250ºC of heat for a few minutes to decompose the toxin (Tao et al. 2018). Therefore, most countries have a maximum limit for OTA in nutrients. This limit is 5 ppb for non-processed cereals, 3 ppb for cereals, and 2 ppb for beer and grape juice by the European Commission. It is applied by the Food and Agriculture Organization as 5 μg/kg for barley, wheat, and rye (Wang et al. 2022). According to the Turkish Food Codex Contaminants Regulation, the maximum level of OTA in untreated cereals is 5 μg/kg (Anonymous 2011).

The main source of livelihood in Ardahan province is cattle breeding. Local livestock complete the year by feeding approximately half in grasslands and pastures and the other half in shelters. The period in the shelter environment coincides with the winter and spring seasons. During these periods, cattle are fed stocked meadow grass. In addition, hay and crushed barley obtained by harvesting cereals (especially barley) are among the important forage items. It is very important to human health to feed cattle with safe food. Because animal products (meat and milk) produced from cattle are a major source of nutrients for humans (Doğan and Doğan 2020). The contamination of animal products with toxins gives rise to serious human health problems. Therefore, no substances harmful to human health should be present in animal products. Animal products containing OTA may be a source of toxins for people (Wang et al. 2022). These toxins go into breastfeeding mothers' milk and negatively affect the baby's health. They also build up in tissues and organs, like muscles and the liver, which are important sources of nutrients for humans (Battacone et al. 2010).

Penicillium verrucosum, a fungus that synthesizes OTA, can grow in agricultural products grown in temperate climates (Clark and Snedeker 2006). The Ardahan region has a cool and moderate climate. In addition, the food harvested during summer (August) is stored and kept until winter. This waiting period strengthens the possibility of OTA synthesis in stock feed materials. Literature reviews have found no research on the presence of OTA in livestock feeds in the Ardahan region. The objective of this investigation was to assess the amount of OTA contained in

certain feed materials given to cattle that are fed indoors during winter and spring in the Ardahan region.

MATERIALS and METHODS

The study began with the approval of the Ministry of Agriculture and Forestry (letter dated 07.11.2022 and numbered E-29486769-325.99-7632633). The research material consisted of certain feeds that were fed to cattle in the province of Ardahan. 30 cattle enterprises that are actively operating in the region were identified for this purpose. The determination of enterprises did not take into account the size of the enterprise and the criteria for animals (number, breed, age, and gender). These farms were visited in mid-February, March, and April 2023 (day 15). In each month visited, 30 samples were collected, one feed sample from each farm. During the three-month study period, 90 (30 meadow grass, 30 hay, and 30 crushed barley) feed samples were collected. Approximately 50 grams of the feed materials served to the cattle were taken and the samples were placed in sterile and sealed bags. After being brought to the laboratory in cold conditions, the samples were stored at -18 °C until analysis.

ELISA kit (Cat. No: CSB-EFD027449, CusaBio, U.S.A) was used to determine the OTA level in feed samples. Sample preparation and analysis were conducted according to the method described by the manufacturer. In this method, 5 grams of crushed samples were placed in a beaker. 25 ml of 40% ethanol solution was added to it. It was then stirred in the vortex for two minutes. 5 mL of the resulting mixture was centrifuged at 4000 rpm for 5 minutes. After centrifugation, 500 µL of the supernatant was taken and was thoroughly mixed with 500 µL of the sample dilution solvent. Then, 50 µL of the resulting mixture was taken and used for analyzes. The standards and well counts for the samples were determined prior to starting the analysis. 50 ml of standard was added to the standard wells and 50 µL of sample was added to the sample wells. Then, 50 µL of HRP-conjugateand 50 µL of antibody were added to each well. The plate was coated with a new adhesive strip and mixed thoroughly. After that, it was left to incubate at 25°C for 30 minutes. The plate was washed four times with the washing solution after incubation. The plate was placed upside down after the last wash, the solution inside was diverted, and drained with a clean paper towel. Then 100 µL of TMB substrate was added to each well and mixed well. The plate was left to incubate at 25°C for 15 minutes, protected from light. After incubation, 50 µL of stop solution was added to each well and the optical density of the sample and standards were measured at 450 nm in an ELISA reader (BioTek ELx800, USA). The amount of OTA was calculated by comparing the optical density of the samples with the optical density of the standards (Yılmaz and Aksu Elmalı 2016).

Statistical evaluation of the data obtained in this study was done using IBM SPSS 20.0 software. The data was checked for normality using the Shapiro-Wilk test. The comparison of the average OTA amount by month was conducted using an independent sample T-test. The results were presented in the form of an average ($\bar{X}$) and a standard deviation (SD). $P<0.05$ was found to be statistically meaningful for this study.

RESULTS

The amount of OTA in feed collected in the Ardahan region in February, March, and April is presented below in Table 1.

As seen in Table 1 above, OTA was not detected in the meadow grass, hay and crushed barley collected in February. OTA was 0.20 µg/kg in meadow grass samples collected in March and 0.28 µg/kg in April. The difference in mean OTA levels detected in meadow grass in March and April was statistically significant ($p<0.05$). The quantity of OTA in the hay samples was 0.19 µg/kg in March and 0.21 µg/kg in April. For barley crushing, it stood at 0.20 µg/kg in March and 0.21 µg/kg in April. In hay and crushed barley collected in April, the amount of OTA increased compared to March. However, the increase was considered statistically non-significant ($p>0.05$).

Table 1. Amount of OTA in Feed by Months (µg/kg).

Samples	February		March		April		P-value
	n	$\bar{X} \pm$ SD	n	$\bar{X} \pm$ SD	n	$\bar{X} \pm$ SD	
Meadow grass	10	0 ± 0 *	10	0.20 ± 0.03 [a]	10	0.28 ± 0.06 [b]	0.003
Hay	10	0 ± 0 *	10	0.19 ± 0.03 [a]	10	0.21 ± 0.03 [a]	0.29
Barley Crushed	10	0 ± 0 *	10	0.20 ± 0.04 [a]	10	0.21 ± 0.04 [a]	0.63

*: It was not detected in the range of test susceptibility limits.

a, b: Those who have different letters on the same line were statistically significant in the $p<0.05$ range.

DISCUSSION

The effects of OTA have been investigated on a number of animal species. It is reported that when applied to female mice at a dose of 1 mg/kg for one week, it disrupts egg development and causes decreases in the number of offspring (Jia et al. 2020). Moreover, it is pointed out that tumors of the kidneys, liver and mammary glands are the causal agents of female mice (Tao et al. 2018) and kidney tumors in male mice (Clark and Snedeker 2006). In a study, degenerative changes in neuron and glial cells were found in mice fed with feeds containing 10 ppm OTA, adenocarcinoma in the intestines at the 19th month and in the lungs at the 21st month (Stoev 2021). In another study, pigs fed with feed containing OTA showed a decrease in sperm motility and a significant reduction in height on the 40th day following ingestion of toxins (Solti et al. 1999).

Cattle are subject to OTA with raw materials. Susceptibility to the toxin is related to ruminal function. One study indicates that the uptake and adverse effects of OTA increase in cattle fed high-sugar feeds (Pantaya et al. 2016). Healthy livestock with improved rumen function can decompose up to 12 mg/kg of the toxin. Exposure of calves whose ruminal function is undeveloped to the toxin threatens the health of calves. Because calves have a greater absorption of toxins than adult bovine animals. The long half-life of the binding toxin in serum proteins (about 77 hours) increases toxicity. It is reported that 1 mg/kg OTA administered to calves with undeveloped ruminal function results in deaths within 24 hours (Battacone et al. 2010).

A number of studies have been carried out to determine the level of OTA in foods and feedstuffs. In 82 commercial cattle fattening feeds collected from different regions of Türkiye, the highest amount of OTA (4.04 µl / kg) was determined in the Marmara region and the lowest (0.76 µl /kg) was determined in the Aegean region (Akkaya and Bal 2013). In a study conducted in the Sivas region, it is reported that the amount of OTA in December, February, and April is 0.025, 0.024, and 0.024 ppm in compound feeds and 0.022, 0.020, and 0.021 ppm in barley samples respectively (Yılmaz and Aksu Elmalı 2016).

In cattle fed with OTA, secretion of toxins through the milk is of great importance to calves and human health (Turkoglu and Keyvan 2019; Miguel Alfonso et al. 2022; Wang et al. 2022). In one study, it was reported that the average OTA amount of 105 milk samples (35 pasteurized, 35 raw milk, and 35 UHT) was 85 ng/L in UHT milk, 137 ng/L in raw milk, and 135 ng/L in pasteurized milk (Turkoglu and Keyvan 2019). In 40 milk samples collected from milk tanks in Burdur region, the amount of OTA is stated to be in the range of 2-270 ng/L (Keyvan et al. 2018). In one Egyptian study, the average amount of OTA in 10 samples of raw milk was found to be 5.134 ppb (the lowest 0.34 ppb and the highest 13 ppb) (Younis et al. 2016). Three of the 264 milk samples collected at 132 farms in winter and summer (1.1%) contained OTA (Boudra et al. 2007). In a study conducted in Sudan, it is stated that 77.78% of the feed samples given to dairy cattle contained 0.22-1.59 µg/kg and 20% of the milk samples contained 2.73 µg/kg OTA (Elzupir et al. 2009).

OTA was not detected in meadow grass, hay, and barley samples collected in February in this research. OTA levels in meadow grass samples were 0.20 µg/kg in March and 0.28 µg/kg in April. The OTA content of the hay samples was 0.19 µg/kg in March and 0.21 µg/kg in April. The mean level of OTA in crushed barley was 0.20 µg/kg in March and 0.21 µg/kg in April. The results obtained from this study are similar to those reported by Akkaya and Bal (2013), Yılmaz and Aksu Elmalı (2016), Türkoğlu and Keyvan (2019), Keyvan et al. (2018), Younis et al. (2016), Boudra et al. (2007), and Elzupir et al. (2009). In this study, the highest level of OTA was detected in meadow grass collected in April. The difference between the average OTA amounts detected in meadow grass in March and April was statistically significant ($p<0.05$). It is thought that the increase in the amount of OTA in meadow grass in April is influenced by the air temperature and the increased humidity as a result of the melting of the snow. Because in the region, meadow grasses are stored in bales and outdoors. The detection of OTA in these grass piles can be explained as the fact that the fungal species synthesizing OTA are contaminated and that suitable conditions (heat, humidity, oxygen, etc.) are formed for their reproduction.

In this study, it was found that the amount of OTA in the hay and barley crushing samples collected in April was higher than in March. However, this increase was found to be statistically insignificant ($p>0.05$). In April, hay and barley samples had low levels of OTA compared to meadow grass. The low level of OTA in hay samples is mainly due to the way straw is obtained. Because straw is obtained by drying and crushing the stem of cereals (especially barley in the region) thoroughly. Also, in the locality hay is stored in sacks. As a result, it may be argued that straw is drier (at lower moisture) than meadow grass. It may be said that the low moisture content of hay limits fungal growth and toxin synthesis. The low level of toxins in barley crushing may be due to storage conditions. In the Ardahan region, barley crushed is either put in sacks and closed or stored in a closed environment in warehouses. The anaerobe environment created by these storage methods is thought to negatively impact fungal reproduction. Because mushrooms are mandatory aerobics. These storage conditions explain the low OTA content in barley crushed.

CONCLUSION

In the Ardahan region, OTA was not detected in the meadow grass, hay and crushed barley used in cattle feeding in February. OTA was 0.20 μg/kg in meadow grass samples collected in March and 0.28 μg/kg in April. In hay samples, 0.19 μg/kg was detected in March, 0.21 μg/kg in April, and barley crushed 0.20 μg/kg in March and 0.21 μg/kg OTA in April. Concentrations of OTA detected in the meadow grass, hay and crushed barley were found to be below the maximum level set by the Turkish Food Codex Contaminants Regulation. In this research, it was concluded that the meadow grass, hay and crushed barley given to cattle in February, March, and April in the Ardahan region did not pose a risk to human and animal health. It is recommended to take measures to prevent fungal growth in feed materials and to provide the necessary training to the breeders about not using moldy feeds in cattle nutrition.

Conflict of Interest: The author declares that there is no conflict of interest.

Ethics Committee Information: This study was approved by the Ministry of Agriculture and Forestry (letterdated 07.11.2022 and numbered E-29486769-325.99-7632633).

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RESEARCH ARTICLE

A Retrospective Study on Pelvic Fractures in Cats and Dogs (2020-2022)

Emine ÇATALKAYA[1*], Sadık YAYLA[1], Semih ALTAN[2], Berna ERSÖZ KANAY[1], Nahit SAYLAK[1], Şimal HATİPOĞLU[1]

[1]Dicle University, Faculty of Veterinary Medicine, Department of Surgery, Diyarbakır/Türkiye
[2]Dokuz Eylül University, Faculty of Veterinary Medicine, Department of Surgery, İzmir/Türkiye

ABSTRACT

Pelvic fractures occur in many traumas in cats and dogs. In this study, it was aimed to evaluate the treatment (conservative and operative) and its results together in cats and dogs diagnosed with pelvic fracture. The study material consisted of 223 pelvic fractures (15 acetabulum, 40 ischia, 68 ilium, 38 pubis and 62 sacroiliac separation) detected in 27 dogs and 75 cats of different ages, breeds and genders between 2020-2022. Pelvic fracture was diagnosed with clinical and radiological examination. It was treated conservatively and operatively (using iliosacral screw, iliosacral pin, locking plate, cerclage, acetabular C plate). It was observed that cases with pelvic fractures were mostly caused by falling from a height in cats and traffic accidents in dogs. It was also observed that there could be a single fracture in the pelvis as well as multiple fractures. A total of 73 cases were operated. In 55 of the fractures, iliosacral screw, 1 iliosacral pin, 45 locked plate, 9 cerclage application, 10 acetabular C plate and 1 excision arthroplasty were applied. Conservative treatment was applied to 29 cases. No complications were observed in the controls performed in the operated patients. It was concluded that good results were obtained in stabilization with screws in cases of sacroiliac separation, and the use of locking plates in ilium fractures and the use of acetebular C plate in acetabulum fractures could lead to a more comfortable and comfortable postoperative period.
Key words: Acetabular C plate, cats, dogs, locking plate, pelvic fractures

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Kedi ve Köpeklerde Kalça Kırıkları Üzerine Retrospektif Bir Çalışma (2020-2022)

ÖZ

Kedi ve köpeklerde birçok travmada pelvik kırıklar meydana gelir. Bu çalışmada pelvik kırık tanısı konulan kedi ve köpeklerde tedavi (konservatif ve operatif) ve sonuçlarının birlikte değerlendirilmesi amaçlandı. Çalışma materyalini 2020-2022 yılları arasında farklı yaş, cins ve cinsiyetteki 27 köpek ve 75 kedide tespit edilen 223 pelvik kırık (15 asetabulum, 40 ischi, 68 ilium, 38 pubis ve 62 sakroiliak ayrılma) oluşturmuştur. Pelvis kırığı tanısı klinik ve radyolojik muayene ile konuldu. Konservatif ve operatif olarak (iliyosakral vida, iliosakral pin, kilitli plak, serklaj, asetebular C plak kullanılarak) tedavi edildi. Pelvik kırık vakalarının kedilerde en çok yüksekten düşme, köpeklerde ise trafik kazası sonucu meydana geldiği görüldü. Pelviste tek kırık olabileceği gibi birden fazla kırık da olabileceği gözlendi. Toplam 73 vaka ameliyat edildi. Kırıkların 55'ine iliosakral vida, 1'ine iliosakral pin, 45'ine kilitli plak, 9'una serklaj uygulaması, 10'una asetabular C plak ve 1'ine eksizyon artroplastisi uygulandı. 29 olguya konservatif tedavi uygulandı. Ameliyat edilen hastalarda yapılan kontrollerde herhangi bir komplikasyon görülmedi. Sakroiliak ayrılma durumlarında vidalarla stabilizasyonda iyi sonuçlar elde edildiği, ilium kırıklarında kilitli plak, asetabulum kırıklarında ise asetabular C plak kullanımının ameliyat sonrası dönemi daha rahat ve konforlu geçirebileceği sonucuna varıldı.
Anahtar kelimeler: Asetabuler C plağı, kedi, köpek, kilitli plak, pelvik kırıklar

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ORCID ID; *EÇ: 0000-0001-7884-5407; SY: 0000-0001-6734-421X; SA: 0000-0003-3158-3678; BEK: 0000-0001-5165-0618; NS: 0000-0003-2008-5403; ŞH 0000-0003-2230-2731* *Corresponding author e-mail: eminecatalkaya21@gmail.com

INTRODUCTION

Pelvic fractures constitute at least 25% of all fractures seen in small animal practice, therefore they are important in traumatic injuries of dogs and cats (Olmstead 1998; Piermattei et al. 2006; Harasen 2007). The configuration of the pelvis, like a rectangular box, ensures that any trauma that would cause a fracture will result in multiple fractures. On the positive side, the major muscle groups around the pelvis provide significant natural stability to most fractures, often eliminating the need for surgical repair (Olmstead 1998; Denny and Butterwort; 2000, Piermattei et al. 2006; Harasen 2007). Pelvic fractures often develop as a result of major traumas such as traffic accidents (Bouabdallah et al. 2020; Yurtal et al. 2022). One or both sacroiliac joints are usually luxated in animals exposed to posterior trauma. There is an associated sacral fracture or combinations of long, oblique fractures in one or both ilia. In animals exposed to side trauma, however, the femoral head may push towards the acetabulum, which may cause fracture of the ilium, pubis, and acetabulum (Olmstead 1998, Harasen 2007).

Most animals (75%) with pelvic fractures heal without surgery. The most common criteria for recommending surgical repair of a pelvic fracture are:

- Displaced acetabulum fractures, especially involving the 2/3 cranial of the acetabulum
- Narrowing of the pelvic canal by more than 1/3 of the diameter with fracture fragments;
- Neurological impairment including persistent pain attributable to fracture
- İpsilateral fractures of the ilium, the ischium and pubis, resulting in an unstable hip joint (Olmstead 1998; Aksoy & Özsoy 2003; Harasen 2007; Meeson and Geddes 2015).

Conversely, minimally displaced fractures, fractures beyond the cranial 2/3 of the acetabulum, cases in which pain is well-managed, or fractures older than 7-10 days are often best treated with cage rest (Olmstead 1998; Bouabdallah et al. 2020; Bourbos et al. 2020; Harasen 2007).

The most common pelvic fractures for which surgery may be considered are sacroiliac luxations, iliac fractures, and acetabulum fractures (Aksoy and Özsoy 2003; Piermattei et al. 2006; Harasen 2007, Bourbos et al. 2020; Yurtal et al. 2022). Sacroiliac luxations or fractures are a source of pain and instability, and are the most common pelvic fracture associated with neurological deterioration (Piermattei et al. 2006; Harasen 2007; Meeson and Cor 2011; Sadan 2016). The fracture or dislocation causes craniodorsal displacement of the ilium. Surgical repair of bone screws can be performed (Zamirbekowa et al. 2021).

The most common fractures of the pelvis are fractures of the ilium (46% of all pelvic fractures) (Olmstead 1998; Denny and Butterwort 2000; Altunatmaz et al. 2004; Piermattei et al. 2006; Harasen 2007; Meeson and Cor 2011; Zamirbekowa et al. 2021; Yurtal et al. 2022). These fractures are important in terms of narrowing the pelvic canal and causing trauma to the lumbosacral nerve trunk that branches into the sciatic nerve. Bone plate repair is the most common and successful method of surgical treatment (Olmstead 1998; Denny and Butterwort 2000; Altunatmaz et al. 2004; Piermattei et al. 2006; Harasen 2007; Meeson and Cor 2011; Yurtal et al. 2022).

In addition, surgical repair is indicated for fractures of the acetabulum. Surgical repair of acetabular fractures with bone plates or bone screws, interfragmentary wire can yield good clinical results if rigid fixation and anatomical reduction are provided. If surgical repair of the acetebulum is not possible, salvage procedures such as femoral head and neck ostectomy are performed (Piermattei et al. 2006).

In this study, it was aimed to evaluate the treatment (conservative or operative) and its results in cats and dogs with pelvic fractures brought to our hospital with various etiologies.

MATERIAL and METHODS

Medical records and radiographs of cats and dogs with pelvic fractures in Dicle University Veterinary Faculty Animal Hospital Surgery Clinic between 2020 and 2022 were reviewed. According to data obtained from medical records, gender, age, body weight, gender, and recovery status after surgical treatment were evaluated. The number and location of pelvic fractures were determined on preoperative lateral and ventrodorsal radiographs of the pelvis.

Surgical techniques were determined according to the size and location of the fracture. For sacroiliac luxation, stabilization of the sacroiliac joint was achieved with the application of one or two long cortical screws or a U-shaped percutan pin (Figure 1). Locking plates or wires were applied to stabilize the iliac fracture (Figure 2). Acetabular C plate was used for stabilization of acetabular fractures (Figure 3). Femoral head and neck osteotomy (FHO) was performed in cases where the caput femoris was fractured together with the acetabulum. Conservative treatment and cage rest were recommended for ischial or pubic fractures.

Postoperative clinical follow-up and evaluation of complications were based on two considerations. The first of these was related to the incision wound. The second was the evaluation of permanent lameness. The degree of lameness was scored from 0 to 4 (0= no lameness; 1= subtle lameness; 2= obvious lameness; 3= intermittent, non-weight-bearing lameness; 4= non-weight-bearing lameness) (Kim et al. 2011).

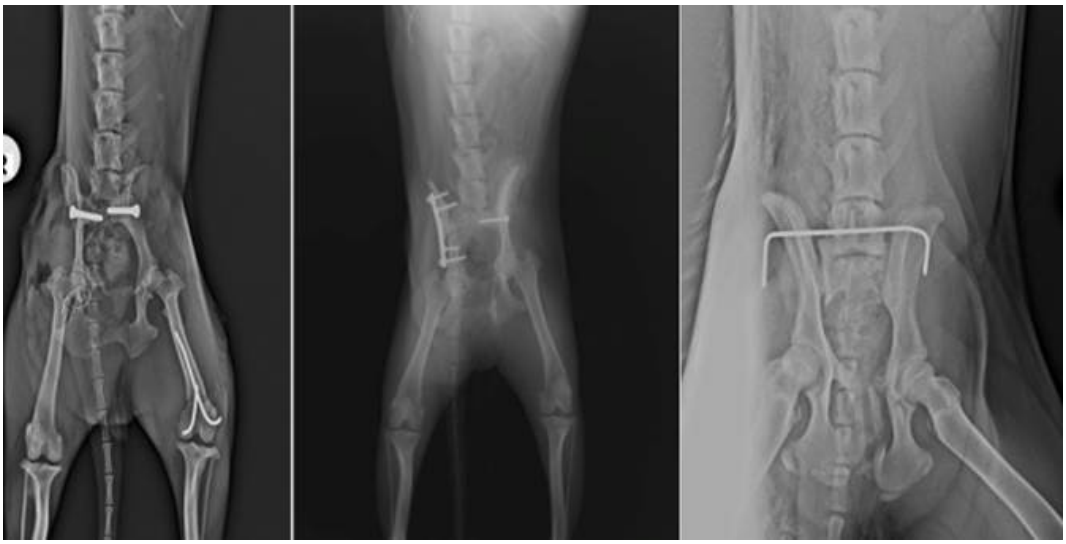

Figure 1: Sacroiliac separations. It is also possible to place one or two screws lateral to the sacroiliac joint. In addition, especially in bilateral cases, a pin passed percutaneously from the ala ossis ilium from the lateral side can be advanced until it emerges from the ala ossis ilium on the opposite side and placed in a U shape.

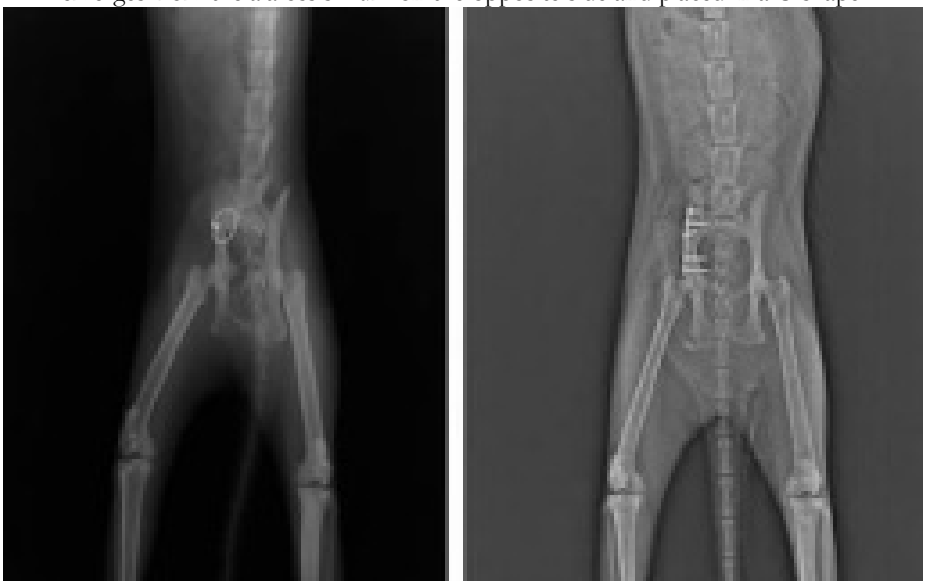

Figure 2: It is doubtful that the application of cerclage wire, which is passed through the holes drilled into the fragments in os ilium fractures, will provide adequate immobilization, but nail, plate and screw applications can also be performed depending on the localization of the fracture. In the study, plaque was applied in 45 cases and cerclage was applied in 5 cases. Functional improvement was achieved in both applications. However, it can be said that the decrease in the costs of vinyl with the developing technology in recent years has contributed to the widespread use of this practice.

Figure 3: Fractures in which the acetabulum is affected. The acetabulum is very important for the function of the coxofemoral joint, and the application of acetabular C plate is more recommended in order to avoid complications such as coxoarthrosis in fractures of the acetabulum that include the articular surface and have collapsed.

RESULTS

It was obtained from the registration information that among the total of 512 patients with trauma-induced orthopedic fractures in 2020-2022, the number of patients with pelvic fractures was 102 (19.92%). These pelvic fractures were reported in 27 dogs (26.47%) and 75 cats (%73.52). Body weights ranged from 12 kg to 60 kg in dogs and between 1.2 kg and 6.2 kg in cats. The average body weight is 23.03 kg for dogs and 2.4 kg for cats. The mean age of dogs at the time of fracture was 2.65 years (1 to 5 years) and 1.95 years (4 months to 5 years) in cats. 14 dogs (13.72%) and 46 cats (45.09%) were male, 13 dogs (12.74%) and 29 cats (28.43%) were female. In the clinical examination of dogs and cats, there were different degrees of lameness from grade 1 to grade 4.

While the cause of pelvic fractures in cats was falling from a height (n=69, 67.64%), traffic accident (n=4, 3.92%), unknown (n=2, 1.96%), it was observed that this condition occurred in dogs in a traffic accident (n=21, 20.58%) and the cause of unknown origin (n=6, 5.88%).

The distribution of the pelvic fractures of the cats and dogs included in the study and the treatments applied are summarized in table 1 and table 2. In this study, which was conducted on 75 cats and 27 dogs, 223 fractures were evaluated. Because more than one fracture was found in the pelvis of the same animal. In addition, there were patients with bilateral iliosacral separation (85.21% in cats, 76.92% in dogs) and bilateral iliac fractures (32.25% in cats, 13.33% in dogs).

Table 1. Pelvic fracture and treatment distributions of 75 cats

Fractures		**Treatment selection**	**Operative**	**Conservative**
Sacroiliac separations	39	Iliosacral screw	35 (19 cats)	4 (2 cats)
Ilium fractures	51	Locked plate	34 (29 cats)	14 (9 cats)
		Cerclage	3 (3 cats)	
Acetabulum	14	Acetabular C plate	10 (10 cats)	2 cats
		Other (Cerclage or excision arthroplasty)	2 (2 cats)	
Ischia	28	Cerclage	3 (3 cats)	25 (21 cats)
Pubic	27	-	**-**	27 (21 cats)
Total	159	87 fractures (54 cats)		72 fracture (21 cats)

An animal may have more than one fracture. Also, one animal had bilateral fractures.

Table 2. Pelvic fracture and treatment distributions of 27 dogs.

Fractures		**Treatment selection**	**Operative**	**Conservative**
Sacroiliac separations	23	Iliosacral screw	19 (11 dogs)	2 (1 dog)
		U pin	2 (1 dog)	
Ilium fractures	17	Locked plate	11 (9 dogs)	4 (4 dogs)
		Cerclage	2 (2 dogs)	
Acetabulum	1	Cerclage	1 (1 dog)	-
Ischia	12	-	-	12 (8 dogs)
Pubic	11	-	**-**	11 (7 dogs)
Total	64	35 fractures (19 dogs)		29 fracture (8 dogs)

An animal may have more than one fracture. Also, one animal had bilateral fractures.

In cases with a single fracture, patients were discharged on the same day, while patients with multiple fractures were discharged a day later after being checked. Discharge time for both cats and dogs who underwent unilateral surgery was significantly shorter than those with multiple fractures or bilateral surgery. No implant-related complications were observed in any of the pelvic fractures in cats and dogs, or a second surgery was not required.

Among the cases, complications such as blood in urine and feces were seen in 20 cats (26.6%) and 7 dogs (25.9%) immediately after trauma, but all of them recovered. At the initial evaluation, 3 cats (4%) and 1 dog (3.7%) had neurological symptoms and recovered. Among the postoperative complications, local symptoms such as accumulation of surgical site serosity and opening of the sutures were observed in 12 cats (%16) and 3 dogs (%11). 5 cats (%6.66) with iliac fractures showed signs of paralysis in the relevant leg, but these symptoms disappeared spontaneously 15 days after the operation. Such a situation was not observed in dogs.

Femoral head resection was performed in 1 cat with displaced ilium and acetabulum fractures, but constipation developed in the 4th postoperative month and megacolon developed in the following period.

Among the cases with sacroiliac separations, those who were fixed with screws started pressing the next day and their lameness scores were 0 at the end of the first postoperative week. In iliac and acetabulum fractures, the healing was better in plate-fixed cases, but the same was not possible in wire-placed cases. It took 2 months for the lameness score to be "0" in 1 cat who developed paralysis with only iliac fracture. In cases who received conservative treatment, it took 2 or 3 weeks to be "0" according to the lameness score.

Despite functional improvement in all cases, permanent lameness remained in 2 dogs (one case in which a cerclage wire was applied to the ilium and another case with multiple fractures treated conservatively). It was seen at the end of the 4th month that all the cats recovered.

DISCUSSION

Pelvic fractures are frequently encountered in cats and dogs. These fractures, which make up about 25% of all fractures in small animals, affect cats and dogs of all ages, but this mostly occurs in young people (Kim et al. 2011; Gant and Asztalos 2019; Arıcan (a) 2019; Arıcan (b) 2019; Bourbos et al. 2020). In this study, pelvic fractures constituted approximately 19.92% of all fracture cases encountered during the two-year period of the study. The age population in previous studies is similar to our study. Because both the active state of young cats or dogs during their walks and their inexperience are thought to be effective.

In previous studies (Aksoy and Özsoy 2003; Altunatmaz et al. 2004; Kim et al. 2011; Sağlam et al. 2016; Bouabdallah et al. 2020; Leffman and Prittie 2022), it has been reported that the etiology of pelvis fractures is generally caused by falling from a height in cats and traffic accidents in dogs. In our study, similar results were obtained in previous studies. It was determined that 67.64% of all cases occurred as a result of falling from a height in cats, and 20.58% occurred as a result of traffic accidents in dogs. In this case, it can be said that the problem of cats falling from heights contributes significantly to pelvic fractures.

The first two common causes of pelvis fractures are falls from height and traffic accidents (Altunatmaz et al. 2004; Hammer et al. 2019; Bouabdallah et al. 2020). They are also a cause of trauma. Therefore, trauma management should be considered. and the vital values necessary to keep the animal alive should be constantly monitored. In addition to the evaluation of the respiratory system and cardiovascular system, neurological functions should be followed up. In addition, the patient should be followed up as a whole, especially in terms of thoracic trauma and urinary system trauma (Meeson and Cor 2011; Kim et al. 2011; Sadan et al. 2016; Gant and Asztalos 2019; Parlak et al. 2021).

Treatment should be planned surgically in sacrum fractures, ilium fractures and 2/3 cranial part (weight bearing region) fractures of the acetabulum, displaced fractures that cause 50% or more narrowing of the pelvic canal (Meeson and Geddes 2015; Arıcan (a) 2019; Arıcan (b) 2019). The surgical procedure varies according to the location and type of fracture. Appropriate implant selection and correct reduction of anatomical placement are associated with a favorable prognosis after surgery (Kim et al. 2011; Arıcan (a) 2019; Arıcan (b) 2019). Conservative treatment is considered when a fracture occurs in the pubis, ischia or caudal 1/3 of the acetabulum (Arıcan (a) 2019; Arıcan (b) 2019; Hammer et al. 2019). In our study, the cases were selected by considering these considerations while planning the surgery. In this study, fixation with a cerclage wire was provided in some of the cases where surgery was recommended due to the financial burden of the owner, but the wire alone never provided an adequate fixation. For this reason, a more comfortable postoperative period was passed in fractures where plate was applied.

In sacroiliac separations, repair failure may occur if the screw length is too short or the screw is applied suboptically. Screw migration has been reported in some studies (Meeson and Cor 2011; Kim et al. 2011; Hammer et al. 2019). In our study, no complications related to screw applications were observed in cases of sacroiliac separation. In addition, these patients had better postoperative comfort than others. In our study, "U pin application" was performed percutaneously in a dog weighing 60 kg and it was observed that this dog recovered with a lameness score of "0". It can be said that sacroiliac separations are more comfortable than other fractures of the pelvis.

Acetabulum fractures are more difficult among pelvic fractures and implant failure and bone fragment displacement can be seen as a complication in these fractures. The acetabular C plate application has found application for this type of fracture (Arıcan (a) 2019; Arıcan (b) 2019). We also used the acetabular C plate in our study and had excellent results. The postoperative comfort of the patients was very good, including their gait. In only 1 cat, the acetabulum was fixed with a cerclage wire due to C plate incompatibility. And although this patient recovers functionally, the result is doubtful because if a fully compatible joint cannot be achieved in terms of osteoarthritis, it is always risky in the long term.

Blood in the urine and stool can be seen in hip fractures. Trauma follow-up should be performed in these patients. Sometimes constipation is also possible. Bowel movements of a patient who cannot walk normally are also affected. In addition, if there is a narrowing in the pelvic canal, the colon is adversely affected (Kim et al. 2011; Kipfer and Montavon 2011; Meeson and Geddes 2015; Pinna et al. 2021). In one of the cat cases, which underwent excision arthroplasty and planned conservative treatment, constipation was observed at the beginning of the postoperative period and megacolon development 4 months later, although the pelvic canal did not completely narrow. Therefore, in cases where the pelvic canal is affected, a good reduction and fixation must be ensured.

It is known that the sciatic nerve may be affected, especially in ilium fractures (Meeson and Cor 2011; Zamirbekova et al. 2021). Some of our cases had neural symptoms associated with the ilium fracture and these disappeared spontaneously, but a cat with a unilateral ilium fracture gave neurological symptoms after plaque placement. This situation was alarming, but after a while this situation disappeared.

CONCLUSION

As a result, we think that good results are obtained in stabilization with screws in sacroiliac separation cases for pelvic fractures, the use of locking plate in ilium fractures gives more effective results and the use of acetabular C plate for acetabular fractures contributes to the postoperative process significantly. In addition, conservative treatment may be considered for ischial and pubic fractures with fractures for which surgery is not indicated.

Conflict of Interest: No potential conflict of interest was reported by the authors.

Authorship Contributions: EÇ, SY, SA and BEK conceived and supervised this study. EÇ, SY, SA, BEK, NS and ŞH collected and analyzed data. EÇ wrote the first draft of manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

The Horse Behaviour toward Grooming

Yavuzkan PAKSOY[1*]

[1]*Department of Plant and Animal Production, Konya Ereğli Kemal Akman Vocational School, Necmettin Erbakan University, Konya, Türkiye*

ABSTRACT

The domestication and artificial selection of horse for various purposes have led to the emergence of various horse breeds, often associated with specific physical characteristics and behavioural tendencies. Understanding individual differences in horse behaviour, particularly during interactions with humans, is essential for promoting positive experiences and welfare. Mane grooming, a common human-horse interaction, provides valuable information about the horse's emotional responses and the preferences. This study was conducted to determine the behaviour of 350 horse seven different horse breeds (101 Thoroughbred horses, 96 Arabian horses, 123 Hanoverian horses, 10 Belgian horses, 6 French horses, 8 Dutch horses, 2 German horses, and 1 Hungarian horse) during mane grooming sessions. The study involved observing the horse from the front and both sides during the mane grooming process and face-to-face interviews with horse owners. The research aims to obtain information about the emotional reactions of the horses by focusing on approach and avoidance behaviours, and it investigates the effects of characteristics such as breed, age, gender and mane direction. Research has revealed that personality differences among seven different horse breeds. Also, it shows that only 10.28 % exhibited aggressive behaviours during the mane grooming. Some differences have been observed among breeds in terms of mane direction. According to the results, it was concluded that breed-specific and individual characteristics should be taken into account in care practices in order to enhance horse welfare and strengthen the human-horse bond.

Keywords: Aggressive behaviour, grooming, horse mane

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Atın Bakıma Karşı Davranışı

ÖZ

Atların çeşitli amaçlar için evcilleştirilmesi ve seleksiyon, farklı fiziksel özelliklere ve davranış eğilimlerine sahip özel at ırklarının ortaya çıkmasına yol açmıştır. Özellikle insanlarla etkileşimde at davranışlarındaki bireysel farklılıkları anlamak pozitif deneyimleri arttırmak ve hayvan refahı bakımından önemlidir. Yaygın bir insan-at etkileşimi olan yele bakımı, atların duygusal tepkileri ve tercihleri hakkında değerli bilgiler sağlar. Bu çalışma, yedi farklı at ırkından 350 atın (101 İngiliz atı, 96 Arap atı, 123 Avrupa atı, 10 Belçika atı, 6 Fransız atı, 8 Hollanda atı, 2 Alman atı ve 1 Macar atı) yele bakım seansları sırasındaki davranışlarını belirlemek amacıyla gerçekleştirilmiştir. Çalışma, yele bakımı sırasında atın önden ve her iki yandan gözlemlenmesini ve at sahipleriyle yüz yüze görüşmeleri içermektedir. Araştırma, yaklaşma ve kaçınma davranışlarına odaklanarak atların duygusal tepkileri hakkında bilgi edinmeyi amaçlamakta ve ırk, yaş, cinsiyet ve yele yönü gibi özelliklerin etkilerini araştırmaktadır. Araştırma, yedi farklı at ırkı arasında kişilik farklılıkları olduğunu ortaya koymuştur. Ayrıca, sadece %10,28'inin yele tımarı sırasında agresif davranışlar sergilediğini göstermektedir. Yele yönü bakımından ırklar arasında bazı farklılıklar gözlenmiştir. Araştırma sonucuna göre, at refahını artırmak ve insan-at bağını güçlendirmek amacıyla bakım uygulamalarında ırka özgü ve bireysel özelliklerin dikkate alınması gerektiğini sonucuna varılmıştır.

Anahtar Kelimeler: Agresif davranış, at yelesi, tıraş

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ORCID ID; *YP: 0000-0002-0935-7693*

*Corresponding author e-mail: yavuzkan7@gmail.com

INTRODUCTION

During the domestication of horses, certain traits were selected through artificial selection to serve different purposes, leading to the emergence of various domestic horse breeds. The process of artificial selection often focuses on physical traits such as size, strength and speed (Clutton-Brock 1999). However, desired behaviours have also been taken into consideration (Hislop 1992; Houpt and Kusunose 2000). Different horse breeds are commonly associated with specific behavioural tendencies, and such claims are often supported by anecdotal evidence from passionate breed enthusiasts. Breeds promote the group by describing its typical temperament and personality. For example, the Highland pony is known for its "kindly nature and even temperament" (Highland Pony Society 2006), while the Irish draught horse is described as having "an intelligent and gentle nature and is noted for its docility and sense" (Irish Draught Horse Society of Great Britain 2006). As a result, it is anticipated that different horse breeds have different personalities. The term "personality" in this context refers to the consistent patterns of feeling, thinking, and behaving in an individual (Pervin and John 1997). While the application of this definition to animals has limitations, studies on horse personality have focused on observing behaviours to establish individual differences rather than attempting to assess feelings or thoughts, which is challenging or impossible to do. Recent studies have explored that assessing individual differences through behaviour tests and through ratings provided by handlers (Le Scolan et al. 1997; Wolff et al. 1997; Anderson et al. 1999; Seaman et al. 2002; Creighton 2003; Momozawa et al. 2003; Visser et al. 2003b; Momozawa et al. 2005). These studies have shown that it is possible to reliably evaluate individual differences in horse behaviour, and there have been discussions about potential applications of this knowledge (Mills 1998).

Horse behaviour toward groomers and mane grooming is a type of interaction with humans that can vary based on individual temperament and past experiences (Lansade et al. 2021; Merkies and Franzin 2021). Mane grooming involves combing, detangling, and styling the mane, which is the long hair along the top of the horse's neck. For many horses, the relationship with their groomers plays a significant role in how they respond to mane grooming. Some horses thoroughly enjoy the attention and relaxation that comes with the grooming process and may stand still and content during mane grooming sessions. They may even nuzzle the groomer or show signs of pleasure, such as lowering their heads or gently swaying their bodies (Lansade et al. 2019). These horses have likely developed a positive association with grooming and trust the groomer's touch. Conversely, some horses may feel uneasy or anxious about mane grooming, especially if they are not familiar with the process or have had negative experiences in the past (Lansade et al. 2018). These horses may display signs of resistance, such as moving away, raising their heads, or tensing their muscles (Lansade et al. 2019). Mane grooming can be particularly sensitive for some horses, as the mane can tangle easily, and detangling might cause discomfort or pain.

The direction of a horse's mane is primarily determined by genetics and the development of hair follicles on the neck (Svensson et al. 2012; Whishaw & Kolb 2016). In most horses, the mane typically grows and falls on either the left or right side of the neck. Some horses may have deviations from the standard left or right mane, with hair growing on both sides or in a non-conforming manner.

The enchanting and unique aspect of horses lies in their beautiful and lustrous mane and tail. Similar to how people have various hairstyles, horses can exhibit different mane "styles" (Jo King 2021). Besides being visually attractive, horse manes serve several purposes, such as pest control, protection, and providing a grip for riders during the early stages of domestication (Jo King 2021). It's important to note that the direction of the mane does not have any impact on a horse's behaviour or performance; rather, it is mainly an aesthetic characteristic (Whishaw & Kolb, 2016). Horse behaviour toward groomers and mane grooming can range from cooperative and relaxed to apprehensive and resistant (Lansade et al. 2018). Building trust and understanding the horse's preferences and comfort levels are essential for creating a positive and enjoyable grooming experience. Mane grooming with patience, kindness, and sensitivity can become a rewarding part of the human-horse relationship, contributing to the horse's overall welfare and fostering a deeper bond with their human caretakers (Bastian 2022).

The aim of this study is to investigate the various behaviours exhibited by seven different horse breeds during mane grooming sessions. The main purpose of this research is to evaluate horses' behavioural responses to grooming, specifically by observing approach and avoidance behaviours. Additionally, the study investigates the impact of characteristics such as horse breed, age, gender, and mane direction on grooming responses.

MATERIALS and METHODS

Animal Materials

The study's animal materials consisted of horses from equestrian clubs and stud farms located in different cities in Turkey (Mersin 2 clubs, Adana 4 clubs, Konya 1 club, Eregli 2 clubs, Osmaniye 2 clubs, Istanbul 2 clubs, Antalya 2 clubs, Tarsus 1 club, Gaziantep 1 club, and Ankara 1 club). Behaviour data from 350 horses from seven different horse breeds

were collected and compared. Horses' breed, age, gender, and coat colour were obtained from the pedigree records kept by the High Council of Stewards of the Ministry of Agriculture and Forestry. The horses diet included oats, barley, hay, apples, and carrots. Table 1 shows the horses breed age and gender information used in the study.

Table 1. The numbers, ages, genders, and breeds of the horses used in the study

Horse Breed	Male Horse Number	Female Horse Number	Minimum Age (Year)	Maximum Age (Year)	Average Age (Year)
Thoroughbred	52	49	1	22	6.24
Arabian	59	37	2	17	6.54
Hanoverian	80	43	2	30	12.14
Belgian	6	4	6	16	13.4
French	3	3	10	16	12.5
Dutch	6	2	2	11	6.875
German	2	-	14	18	16
Hungarian	-	1	16	16	16

Observations

Horse behaviours

The horse behaviour consisted of two behavioural categories: aggressive behaviour and relaxed behaviour. Interview with the horse owners on the behaviour of their horses during the grooming process and specifically noted whether the horses displayed any signs of aggression. Among the behaviours associated with aggression were biting, striking, rearing, not permitting grooming, and even throwing themselves on the ground. When horses exhibited resistance during the grooming process, various strategies and methods were employed to overcome this resistance. Typically, when the grooming process commenced, horses attempted to endure the procedure patiently for the first 10 minutes. If a horse continued to resist, approaches such as increasing the number of caregivers were attempted. In such cases, additional individuals were brought in to participate in the grooming process to help the horse remain calmer. However, if the horse continued to resist, rope twitch was used, attached to the horse's nose to gain control over the horse. The rope twitch was employed to encourage the horse to remain calm and cooperative. The grooming process extended to an average of 30 minutes. If, after this 30-minute duration, the horse still did not permit the grooming process, two main options were considered. The first was sedating the horse, and the second was discontinuing the grooming process.

Horse Mane Direction

The horse mane direction was categorized into three types: left mane root, right mane root, and split (where the mane falls on both sides). The grooming process was done using horse clipper (Heiniger Delta 3 Clipper, Switzerland). The study documented the direction of the manes of the horses, as well as the direction in which the manes grew back after being groomed. Mane directions were determined through a combination of observation and face-to-face interviews with horse owners. The examination involved observing the horse from the front and both sides.

RESULTS

Horse behaviours

The Dutch, Belgian, French, and Hungarian horse breeds did not show any sign of aggression. During mane grooming session, cases of aggression were observed in Thoroughbred horses mostly. Out of a total of 52 male Thoroughbred horses, 15 of them displayed aggressive behaviour when their manes were shaved. Out of 49 female Thoroughbred horses 11 horses have shown aggressive behaviour during mane grooming. This indicates that 25.74% of Thoroughbred horses expressed aggressive behaviour.

Among a group of 59 Arabian male horses, only 3 horses exhibited aggression during the grooming session. Out of 37 Arabian female horses, 6 of them showed aggression during the grooming session. Thus 9.375% of Arabian horses expressed aggression.

Out of a 123 Hanoverian horses, only one female horse with a left mane root showed aggression, representing 0.81% of the Hanoverian horse subjects.

Among the two German male horses observed, one horse exhibited aggression during the grooming session. This particular horse had a mane directed to the right. Table 2 provides information about the aggression displayed by horses during mane grooming sessions.

Table 2. Numbers of the aggressive horses by breed, age, gender, and coat colour

Variables	Horse Breeds			
	Thoroughbred	Arabian	Hanoverian	German
Age				
1-4	17	6	1	-
5-9	6	3	-	-
10-18	3	-	-	1
Gender				
Male	15	3	-	1
Female	11	6	1	-
Coat colour				
Bay	14	1	-	-
Black	5	-	-	-
Chestnut	2	5	1	-
Flea-Bitten Gray	-	1	-	-
Dapple Gray	-	2	-	1
Mahogany Bay	5	-	-	-

Horse Mane Direction

Among the 15 Thoroughbred aggressive male horses, 6 had a right mane, 6 had a left mane, and 3 had a split mane. In total 23 of the 52 male Thoroughbred horses had left mane, 19 right mane and only 10 with split mane. 4 of the 11 aggressive female horses had right mane and 7 of the 11 aggressive female horses had left mane. In total 25 of the 49 female Thoroughbred horses had left mane, 21 right mane and only 3 with split mane. The manes of five Thoroughbred horses were completely shaved from the root, and no change in the direction of the manes was observed.

Out of 59 Arabian male horses observed, 19 had a left mane, 35 had a right mane, and 5 had split mane. Among the aggressive Arabian male horses, 2 had a left mane, and 1 had a right mane. Similarly, among the 37 observed Arabian female horses, 13 had a left mane, 23 had a right mane, and 1 had split mane. Among the aggressive Arabian female horses, 2 had a left mane, and 4 had a right mane. The manes of eight Arabian horses were completely shaved from the root, and no change in the direction of the manes was observed.

Among the 123 Hanoverian horses observed, only one female horse with a left mane root displayed aggression. Out of the 80 male Hanoverian horses, 34 had a left mane, 40 had a right mane, and 6 had a split mane. Among the 43 female Hanoverian horses, 14 had a left mane, 25 had a right mane, and 4 had a split mane. The manes of four Hanoverian horses were completely shaved from the root, and no change in the direction of the manes was observed.

In the observed sample of horses, there were 6 male Dutch horses, with 3 having a right mane, 2 having a left mane, and 1 having a split mane. Among the 2 female Dutch horses, both had a left mane. Two German male horses were also observed, one with a right mane and the other with a left mane. In the case of the 10 Belgian horses, there were 3 male horses with a right mane and 3 male horses with a left mane. Among the 4 female horses, 3 had a right mane, and 1 had a left mane. Among the 6 French horses, 3 male horses were observed, all of which had a right mane. Out of the 3 female horses, 2 had a left mane, and 1 had a split mane. The mane of one French horse was completely shaved from the root, and no change in the direction of the mane was observed. Additionally, one female horse from Hungary had a left mane. Table 3 provides the number of horses according to mane direction.

Table 3. Number of horses according to mane direction

Horse Breeds	Gender	Left Mane	Right Mane	Split Mane
Thoroughbred	Male	23	19	10
	Female	25	21	3
Arabian	Male	19	35	5
	Female	13	23	1
Hanoverian	Male	34	40	6
	Female	14	25	4
Belgian	Male	3	3	-
	Female	1	3	-
French	Male	-	3	
	Female	2	-	1
Dutch	Male	2	3	1
	Female	2	-	-
German	Male	1	1	-
	Female	-	-	-
Hungarian	Male	-	-	-
	Female	1	-	-

DISCUSSION

Lansade et al. (2019) conducted a study involving 69 horses during grooming sessions to evaluate their behaviour. Their findings indicated that only 5% of the observed horses showed mutual grooming, approach, or relaxed behaviour, while avoidance and threatening behaviours were observed in a significantly higher number of horses. The study revealed that the expression of avoidance or approach behaviours was not influenced by the gender or breed of the horses. This suggests that these behaviours might be more attributable to the grooming approach used rather than inherent characteristics of the horses themselves. Notably, the observation method used in the study was consistent with a previous study by Lansade et al. (2018), where 100% of the horses exhibited an approach response during gentle grooming adapted to their individual reactions.

The results suggest that there may be personality differences among the seven horse breeds evaluated in this study. In total out of 350 horses 36 horses had aggressive behaviour. This indicates that only 10.28% of horses expressed aggressive behaviours. Gender, age, and mane direction had no impact on the number of horses expressing aggressive behaviour and relaxed behaviour. It can be argued that the behaviour of horses during the grooming session could influenced by their breed, particularly evident in Thoroughbred horses. Also, the character of the horse and the way the mane was shaved may influence the behaviour of the horses.

Improving grooming practices to elicit positive behaviours in horses is relatively straightforward. In a study by Feh and de Mazières (1993), they found that massaging the horse in its preferred zones led to many positive behaviours. Discovering the horse's preferred zones involves being observant of their approach and avoidance behaviours, as well as paying attention to facial expressions, which can be particularly sensitive indicators of the horse's emotional state (Hintze et al. 2016; Lansade et al. 2018).

Positive emotional states during grooming can be identified by a raised eyebrow, low neck carriage, half-closed eyes, and extended lips. These signs should encourage the handler to continue brushing that area. Conversely, negative emotional states are indicated by a raised neck, wide-open eyes, and even slight tensing of the mouth corners. If these signs are noticed, the person should adjust their grooming or grooming approach accordingly. Additionally, it is crucial for the handler to be aware of any grimaces of pain, as described by Dalla Costa et al. (2014), which should immediately prompt them to stop and assess the situation.

During the study, it was observed that Thoroughbred horse subjects tended to slightly have more left manes with percentage of 47.52%, whereas Arabian horses showed a higher prevalence of right manes with a percentage of 60.42%. Similarly, Hanoverian horses displayed a slightly more noticeable prevalence of right manes with a percentage of 51.22%. In contrast, Dutch horse subjects exhibited a greater occurrence of left manes, while Belgian horse subjects had a higher prevalence of right manes.

It is important to acknowledge that these variations in mane direction among different horse breeds were observed but did not exhibit substantial differences.

CONCLUSION

In conclusion, this study provides valuable insights into the behaviours of seven horse breeds during mane grooming sessions. We examined potential influences on their grooming reactions, such as gender, age, mane direction, and breed characteristics. Compassionate grooming practices are essential for enhancing horse welfare and strengthening the human-horse bond. Differences in personality among breeds were observed, but not all pairs showed significant distinctions. Only a small percentage of horses exhibited aggressive behaviours (10.28%). Mane direction is primarily influenced by genetics, and while variations were noted among breeds, they were not significant. Considering individual differences and breed-specific traits can lead to a positive and enjoyable grooming experience for both horses and groomers, fostering a deeper bond between humans and horses.

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

Authors' Contributions: YP contributed to the project idea, design and execution of the study. YP contributed to the acquisition of data. YP analyzed the data. YP drafted and wrote the manuscript. YP reviewed the manuscript critically. The author has read and approved the finalized manuscript.

Ethical approval: "This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules."

Explanation: The author of the study ensured compliance with animal ethics policies after carefully reviewing them. The research solely involved observing horses during grooming sessions in a natural field setting, and no specific experimental interventions were conducted on the animals for this investigation.

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RESEARCH ARTICLE

Determination of Lactic Acid Bacterial Numbers of Lyophilized or Frozen Natural Lactic Acid Bacterial Liquids Prepared with Different Methods and Stored for Different Times

Sadık Serkan AYDIN[1*], Nihat DENEK[1]

[1]*Department of Animal Nutrition and Nutritional Disease, Faculty of Veterinary Medicine, Harran University, Şanlıurfa, Türkiye*

ABSTRACT

In this study, it was aimed to determine the lactic acid bacterium (LAB) counts and viability of fermented lactic acid bacteria liquids (pre-fermented juice (PFJ)) prepared with different levels of sucrose (3%, 5% and 10%) addition and incubation at different times (2, 5 and 10 days), using different cryoprotectants (trisodiumcitrate (TRIS) and dimethylsulfoxide (DMSO)), freezing and lyophilization, and at the end of different storage periods (one and three months). In frozen PFJs, the highest LAB counts were obtained in the TRIS and 5% sucrose supplemented group incubated for 5 days in a one-month storage period, and in the TRIS and 10% sucrose supplemented group incubated for 5 days at the end of the three-month storage period ($p<0.01$). At the end of one month of storage, the highest LAB numbers in lyophilized PFJs were obtained in groups incubated for 5 days and supplemented with 10% sucrose with TRIS and DMSO. In lyophilized PFJs subjected to 3-month storage, the highest LAB numbers were determined in the TRIS and 5%-10% sucrose-supplemented groups incubated for 5 days and in the DMSO and 5% sucrose-supplemented group incubated for 2 days ($p<0.01$). When the results obtained in the study were evaluated in general, it was observed that the viability ratios of LAB decreased due to the sucrose level and the prolongation of incubation and storage time. It can be said that cryoprotectant additive has a positive effect on the preservation of LAB and lyophilization process is advantageous compared to freezing in the deep freezer.

Keywords*:* Freezing, lactic acid bacteria, lyophilization

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Farklı Şekillerde Hazırlanarak Değişik Sürelerde Depolanan Liyofilize Edilmiş ve Dondurulmuş Doğal Laktik Asit Bakteri Sıvılarının Laktik Asit Bakteri Sayılarının Belirlenmesi

ÖZ

Bu çalışma kapsamında, farklı seviyelerde sükroz ilavesi (%3, %5 ve %10) ve farklı sürelerde inkübasyonla (2, 5 ve 10 gün) hazırlanmış fermente edilmiş laktik asit bakteri (LAB) sıvılarının (pre-fermented juice (PFJ)), farklı kryoprotektan maddeler (trisodyum sitrat (TRIS) ve dimetil sülfoksit (DMSO)) kullanılarak, dondurma ve liyofilizasyon ile farklı depolama süreleri (bir ve üç ay) sonunda LAB sayıları ve canlılığının belirlenmesi amaçlanmıştır. Dondurulan fermente edilmiş LAB sıvılarında en yüksek LAB sayıları bir aylık depolamada 5 gün inkübe edilen TRIS ve %5 sükroz katkılı grupta elde edilirken, üç aylık depolama süresi sonunda ise 5 gün inkübe edilen TRIS ve %10 sükroz katkılı grupta elde edilmiştir ($p<0.01$). Liyofilize edilmiş PFJ'lerde en yüksek LAB sayıları bir aylık depolama süresi sonunda TRIS ve DMSO katkılı %10 sükroz eklenerek 5 gün inkübe edilen gruplarda elde edilmiştir. Üç aylık depolama uygulanan liyofilize edilmiş PFJ'lerde ise en yüksek LAB sayıları 5 gün inkübe edilen TRIS ve %5-%10 sükroz katkılı gruplarda ve 2 gün inkübe edilen DMSO ve %5 sukroz katkılı grupta belirlenmiştir ($p<0.01$). Çalışmada elde edilen sonuçlar genel olarak değerlendirildiğinde; sükroz seviyesi ile inkübasyon ve depolama süresinin uzamasına bağlı olarak LAB'nin canlılık oranlarında azalmalar görülmüştür. Kryoprotektan katkısının LAB'nin korunmasında olumlu etki yaptığı, liyofilizasyon işleminin ise derin dondurucuda dondurulma işlemine göre avantajlı olduğu söylenebilir.

Anahtar Kelimeler: Dondurulma, laktik asit bakterisi, liyofilizasyon

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ORCID ID; *SSA: 0000-0002-3252-3944, ND: 0000-0003-0904-8943*

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

INTRODUCTION

Short-term (simple methods) and long-term preservation methods (modern methods) are used for the preservation of lactic acid bacteria inoculants (Haigh et al. 1987). Short-term preservation methods include transfer, preservation under liquid paraffin, preservation in distilled water and drying, while long-term preservation methods include lyophilization and deep freezing. Nowadays, lyophilization and deep-freeze preservation are preferred among long-term drying techniques for the preservation of both commercial lactic acid bacteria (LAB) inoculants used as silage additives and microorganisms used in microbiology (Öztürk and Çakır 2015). Lyophilization is the drying process of biologically derived materials with high moisture content or aqueous solutions under very low pressure by removing (desorption) the frozen free water particles in the material to be lyophilized by creating suitable conditions by sublimation (Meryman 1960). There are many factors affecting microbial viability and activity in the lyophilization process. These factors can be listed as the genus, species, strain, age, medium, cell density before lyophilization, composition of the protective medium used, the atmosphere in which the culture is protected after lyophilization (vacuum, inert gases, etc.), the amount of moisture remaining in the culture, storage conditions (temperature, time, light), the composition and temperature of the solution used in dilution. Apart from these, factors such as the system used in lyophilization, pre-freezing temperature, etc. are also known to be effective on microbial viability and activity. Bacterial cultures can be preserved as frozen and as lyophilized. When frozen cultures are preserved under appropriate conditions, they maintain their viability during storage. When stored under optimum conditions, they can remain viable for years (Tedeschi and De Paoli, 2011; Peiren et al. 2015). The purpose of this study was to ascertain the LAB numbers of fermented LAB liquids that had been made with varying amounts of sucrose addition, incubated for varying lengths of time by freezing in a deep freezer, dried using the lyophilization technique, and stored for one and three months.

MATERIALS and METHODS

In the study, fermented LAB liquids were prepared by adding sucrose at different levels (3%, 5% and 10%) and incubated for different times (2, 5 and 10 days). At the end of the incubation times, TRIS and DMSO were added to the fermented LAB liquids and the frozen or lyophilized dried LAB liquids were stored for one and three months and LAB numbers were determined.

Preparation of fermented lactic acid bacteria liquid

The fermented LAB liquid was prepared according to the method reported by (Masuko et al. 2002). However, the amount of pure water used was reduced to increase the microbial density. For this purpose, 1000 ml of pure water was added to 3000 g of fresh alfalfa plants and the mixture was disintegrated for 2 minutes with the help of a mixer. The plant liquid mixture obtained after the process was filtered through two layers of cheesecloth. The filtrate was transferred to 50 ml sterile plastic falcon tubes with screw caps and the groups without additive, with 3%, 5% and 10% sucrose addition were formed. The groups without additive, with 3%, 5% and 10% sucrose addition were incubated at 30 °C for 2, 5 and 10 days. After each incubation period, the fermented LAB liquids were centrifuged at 2000 rpm for 4 minutes to increase the LAB density. After centrifugation, 20% of the liquid remaining on top was removed. At the end of each incubation period, 1 ml of fermented LAB liquid was placed in 10 ml sterile bottles for lyophilization and freezing. No cryoprotectant was added to the groups without additives, 20% (v/v) of 50% TRIS solution was added to the TRIS groups, and 0.1% (v/v) DMSO was added to the DMSO groups, and lyophilization as well as deep freezing were performed. Thus, for each incubation period (2, 5 and 10 days), a total of 324 sterile bottles were formed with three replicates for the lyophilization and deep-freezing groups by adding TRIS and DMSO to the groups without additives, with 3%, 5% and 10% sucrose additions.

Lyophilization and deep-freezing process

Sterile special lyophilization bottles were replicated in 15 replicates, and the slit stoppers of the lyophilization bottles were half closed to allow the lyophilization process. In the first stage of the lyophilization process (pre-freezing phase), the lyophilization bottles were frozen at -80 °C for 24 hours and transported under liquid nitrogen vapor to the lyophilization device (Scanvac Coolsafe 55-4) which was started one hour before the start of the lyophilization process and placed on the shelves of the lyophilization device. The vacuum pump of the lyophilization device was operated to start the lyophilization process and the primary drying phase was carried out for 30 hours. At the end of the lyophilization process, the slit lids of the lyophilization bottles were closed without letting air into the system thanks to the mechanism pre-adapted to the lyophilization device. After the completion of the lyophilization process, each sucrose level, incubation time and moisture contents of the lyophilized LAB liquids for the groups without additives, with DMSO and TRIS additives were determined in 3 replicates for each group. The

lyophilized products were preserved in the refrigerator at +4 °C for one and three months.
LAB liquids to be stored using the freezing process were prepared in the same way. Before freezing, the air remaining in the closed bottles was removed with the help of a vacuum motor fitted with a syringe needle. The bottles containing the vacuumed LAB liquids were preserved in a deep freezer at -21 °C for one- and three-months during storage.

Lactic acid bacteria counting
LAB counting of the fermented LAB liquids obtained before incubation, after incubation, and after storage for one and three months was carried out according to the Tempo automatic bacterial counting device test method in 3 replicates for each group. For this purpose, the Tempo LAB device (bioMerieux, MarcyI'Etoile France) was used for LAB counting according to the recommended method (REF-80 071).

Statistical analysis
Statistical analysis was applied according to 3x3x3x2x2 (incubation time x sucrose level x protector x storage time x storage method) factorial experimental design in the evaluation of the data obtained as a result of the study. In this context, the effects of sucrose level, incubation periods, cryoprotectant type, lyophilization and deep-freezing processes and storage periods of one and three months were analyzed. General Linear Model (GLM) was used to determine the significance of the mean of the data and was determined by Duncan's multiple comparison test ($P<0.01$). For this purpose, SAS (1989) package program was used.

RESULTS

The dry matter, crude ash, crude protein, acid detergent fiber (ADF) and neutral detergent fiber (NDF) values of fresh alfalfa plant (*Medicago sativa*) used in the preparation of fermented LAB liquids in the study were determined as 19.21%, 13.19%, 31.11%, 18.70% and 29.59%, respectively; buffering capacity as 670 meq/kg; water-soluble carbohydrate (WSC) content as 64.6 g/kg and LAB number as $1x10^5$ cfu/g. Interaction analyses and sources of interactions for one and three months of storage of LAB liquids incubated at different levels of sucrose addition (3%, 5% and 10%) and for different periods (2, 5 and 10 days), frozen in deep freezer with TRIS and DMSO additives and dried by lyophilization process are presented in Table 1 and Table 2. When Table 1 and Table 2 were examined, sucrose level, incubation time, cryoprotectant type, storage method and storage time showed significant differences ($P<0.01$). It has been observed that incubation time, storage method, storage period and cryoprotectant type affect the survivability of lactic acid bacteria.

The LAB numbers of fresh material, non-additive, TRIS and DMSO-additive, deep-frozen and lyophilized and dried LAB liquids obtained by incubating with different levels of sucrose for different periods are presented in Table 3 (cfu/ml) before one- and three-months storage. The highest LAB numbers in fermented LAB liquids frozen in deep freezer with TRIS and DMSO addition were obtained from 10% sucrose added groups ($9.33x10^{10}$ and $4.67x10^9$ cfu/ml) in 2 days incubation. In the lyophilized and dried groups, the highest LAB values were obtained from the groups to which 5% and 10% sucrose were added ($8.67x10^{10}$ and $2.67x10^{11}$cfu/ml) in 5 days of the incubation period. In the fermented LAB liquids frozen in the deep freezer by adding TRIS and DMSO, it was observed that the LAB numbers increased with the increase in sucrose level in 2 days of incubation time, while LAB number decreased with the increase in sucrose level in 5- and 10-day incubation times ($P<0.01$).
In the fermented LAB liquids dried by lyophilization with the addition of TRIS and DMSO, the LAB number decreased with the increase in sucrose level in the 2 days of incubation period. In the 10 days of incubation period, it was observed that the LAB number decreased due to the increase in sucrose level in the TRIS additive groups. In the DMSO additive group, it was observed that there was a decrease in the group with 5% sucrose addition ($P<0.01$).
The LAB viability ratios of lyophilized and dried LAB liquids without additives, with TRIS and DMSO additives, obtained by incubating for different periods of time with different levels of sucrose addition, compared to the freshly prepared group before storage for one and three months are given in Table 4.
The LAB values (cfu/ml) of the fermented LAB liquids made by adding various amounts of sucrose and incubating for various lengths of time after freezing in the deep freezer and lyophilized and stored for one month are presented in Table 5. In the samples stored in the deep freezer for one month, the highest LAB values were obtained with 5% sucrose ($5.33x10^{10}$ cfu/ml) in 5-day incubation time in the group which TRIS was added. In the DMSO added group, it was determined with the addition of 10% sucrose ($1.47x10^9$ cfu/ml) in 2-days incubation time ($P<0.01$). In the samples dried by lyophilization and stored for one month, the highest LAB values were obtained in the group with 10% sucrose in 5-day incubation time in both the TRIS- (9.00×10^{10} cfu/ml) and DMSO-added (1.00×10^{11} cfu/ml) groups ($P<0.01$).
The lowest LAB numbers of lyophilized and dried LAB bacterial liquids after one month of storage were obtained with 10 days of incubation and 3% sucrose ($2.67x10^6$ and $3.00x10^7$ cfu/ml) addition in TRIS and DMSO additive groups ($P<0.01$). Similarly, it was observed that 10 days of incubation period had a negative effect on the number of viable LAB after

one month of storage in LAB liquids frozen in deep freezer with TRIS and DMSO addition.

At the end of one month of storage in deep freezer, the lowest LAB numbers were determined in the group with 3% sucrose ($3.33x10^6$ cfu/ml) addition with TRIS additive in 10 days of the incubation period. In the DMSO additive group, it was obtained as a result of 5% and 10% sucrose additions in 5 and 10 days of incubation periods ($P<0.01$). The highest LAB values were obtained with 5% sucrose addition ($5.33x10^{10}$ cfu/ml) in 5 days of incubation and with TRIS additive, and with 10% sucrose ($1.00x10^{11}$ cfu/ml) addition in 5 days of incubation in the deep-frozen, lyophilized and dried samples. While the values obtained from the samples frozen in deep freezer with DMSO additive were generally low, the LAB values obtained from lyophilized and dried samples in 2 and 5 days of daily incubation and 10% sucrose addition were found to be the highest ($P<0.01$).

The moisture contents of the LAB liquids obtained by lyophilizing and drying the fermented LAB liquids obtained by adding different levels of sucrose (3%, 5% and 10%) and incubating for different periods (2, 5 and 10 days) are presented in Table 6. While the lowest moisture contents of the lyophilized LAB liquids were obtained with 3% sucrose addition and in 2 days of incubation (8.22%, 8.39% and 8.95%), the highest moisture contents were obtained with 10% sucrose addition in 10 days of incubation (15.09%, and 16.64%) in non-additive and DMSO groups ($P< 0.01$). In TRIS groups, the highest moisture content was observed in the 5D10%S group ($P<0.01$).

The LAB viability ratios of TRIS and DMSO additive deep frozen and lyophilized and dried LAB liquids obtained by incubating with different levels of sucrose for different periods of time are given in Table 7.

When the fermented LAB liquids lyophilized and frozen in the deep freezer with TRIS and DMSO additives were compared with the freshly prepared group, the highest LAB viability ratios were obtained from TRIS additive groups. In DMSO additive groups, the microbial viability ratio decreased up to 28% due to the prolonged incubation period. In lyophilized and dried liquids, it can be said that generally the LAB viability ratios obtained from TRIS and DMSO additive groups in 2 and 5 days of incubation were higher than the viability ratios decreased in 10 days of incubation.

The results of the 3-month trial are presented in Table 8 (cfu/ml). In the samples stored in the deep freezer for three months, while the highest LAB value was obtained with 10% sucrose ($8.33x10^9$ cfu/ml) addition in the TRIS additive group in 5 days of incubation, the highest LAB value was obtained with 10% sucrose ($3.67x10^6$ cfu/ml) addition in the DMSO additive group in 2 days of incubation ($P<0.01$).

While the highest LAB values in LAB liquids dried by lyophilization process and stored for three months were obtained with 10% sucrose ($4.60\ x10^{10}$ cfu/ml) addition in the TRIS additive group in 5 days of incubation, the highest LAB values were obtained with 5% sucrose ($7.33x10^{10}$ cfu/ml) addition in DMSO additive group in 2 days of incubation ($P<0.01$). In general, it was observed that 10 days of incubation in TRIS addition as well as 5 and 10 days of incubation in DMSO addition had a negative effect on the number of viable LAB after three months of storage in lyophilized and dried LAB liquids. While the lowest viable LAB numbers after three months of storage in lyophilized and dried LAB liquids were obtained with 10 days of incubation and the addition of 3%, 5% and 10% sucrose ($1.23x10^6$, $1.50x10^6$ and $1.27x10^6$ cfu/ml) in TRIS additive groups, it was obtained with 10 days of incubation and 3% sucrose level ($7.00x10^6$ cfu/ml) in DMSO additive groups. Similarly, it was observed that three months of storage time had a negative effect on LAB number in all incubation times (2, 5 and 10 days) in deep frozen LAB liquids with DMSO addition.

The lowest LAB number in deep frozen LAB liquids after three months of storage was obtained in 5 days of incubation and with 3% sucrose ($6.00x10^6$ cfu/ml) in TRIS additive group and in 10 days of incubation with 3%, 5% and 10% sucrose in DMSO additive group ($P<0.01$). LAB values obtained in 5 days of incubation and with 10% sucrose addition ($8.33x10^9$ and $4.60x10^{10}$ cfu/ml) were the highest ($P<0.01$) in the groups prepared with TRIS additive and frozen in deep freezer and lyophilized and stored for three months. While the LAB values obtained from the samples prepared with DMSO additive and frozen in the deep freezer were generally low, the value obtained in 2 days of incubation and with 5% sucrose addition ($7.33x10^{10}$ cfu/ml) was the highest in the lyophilized dried samples ($P<0.01$). When the LAB values obtained at the end of the three-month storage period (Table 8) were examined, it was found that the LAB numbers obtained in 2- and 5-day incubation periods in the groups dried by lyophilization process with TRIS additive were close to the values obtained from freshly prepared LAB liquid. It was also observed that LAB loss was less due to TRIS addition. On the contrary, the values obtained from the TRIS additive groups, which were lyophilized, dried and stored for three months by adding different sucrose levels (3%, 5% and 10%) at the end of the 10 day of the incubation period, were found to be lower than the values obtained from freshly prepared LAB liquid, and LAB loss was observed to be higher in these groups. When the values presented in Table 8 at the end of the three-month of storage period were examined, it was observed that the number of LAB decreased in the groups stored in the deep freezer with the addition of DMSO due to the prolongation of the incubation time ($P<0.01$).

The LAB viability ratios of TRIS and DMSO additive deep-frozen and lyophilized and dried LAB liquids obtained by incubating with different levels of sucrose for different periods are given in Table 9. When the fermented LAB liquids frozen in deep freezer with TRIS and DMSO additives were compared with the freshly prepared group, while the highest LAB viability ratios were obtained from the TRIS additive groups, the viability ratio decreased from 59% to 17% in the DMSO additive groups due to the prolonged incubation period. In lyophilized and dried LAB liquids, while the highest viability ratios were obtained from TRIS and DMSO additive groups (74%-99%) in 2 and 5 days of incubation, the viability ratios decreased in 10 days of incubation.

Table 1. Interaction analyses (Log10) for one- and three-month storage of frozen and lyophilized LAB liquids with different levels of sucrose addition and incubated for different periods of time.

			1 Month Storage Time		3 Month Storage Time	
			Storage Type		Storage Type	
Incubation Time, Days	Sucrose Level %	Protector Type	Freezing	Lyophilized	Freezing	Lyophilized
2	3	TRIS	9.42	10.45	8.60	10.44
		DMSO	8.63	10.00	5.00	9.97
	5	TRIS	9.59	10.71	8.00	10.00
		DMSO	8.90	10.64	5.48	10.62
	10	TRIS	9.80	10.00	8.88	10.10
		DMSO	9.15	10.98	6.56	10.86
5	3	TRIS	7.48	10.48	6.78	10.48
		DMSO	8.20	10.55	3.00	9.90
	5	TRIS	10.73	10.71	9.52	10.60
		DMSO	3.00	10.10	3.00	8.95
	10	TRIS	10.00	10.95	9.92	10.66
		DMSO	3.00	11.00	3.00	8.59
10	3	TRIS	6.52	6.42	6.48	6.09
		DMSO	4.48	7.48	2.00	6.84
	5	TRIS	10.10	6.82	9.52	6.18
		DMSO	3.00	7.75	2.00	7.75
	10	TRIS	10.18	6.63	7.10	6.09
		DMSO	3.00	8.07	2.00	8.00

Incubation Time: 2, 5 and 10 days; Sucrose Level: 3%, 5% and 10%; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide.

Table 2. Interaction sources for one- and three-month storage of frozen and lyophilized LAB liquids with different levels of sucrose addition and incubation for different periods of time.

Interaction Source	F Value	Significance	p
Month	12750.53	0. 000	**
Sucrose	699.23	0. 000	**
Incubation time	40514.93	0. 000	**
Protector	49654.93	0. 000	**
Storage	79540.26	0. 000	**
Month*Sucrose	145.95	0. 000	**
Month*Incubation time	83.27	0. 000	**
Month*Protector	1619.90	0. 000	**
Month*Storage	4236.08	0. 000	**
Sucrose*Incubation time	358.85	0. 000	**
Sucrose*Protector	2440.17	0. 000	**
Sucrose*Storage	158.72	0. 000	**
Incubation time* Protector	5402.52	0. 000	**
Incubation time*Storage	5846.40	0. 000	**
Protector*Storage	62162.50	0. 000	**
Month*Sucrose*Incubation time	278.06	0. 000	**
Month*Sucrose*Protector	923.60	0. 000	**
Month*Sucrose*Storage	414.73	0. 000	**
Month*Incubation time*Protector	424.80	0. 000	**
Month*Incubation time*Storage	701.76	0. 000	**
Month*Protector*Storage	592.70	0. 000	**
Sucrose*Incubation time*Protector	1947.08	0. 000	**
Sucrose*Incubation time*Storage	172.32	0. 000	**
Sucrose*Protector*Storage	3272.60	0. 000	**
Incubation time*Protector*Storage	6270.91	0. 000	**
Month*Sucrose*Incubation time*Protector	197.33	0. 000	**
Month*Sucrose*Incubation time*Storage	595.45	0. 000	**
Month*Sucrose*Protector*Storage	949.65	0. 000	**
Month*Incubation time*Protector*Storage	420.67	0. 000	**
Sucrose*Incubation time*Protector*Storage	666.44	0. 000	**
Month*Sucrose*Incubation time*Protector*Storage	256.95	0. 000	**

Month: Storage period 1 and 3 months; Sucrose: Sucrose levels %3, %5 and %10; Incubation time: 2, 5 and 10 days; Protector: Trisodium citrate (TRIS) and Dimethyl sulfoxide (DMSO), Storage: Storage type Freezing or Lyophilization, **: $p<0.001$

Table 3. LAB numbers (cfu/ml) of LAB liquids prepared in different ways, frozen and lyophilized before one and three months of storage.

		Frozen			Lyophilized			
Groups	**Fresh**	**Non-additive**	**TRIS**	**DMSO**	**Non-additive**	**TRIS**	**DMSO**	**SEM**
2D3%S	$3.01x10^{10f\ A}$	$1.00x10^{9c\ D}$	$1.67x10^{10bc\ AB}$	$1.10x10^{9d\ E}$	$2.33x10^{9f\ C}$	$1.33x10^{10c\ B}$	$1.97x10^{10cd\ AB}$	0.164
2D5%S	$5.43x10^{10de\ A}$	$2.33x10^{9b\ D}$	$3.00x10^{10b\ B}$	$2.33x10^{9b\ D}$	$9.33x10^{9cd\ C}$	$4.00x10^{10b\ AB}$	$7.33x10^{9e\ C}$	0.123
2D10%S	$1.20x10^{11bc\ A}$	$2.00x10^{10a\ B}$	$9.33x10^{10a\ A}$	$4.67x10^{9a\ D}$	$6.33x10^{9de\ CD}$	$1.00x10^{10c\ BC}$	$4.00x10^{9e\ D}$	0.131
5D3%S	$7.67x10^{10cd\ A}$	$1.00x10^{8e\ E}$	$4.67x10^{9d\ C}$	$7.00x10^{8c\ D}$	$5.33x10^{9e\ C}$	$5.00x10^{10b\ B}$	$4.00x10^{10c\ B}$	0.221
5D5%S	$6.00x10^{10de\ A}$	$1.00x10^{9c\ D}$	$2.33x10^{9de\ C}$	$4.33x10^{8d\ E}$	$3.00x10^{10b\ B}$	$8.67x10^{10a\ A}$	$2.00x10^{10d\ B}$	0.194
5D10%S	$4.20x10^{11a\ A}$	$9.23x10^{9a\ C}$	$1.17x10^{9e\ D}$	$2.33x10^{7g\ E}$	$6.33x10^{10a\ B}$	$1.00x10^{10c\ C}$	$2.67x10^{11a\ A}$	0.313
10D3%S	$2.83x10^{11a\ A}$	$2.00x10^{9bc\ E}$	$2.33x10^{10bc\ CD}$	$3.00x10^{8d\ F}$	$1.33x10^{10c\ D}$	$3.33x10^{10b\ C}$	$8.00x10^{10b\ B}$	0.214
10D5%S	$1.47x10^{11b\ A}$	$1.33x10^{9bc\ D}$	$3.00x10^{9d\ C}$	$2.00x10^{8e\ F}$	$6.67x10^{8g\ E}$	$1.00x10^{10c\ B}$	$1.67x10^{10d\ B}$	0.203
10D10%S	$4.33x10^{10ef\ A}$	$2.17x10^{8d\ E}$	$1.37x10^{10c\ B}$	$1.00x10^{8f\ F}$	$4.67x10^{8g\ D}$	$1.00x10^{9d\ C}$	$3.00x10^{10cd\ A}$	0.234
SEM	0.071	0.134	0.123	0.132	0.134	0.114	0.103	

a-g: Values with different letters in the same column were found to be statistically different ($p<0.01$); A-E: Values with different letters in the same line were found to be statistically different. ($p<0.01$); 2D: 2 days; 5D: 5 days; 10D: 10 days; %3S: %3 sucrose; %5S: %5 sucrose; %10S: %10 sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide.

Table 4. LAB viability ratios of LAB liquids prepared in different ways, frozen and lyophilized group before storage for one and three months %.

		Frozen			Lyophilized		
Groups	**Fresh**	**Non-additive**	**TRIS**	**DMSO**	**Non-additive**	**TRIS**	**DMSO**
2D3%S	100	86	97	81	89	96	98
2D5%S	100	87	97	87	93	99	92
2D10%S	100	93	99	87	88	90	87
5D3%S	100	74	89	81	89	98	97
5D5%S	100	84	87	80	97	99	95
5D10%S	100	86	78	63	93	86	98
10D3%S	100	81	90	74	88	92	95
10D5%S	100	82	85	74	79	90	91
10D10%S	100	78	95	75	82	85	98

2D: 2 days; 5D: 5 days; 10D: 10 days; %3S: %3 sucrose; %5S: %5 sucrose; %10S: %10 sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide

Table 5. LAB values (cfu/ml) at the end of one month storage period of LAB liquids prepared in different ways, frozen and lyophilized.

	Frozen		Lyophilized		
Groups	**TRIS**	**DMSO**	**TRIS**	**DMSO**	**SEM**
2D3%S	$2.67x10^{9d\ C}$	$4.33x10^{8c\ D}$	$2.83x10^{10c\ A}$	$1.00x10^{10c\ B}$	0.206
2D5%S	$4.00x10^{9d\ B}$	$8.00x10^{8b\ C}$	$5.10x10^{10b\ A}$	$4.43x10^{10b\ A}$	0.228
2D10%S	$6.33x10^{9c\ B}$	$1.47x10^{9a\ C}$	$1.00x10^{10d\ B}$	$9.47x10^{10a\ A}$	0.198
5D3%S	$3.03x10^{7e\ C}$	$1.67x10^{8d\ B}$	$3.00x10^{10c\ A}$	$3.53x10^{10b\ A}$	0.411
5D5%S	$5.33x10^{10a\ A}$	$1.00x10^{3f\ C}$	$5.13x10^{10b\ A}$	$1.33x10^{10c\ B}$	0.984
5D10%S	$1.00x10^{10b\ B}$	$1.00x10^{3f\ C}$	$9.00x10^{10a\ A}$	$1.00x10^{11a\ A}$	1.001
10D3%S	$3.33x10^{6f\ B}$	$3.00x10^{4e\ C}$	$2.67x10^{6g\ B}$	$3.00x10^{7f\ A}$	0.329
10D5%S	$1.33x10^{10b\ A}$	$1.00x10^{3f\ D}$	$6.67x10^{6e\ C}$	$5.67x10^{7e\ B}$	0.772
10D10%S	$1.50x10^{10b\ A}$	$1.00x10^{3f\ D}$	$4.33x10^{6f\ C}$	$1.20x10^{8d\ B}$	0.782
SEM	0.256	0.538	0.366	0.266	

a-g : Values with different letters in the same column were found to be statistically different. ($p<0.01$). A-D: Values with different letters in the same line were found to be statistically different. ($p<0.01$). 2D: 2 days; 5D: 5 days; 10D: 10 days; %3S: %3 sucrose; %5S: %5 sucrose; %10S: %10 sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide

Table 6. Moisture Content of LAB Liquids Obtained by Being Dried with Lyophilization Process, %

Groups	**Non-additive**	**TRIS (Ts)**	**DMSO (Ds)**	**SEM**
2D3%S	8.22^{e}	8.39^{e}	8.95^{f}	0.225
2D5%S	$10.97^{bcd\ A}$	$9.20^{e\ B}$	$11.27^{d\ A}$	0.350
2D10%S	$10.02^{d\ B}$	$11.46^{b\ A}$	$9.88^{e\ B}$	0.272
5D3%S	$10.56^{cd\ AB}$	$9.37^{e\ B}$	$11.16^{d\ A}$	0.297
5D5%S	11.44^{bcd}	12.05^{ab}	12.42^{bc}	0.304
5D10%S	12.42^{b}	12.65^{a}	13.06^{b}	0.131
10D3%S	$10.63^{cd\ B}$	$12.23^{ab\ A}$	$12.36^{bc\ A}$	0.289
10D5%S	12.20^{bc}	11.92^{ab}	12.11^{c}	0.122
10D10%S	$15.09^{a\ B}$	$11.59^{ab\ C}$	$16.64^{a\ A}$	0.752
SEM	0.368	0.296	0.407	

$^{a-f}$:Values with different letters in the same column were found to be statistically different. ($p<0.01$). $^{A-C:}$ Values with different letters in the same line were found to be statistically different. ($p<0.01$). 2D: 2 days; 5D: 5 days; 10D: 10 days; 3%S: 3% sucrose; 5%S: 5% sucrose; 10%S: 10% sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide

Table 7. LAB viability ratios as a result of freezing and lyophilization of LAB liquids incubated for different periods of time with different levels of sucrose addition and storage for one month, %

		Frozen		Lyophilized	
Groups	**Fresh**	**TRIS**	**DMSO**	**TRIS**	**DMSO**
2D3%S	100	90	82	99	95
2D5%S	100	89	83	99	99
2D10%S	100	88	83	90	99
5D3%S	100	67	75	96	97
5D5%S	100	99	28	99	94
5D10%S	100	86	26	94	95
10D3%S	100	57	39	56	68
10D5%S	100	91	27	61	70
10D10%S	100	96	28	62	76

2D: 2 days; 5D: 5 days; 10D: 10 days; 3%S: 3% sucrose; 5%S: 5% sucrose; 10%S: 10% sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide

Table 8. LAB values (cfu/ml) at the end of three months of storage period of LAB liquids prepared in different ways, frozen and lyophilized.

	Frozen		**Lyophilized**		
GRUP	**TRIS**	**DMSO**	**TRIS**	**DMSO**	**SEM**
2D3%S	$4.00x10^{8d\ C}$	$1.00x10^{5c\ D}$	$2.80x10^{10c\ A}$	$9.33x10^{9c\ B}$	0.621
2D5%S	$1.00x10^{8e\ C}$	$3.00x10^{5b\ D}$	$1.00x10^{10d\ B}$	$7.33x10^{10a\ A}$	0.550
2D10%S	$7.67x10^{8c\ C}$	$3.67x10^{6a\ D}$	$1.27x10^{10d\ B}$	$4.13x10^{10b\ A}$	0.469
5D3%S	$6.00x10^{6g\ C}$	$1.00x10^{3d\ D}$	$3.00x10^{10bc\ A}$	$8.00x10^{9c\ B}$	0.756
5D5%S	$3.33x10^{9b\ B}$	$1.00x10^{3d\ D}$	$4.00x10^{10ab\ A}$	$9.00x10^{8d\ C}$	0.770
5D10%S	$8.33x10^{9a\ B}$	$1.00x10^{3d\ D}$	$4.60x10^{10a\ A}$	$4.00x10^{8e\ C}$	0.850
10D3%S	$3.00x10^{6h\ B}$	$1.00x10^{2e\ D}$	$1.23x10^{6e\ C}$	$7.00x10^{6h\ A}$	0.447
10D5%S	$3.47x10^{9b\ A}$	$1.00x10^{2e\ D}$	$1.50x10^{6e\ C}$	$5.67x10^{7g\ B}$	0.632
10D10%S	$1.00x10^{7f\ B}$	$1.00x10^{2e\ D}$	$1.27x10^{6e\ C}$	$1.00x10^{8f\ A}$	0.556
SEM	0.238	0.314	0.396	0.255	

a-h :Values with different letters in the same column were found to be statistically different. ($p<0.01$). A-D: Values with different letters in the same line were found to be statistically different. ($p<0.01$). 2D: 2 days; 5D: 5 days; 10D: 10 days; 3%S: 3% sucrose; 5%S: 5% sucrose; 10%S: 10% sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide

Table 9. LAB viability ratios as a result of freezing and lyophilization of LAB liquids incubated for different periods of time with different levels of sucrose addition and storage for three months, %

		Frozen		Lyophilized	
Groups	**Fresh**	**TRIS**	**DMSO**	**TRIS**	**DMSO**
2D3%S	100	82	48	99	95
2D5%S	100	75	51	93	99
2D10%S	100	80	59	91	96
5D3%S	100	62	28	96	91
5D5%S	100	88	28	98	83
5D10%S	100	85	26	92	74
10D3%S	100	39	17	53	60
10D5%S	100	85	18	55	70
10D10%S	100	67	19	57	75

2D: 2 days; 5D: 5 days; 10D: 10 days; 3%S: 3% sucrose; 5%S: 5% sucrose; 10%S: 10% sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide.

DISCUSSION

The fresh alfalfa plant employed in this study's manufacture of fermented LAB liquids had a LAB number of $1x10^5$ cfu/ml. It was found to be compatible with the reports of Ohshima et al. (1997c) on LAB numbers of alfalfa plants ($3x10^5$ cfu/ml).

Before harvest, the amount of LAB infecting the plant can range from $1x10^1$ cfu/ml to $1.0x10^7$ cfu/ml. There are also discrepancies in the number and types of LAB contaminating the plants that will be used to make silage. These variations can be attributed to a variety of factors, including ambient temperature, UV light, environmental humidity, and other aspects of the plant itself. In this study, in the freshly prepared fermented LAB liquids, the total number of LAB decreased at the end of the 10 days of incubation due to the increase in sucrose level; on the contrary, a general increasing trend was observed in the total number of LAB in 2 and 5 days of incubation (Table 3). Depending on the sucrose level and incubation time, it is thought that the logarithmic increase period of LAB continued in 2, 5 and 10 days of incubation in 3% and 5% sucrose additive groups and in 5 days of incubation in 10% sucrose additive groups. It was observed that there was a decrease in the number of LAB in the 10% sucrose additive group in the 10 days of incubation ($P<0.01$).

The reason for the decrease in the number of LAB during the 10-day incubation period and in the group with 10% sucrose addition may be related to the fact that the environment no longer provided the nutrients that the growing population of LAB required, as well as the rise in the number of toxic substances (lactic acid, acetaldehyde, peroxide, etc.) and the decline in the pH level of the environment. This results in a decrease in the number of bacteria that can survive in the environment and an increase in the number of microorganisms that cease to function (Brock et al. 2003).

The type of freezing process and the rapid cooling process applied in the cryopreservation of microorganisms are important in ensuring maximum cell viability (Tedeschi and De Paoli 2011; Fonseca et al. 2016). It has been reported that damage to the bacterial cell membrane caused by the freezing process may be due to the high solution concentration of the environment in which the cell is located and due to intracellular ice crystals (Dumont et al. 2004; Chang and Zhao 2021). The reason for the use of cryoprotectants in the cryopreservation of microorganisms is that they prevent the formation of large ice crystals inside and outside the cell during the freezing process, allowing frozen microorganisms to reach high microbial viability (Dumont et al. 2004). In 2- and 5-day incubation time, it was observed that the viability ratio increased depending on the increase in sucrose level and the highest viability ratios were obtained from the 10% sucrose additive group. According to Suharman et al. (2021), there were more lactic acid bacteria when the amount of sucrose increased. In the 10-day incubation time, the highest viability ratio was obtained from the 5% sucrose additive group (82%) (Table 4). When the LAB viability ratios of the deep-frozen LAB liquids obtained by adding TRIS and DMSO cryoprotectants to the LAB liquids obtained by adding sucrose at different levels and incubating for different periods (Table 4) before one- and three-month storage were compared with fresh LAB liquid, the highest viability ratios were determined in 2D3%S, 2D5%S, and 2D10%S groups. In the same groups (2D3%S,

2D5%S and 2D10%S), when compared to fresh LAB liquid, it was determined that there were decreases in microbial viability rates due to longer storage periods with the addition of TRIS (Tables 7 and 9). Likewise, it was determined that there were decreases in microbial viability rates as storage time increased in the same groups prepared with the addition of DMSO compared to fresh LAB liquid (Tables 7 and 9).

In this study, the viability ratio of the 5D5%S group decreased from 99% to 88% and the viability ratio of the 5D10%S group decreased from 86% to 85% among the LAB liquids prepared after one- and three-month storage period by freezing in deep freezer by adding TRIS as cryoprotectant. Oluwatosin et al. (2022) reported that sucrose showed the best performance among other cryoprotectant substances for the protection of microorganisms stored frozen. In this study, the high viability ratios determined due to the increase in sucrose level were found to be consistent with the report. In the study conducted by Bâati et al. (2000) regarding storage of *Lb. acidophilus* by being frozen, they reported that *Lb. acidophilus* maintained microbial viability between 24% and 89% depending on the temperature of the freezing process applied to *Lb. acidophilus* and the suspension environment. They stated that a pre-freezing process before the freezing process to be applied to microorganisms is important in increasing microbial viability.

In this study, the microbial viability ratios obtained by freezing in deep freezer for one and three months were determined between 74% and 93% in the groups without additives, 85% and 99% in the groups with TRIS additives, and 63% and 87% in the groups with DMSO additives. In the fermented LAB groups frozen in this study, the microbial viability ratios obtained from the TRIS additive groups were found to be higher than the groups without additives and with DMSO additive due to the prolongation of the storage period (1 and 3 months). In the deep-frozen groups, microbial viability decreased in the TRIS and DMSO additive groups due to the prolongation of the storage period in general. Consistent with this study, Strasser et al. (2009) found that the viability of lactic acid bacteria was negatively affected by the extension of storage time. The moisture contents of LAB liquids obtained by lyophilization being dried are presented in Table 6. In general, the moisture content of lyophilized cultures is required to be 2% or less. In addition, a moisture content of 2% or less is considered as an indicator of the success of the lyophilization process (Rambhatla and Pikal 2003). In this study, the moisture contents of lyophilized and dried LAB liquids were found to be between 8.22% and 16.64%. When the moisture contents of LAB liquids obtained as a result of the lyophilization process in this study were compared with previous studies, it was observed that the moisture values obtained were high (Sparkes and Fenje 1972; Avcıoğlu 2013). In this study, the reason for higher moisture values in LAB liquids obtained by lyophilization process can be explained by keeping the secondary drying time short, which is the last step of the lyophilization process and reduces the relative amount of water in the product (Avcıoğlu 2013).

There are many factors affecting microbial viability and activity in the lyophilized drying process of microorganisms and cell cultures. These factors include the genus, species, strain and age of the bacteria, growth medium, cell density before the process, ambient conditions, type of cryoprotectant, ambient conditions in which the culture is preserved after the process, the amount of moisture remaining in the culture, storage conditions (temperature, time, light), composition and temperature of the solution used in dilution. Apart from these factors, the system used during the lyophilization process, excessive sublimation, initial freezing temperature, bacteriophage risk, etc. are also known to be effective on the viability and activity of lyophilized cultures (Halkman and Doğan 2000). In general, while the highest microbial viability ratio was determined in the TRIS additive groups in the deep freezer, the lowest microbial viability ratio was determined in the DMSO additive groups (63%-87%). When evaluated in general, while the highest microbial viability ratio was maintained in the TRIS additive group, the highest microbial loss ratio was determined in the groups without additives in drying by the lyophilization process. In the groups dried by the lyophilization process in this study, the viability ratios obtained from TRIS and DMSO additive groups decreased due to the prolongation of the storage period (one or three months). The highest loss in viability ratio was observed in 10 days of incubation periods (Tables 7 and 9).

In a study conducted by (Ferry 1995), it was stated that 5% to 10% sugar contribution is the most ideal for microbial culture preservation by lyophilization process. Greaves (1964) stated that the sucrose ratio should not exceed 10%, and when the sucrose ratio used is between 10-20%, the viability ratio of the lyophilized culture decreases over time. In this study, the maintenance of high microbial viability of anaerobic and aerotolerant lactic acid bacteria in lyophilized LAB liquids obtained by lyophilization and drying was found to be consistent with the report that it is easier to preserve and maintain anaerobic and especially aerotolerant microorganisms in the field of food microbiology (Halkman and Doğan 2000). In the preservation of lactic acid bacteria, especially in the drying method by applying lyophilization process, high amounts of microbial culture density are maintained. In accordance with the report of Giulio et al. (2005), it was observed that lyophilization method preserved microbial viability more than deep freezing in this study. Pınarkara (2008) researched the effects of sucrose, TRIS and DMSO cryoprotectant additives on microbial viability

ratios of different types of LAB in drying by lyophilization process and reported that the use of TRIS as cryoprotectant in the lyophilization process was effective in maintaining the viability ratios of LAB. Giulio et al. (2005) found that the viability ratios of *Lactobacillus bulgaricus, Streptococcus salivarius ssp. and Thermophilus* strains was 95% after lyophilization with the addition of 32% sucrose, while Morichi (1965) reported that in contrast to this result, the viability ratio of *Lactobacillus bulgaricus* was maintained at 38% with the addition of sucrose. In this study, compared to freshly prepared fermented LAB liquids, the viability ratios of lyophilized LAB liquids with the addition of 3% and 5% sucrose and TRIS as cryoprotectant were found to be compatible with the results obtained by Giulio et al. (2005) and Pınarkara (2008).

CONCLUSION and RECOMMENDATIONS

According to the findings obtained within the scope of the study, it is thought that 2 or 5 days of incubation period with the addition of 10% sucrose will be sufficient to obtain high levels of LAB in fermented LAB liquids to be used fresh without deep freezing and lyophilization and drying. It was determined that lyophilization of fermented LAB liquids in one month storage period preserved their viability ratios better than deep freezing, and the highest LAB values were determined in the groups lyophilized by adding 10% sucrose and DMSO as the cryoprotectant in 2 and 5 days of incubation period. At the end of the three months of storage period, it was determined that the lyophilization process preserved the viability ratios of LAB better than deep freezing and the highest LAB values were determined in the groups lyophilized with 5% and 10% sucrose addition and TRIS as cryoprotectant for 5 days of incubation period, and in the group lyophilized with DMSO addition and 5% sucrose for 2 days of incubation period.

When the results obtained from this study are evaluated in general, it can be said that the viability ratios of LAB liquids decreased due to the prolongation of incubation and storage time, TRIS and DMSO additives had a positive effect on the viability ratios, and lyophilization process was advantageous compared to freezing in deep freezer. Therefore, it may be recommended to dry the fermented LAB liquids by lyophilization with the addition of TRIS or DMSO cryoprotectants for long-term storage. Considering the high viability ratios obtained from the lyophilization and drying of LAB liquids with TRIS and DMSO cryoprotectant additives, it is seen that the LAB liquids obtained in this study have a high potential to be used and commercialized as silage additives.

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

Authors' Contributions: SSA and ND contributed to the project idea, design and execution of the study. MK, BS and BD contributed to the acquisition of data. SSA and ND analysed the data. SSA and ND drafted and wrote the manuscript. SSA and ND reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

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RESEARCH ARTICLE

Comparison of Perioperative Serum Glucose and Serum Fructosamine Levels in Cats

Özgenur ÜNSUR[1]*, Turan CİVELEK[2]

[1,2]Department of Veterinary Internal Medicine, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Türkiye

ABSTRACT

In this study, the changes in serum glucose and fructosamine levels according to the sampling time in cats anaesthetised for castration and ovariohysterectomy were investigated. The study material consisted of 25 cats aged between 6 months and 2 years. Blood samples were collected from the anaesthetised cats (medetomidine, butorphanol, ketamine) before anaesthesia, at the beginning of anaesthesia, before awakening from anaesthesia and after awakening from anaesthesia. Glucose, fructosamine, cholesterol, triglyceride, total protein and albumin levels were measured in the sera obtained. In the statistical comparison made according to the measurement times, it was found that serum glucose level changed significantly. However, no statistically significant difference was found in serum fructosamine levels. It was evaluated that the variation in glucose levels in the perioperative period may be related to anaesthetic agents, and although there was no statistically significant change in fructosamine levels, one of the reasons for the increase in glucose levels observed in the perioperative period was probably operation stress. The results of the study showed that serum glucose measurements are important in perioperative glycaemic control of cats, and serum fructosamine measurements have diagnostic value in the exclusion of possible perioperative hyperglycemia that may develop for different reasons. The stable serum fructosamine level in the perioperative period can be accepted as an indication that it was not affected by the anaesthetic agents used in this study.

Keywords: Biochemistry, Castration, Feline, Ovariohysterectomy, Postoperative period

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Kedilerde Perioperatif Serum Glukoz ve Serum Fruktozamin Seviyelerinin Karşılaştırılması

ÖZ

Bu çalışmada kastrasyon ve ovariohisterektomi operasyonu amacıyla anesteziye alınan kedilerde örnekleme zamanına göre serum glukoz ve fruktozamin seviyelerinde meydana gelen değişim araştırıldı. Çalışma materyalini 6 ay ila 2 yaş arasında 25 kedi oluşturdu. Anesteziye (medetomidin, butorfanol, ketamin) alınan kedilerden; anestezi öncesinde, anestezi başlangıcında, anesteziden uyandırmadan önce ve anesteziden uyandıktan sonra kan örnekleri alındı. Elde edilen serumlarda glukoz, fruktozamin, kolesterol, trigliserid, total protein ve albümin konsantrasyonları ölçüldü. Ölçüm zamanlarına göre yapılan istatistiksel karşılaştırmada; serum glukoz düzeyinin anlamlı şekilde değiştiği tespit edildi. Bununla birlikte serum fruktozamin seviyelerinde istatistiksel olarak anlamlı bir fark bulunmadı. Perioperatif dönemde glukoz seviyelerinde meydana gelen varyasyonun anestezik ajanlarla ilişkili olabileceği, fruktozamin düzeylerinde istatistiksel olarak önemli bir değişim görülmemesine rağmen, glukoz düzeyinde perioperatif dönemde gözlenen artışın bir nedeninin de olasılıkla operasyon stresi olduğu değerlendirildi. Çalışma sonucu; kedilerin perioperatif glisemik kontrollerinde serum glukoz ölçümlerinin önemli olduğunu, farklı nedenlerle gelişebilecek olası perioperatif hipergliseminin dışlanmasında ise serum fruktozamin ölçümlerinin diyagnostik yönden değer taşıdığını gösterdi. Perioperatif dönem serum fruktozamin seviyesinin stabil oluşu, bu çalışmada kullanılan anestezik ajanlardan etkilenmediğinin bir göstergesi olarak kabul edilebilir.

Anahtar Kelimeler: Biyokimya, Kastrasyon, Feline, Ovariohisterektomi, Postoperatif dönem

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ORCID ID; *ÖU: 0000-0002-5265-7616, TC: 0000-0002-4819-3484.*
*Corresponding author e-mail: ozgenuryumusak@gmail.com

GİRİŞ

Kedilerde kısırlaştırılma işlemi pratikte veteriner kliniklerde yaygın olarak uygulanmaktadır ve genel anestezi prosedürü gerektirir. Medetomidin, butorfanol ve ketamin anestezisinin özellikle barınak ve klinisyen veteriner hekimleri tarafından kastrasyon ve ovariohisterektomi operasyonlarında güvenle kullanılabileceği belirtilmiştir (Yılmaz 2021). α2-adrenoseptör agonistlerden biri olan medetomidin sedasyon sağlaması, sentral ve spinal etkilerine bağlı olarak da analjezi meydana getirmesi sebebiyle kedi ve köpeklerde en sık kullanılan uygulanan α2-adrenoseptör agonistlerinden birisidir (Topal 2005; Lemke 2004). Literatürde medetomidinin serum glukoz seviyesinde artışına sebep olduğu bildirilmiştir (Kanda ve Hikasa 2008). Anestezi sırasında değişen adrenalin, kortizol gibi hormon seviyeleri ile birlikte serum glukoz düzeylerinin yükseldiğini gösteren çalışmalar bulunmaktadır (Kamohara ve ark. 2021). Ketamin, barbitürat türevi olmayan nöroleptanaljezi ile karakterize olan ve genel anestezi meydana getiren hızlı etkili dissosiyatif bir anestezik maddedir (Arıkan ve ark. 1993; Şanlı ve Kaya 1994). Ketamin uygulamasının da medetomidin gibi glukoz seviyelerinde değişikliklere sebep olduğu rapor edilmiştir (Sharif ve Abouazra 2009). Aynı zamanda yüksek doz ketamin uygulamalarının kolesterol seviyesini değiştirmeksizin trigliserid düzeylerini arttırdığı da ifade edilmiştir (Perumal ve ark. 2007). Ayrıca literatürde beşeri hekimlikte operasyon sonrası stres hiperglisemisinin görüldüğü ve bunun negatif klinik komplikasyonlara sebep olduğu belirtilmektedir (Palermo ve ark. 2016). Stres hiperglisemisinin kedilerde sık görülen bir tablo olduğu bilinmektedir (Laluha ve ark. 2004). Yapılan çeşitli çalışmalar serum fruktozamin ölçümünün kedilerde glisemi değerlendirilmesinde faydalı olduğunu savunmaktadır (De Vries 2011; Link ve Rand 2008). Fruktozamin, glukoz ile proteinlerin geri dönüşümsüz bağlanması sonucunda oluşmaktadır. Glikasyonlu bir serum proteinidir ve kedilerde bir glisemik kontrol indeksidir. Stres hiperglisemisinden etkilenmediği için diyagnostik açıdan değerlidir (Gal ve ark. 2017). Serum fruktozamin konsantrasyonları, plazma glukoz konsantrasyonlarına bağlıdır ve diyabetli hayvanlarda glisemik kontrolü değerlendirmek için kullanılmaktadır (Gilor ve ark. 2010). Kedilerde glukoz metabolizmasındaki bozuklukların erken tanısında glukoza kıyasla daha uzun bir kan glukozu seviyesini yansıttığından dolayı fruktozamin kullanılmaktadır (Perez-Lopez ve ark. 2020). Serum fruktozamin konsantrasyonları, ölçümden önceki 2 ila 3 haftanın glukoz ortalamasını verir (De Vries 2011; Link ve Rand 2008). Perioperatif dönem glisemik kontrollerde de fruktozamin seviyesi ölçümlerinin yapılmasının faydalı olduğu literatürde bildirilmiştir (Shohat ve ark. 2017; Shohat ve ark. 2022). Yapılan bu çalışmada perioperatif dönemde glukoz ile fruktozamin seviyelerinin karşılaştırılması ve anestezik ajanlara/operasyona karşı oluşan yanıtın değerlendirilmesi amaçlanmıştır.

MATERYAL ve METOT

Bu çalışmanın materyalini 10'u erkek ve 15'i dişi olmak üzere, genel durumu iyi olan ve dehidre olmayan, 6 ay ila 2 yaş aralığında klinik olarak sağlıklı 25 kedi oluşturdu. Çalışma gerekli yasal izinler alındıktan sonra (Tarım ve Orman Bakanlığı, Antalya İl Müdürlüğü 20.12.2022 tarih ve 8188707 sayılı yazısı; AKÜHADYEK; 16.03.2023 tarih ve 49533702/22 sayılı onay) Vitality Veteriner Kliniği, Antalya'da gerçekleştirildi. Çalışma öncesi hasta sahipleri aydınlatıldı ve "bilgilendirilmiş onam" formları alındı.
İlgili kliniğe kısırlaştırma operasyonu amacıyla getirilen hastaların genel muayeneleri gerçekleştirilmiş, anamnezinde kronik hastalık öyküsü olmayan kedilerden Vena Cephalica Antebrahium' dan EDTA' lı tüplere kan örnekleri alınarak (RBC, MCV, HCT, WBC, HGB, PLT vd.) hematolojik incelemeleri yapılmış ve sağlıklı oldukları belirlenen kediler çalışmaya dahil edilmiştir.
Biyokimya analizleri için; a)anestezi öncesi, b)anestezi başlangıcında, c)anesteziden uyandırmadan önce ve d)anesteziden uyandıktan sonra Gel-Clot Activatör içeren tüplere kan örnekleri alındı. Numuneler 3500 devirde 12-15 dakika santrifüje edilerek kan serumları çıkarıldı. Elde edilen numuneler ölçüm zamanına kadar -20C0' de saklandı. Serum örneklerinde; glukoz (GLU) , fruktozamin (FRU), kolesterol (TCHOL), trigliserid (TG), total protein (TP) ve albümin (ALB) düzeyleri ölçüldü. Örnekleme zamanları arası değerlerin karşılaştırmaları istatistiksel olarak yapıldı. Anestezi öncesi, anestezi başlangıcında, anesteziden uyandırmadan önce ve anesteziden uyandıktan sonra her bir parametreye göre yapılan karşılaştırmada; ilişkili ölçümler için varyans analizi (repeated measures ANOVA) kullanıldı. Her bir zamanın ikili karşılaştırılmasında ise LSD çoklu karşılaştırma testi uygulandı. Sonuçlar ortalama ± standart sapma olarak verildi. İstatistiksel anlamlılık (önemlilik) düzeyi 0.05 olarak alınmış, araştırmada elde edilen verilerin istatistiksel değerlendirmesi Windows için SPSS 26 paket programı kullanılarak yapılmıştır.

BULGULAR

Biyokimyasal analiz sonuçlarının karşılaştırması Tablo1'de verildi. Örnek alım zamanlarına göre yapılan karşılaştırmada GLU seviyesinde anlamlı bir değişim ($p<0.05$) saptandı. Fruktozamin seviyesinde ise örnek alım zamanları arasında numerik bir değişim olmasına rağmen, bu fark ($p>0.05$) istatistiki olarak anlamlı bulunmadı. TG, CHOL, TP, ALB, GLOB ve A/G seviyelerinde de örnekleme zamanına bağlı önemli bir değişiklik ($p>0.05$) belirlenmedi. Hematoloji sonuçlarına göre sağlıklı olan kediler çalışmaya dahil edildi ve analiz sonuçları Tablo 2'de sunuldu.

Tablo 1. Biyokimyasal analizlerin örnekleme zamanına göre karşılaştırılması
Table 1. Comparison of biochemical analyzes according to sampling time

Gruplar	TG (mmol/l)	CHOL (mmol/l)	GLU (mmol/l)	FRU (umol/l)	TP (g/dl)	ALB (g/dl)	GLOB (g/dl)	A/G
1	0.73±0.32	3.72±0.56	6.65^{b}±1.86	149.35±28.43	7.90±1.23	3.21±0.48	4.69±1.16	0.71±0.17
2	0.71±0.35	3.77±0.65	7.50^{b}±2.47	164.81±28.97	7.48±1.04	3.18±0.37	4.30±0.90	0.75±0.16
3	0.71±0.33	3.65±0.55	10.50^{a}±5.18	157.16±24.73	7.50±1.01	3.03±0.31	4.40±1.16	0.73±0.18
4	0.69±0.32	3.71±0.55	11.15^{a}±5.33	156.08±25.52	7.64±1.34	3.09±0.37	7.07±13.16	0.72±0.17
p	0.417	0.838	**0.000***	0.183	0.366	0.117	0.403	0.797

*: p<0.05 a. b: Aynı sütunda farklı harfleri taşıyan ortalamalar arasındaki farklar önemlidir (p<0.05).
1) anestezi öncesi, 2) anestezi başlangıcında, 3) anesteziden uyandırmadan önce, 4) anesteziden uyandıktan sonra. TG: Trigliserid, CHOL: Total kolesterol, GLU: glukoz, FRU: Fruktozamin, TP: Total Protein, ALB: Albümin, GLOB: Globülin, A/G: Albüminin globüline oranı. *: p<0,05 a, b: Differences between means with different letters in the same column are significant (p<0.05).
1) before anaesthesia, 2) at the beginning of anaesthesia, 3) before awakening from anaesthesia, 4) after awakening from anaesthesia. TG: Triglycerides, CHOL: Total cholesterol, GLU: Glucose, FRU: Fructosamine, TP: Total Protein, ALB: Albumin, GLOB: Globulin, A/G: Albumin globulin ratio.

Tablo 2. Hastaların hematoloji sonuçları
Table 2. Hematology results of patients

Parametreler	Birim	Ortalama	Standart Sapma	Referans Aralıklar
WBC	10^9/l	10.84	3.86	5.50-19.50
Neu%	%	58.97	15.83	38.0-80.0
Lym%	%	29.63	14.24	12.0-45.0
Mon%	%	5.78	1.08	1.0-8.0
Eos%	%	6.03	4.04	1.0-11.0
Neu#	10^9/l	6.67	3.31	3.12-12.58
Lym#	10^9/l	2.91	1.35	0.73-7.86
Mon#	10^9/l	0.63	0.24	0.07-1.36
Eos#	10^9/l	0.59	0.42	0.06-1.93
RBC	10^12/l	8.42	2.01	4.60-10.20
HCT	%	34.83	8.70	26.0-47.0
HGB	g/dl	13.20	5.03	8.5-15.3
MCV	fl	43.34	6.19	38.0-54.0
MCH	pg	15.07	2.60	11.8.0-18.0
MCHC	g/l	333.00	63.29	290-360
RDW-CV	%	17.70	1.89	16.0-23.0
RDW-SD	fl	30.48	5.88	26.4-43.1
PLT	10^9/l	277.68	115.82	100-518
MPV	fl	12.12	1.72	9.9-16.3
PDW	10GSD	14.15	0.90	12-17.5
PCT	ml/l	3.45	1.39	0.9-7.0

TARTIŞMA

Bu araştırmada kastrasyon ve ovariohisterektomi operasyonu amacıyla anesteziye alınan kedilerde örnekleme zamanına göre serum glukoz ve fruktozamin seviyelerinde meydana gelen değişim değerlendirildi. Hayvanlarda kısırlaştırma operasyonları için genel anestezi amacıyla kullanılan ketamin ve medetomidin gibi ajanların serum glukoz düzeylerini arttırdığı (Sharif ve Abouazra 2009; Kanda ve Hikasa 2008) bilinmektedir. Ek olarak bu hastalarda operasyon sonrası stres hiperglisemisi de (Palermo ve ark. 2016; Davis ve ark. 2018) görülebilmektedir. Ketamin uygulamaları serum glukoz seviyesini değişitirmekte (Sharif ve Abouazra 2009), öte yandan yüksek doz uygulamalar kolesterol seviyesini değiştirmeksizin trigliserid düzeylerini de arttırabilmektedir (Perumal ve ark. 2007). Sunulan bu çalışmada kolesterol ve trigliserid seviyeleri de değerlendirildi ancak örnekleme zamanına bağlı anlamlı bir fark tespit edilmedi. Ölçüm zamanı öncesi bir-iki haftalık sürecin kan glukoz ortalamasını gözler önüne seren fruktozamin ölçümleri glisemik kontrollerde kullanılabilmektedir. Serum fruktozamin konsantrasyonları, plazma glukoz seviyelerine bağlı olarak değişir ve diyabetik kedilerde glisemik kontrol amacıyla kullanılır (Gilor ve ark. 2010). Araştırmamızda kullanılan anestezik ajanlar örnek alım zamanına göre glukoz seviyesinde bir artışa sebep olurken, fruktozamin seviyelerinde istatistiksel olarak önem taşımayan numerik değişiklikler gözlendi. Literatürde fruktozamin seviyelerinin köpeklerde albümin seviyesi ve kedilerde ise total protein seviyesi ile ilgili ilişkili olduğu bildirilmiştir (Reusch 2001). Bu çalışmada ALB ve TP ölçümleri de gerçekleştirildi fakat bu değerlerde örnekleme zamanına bağlı anlamlı bir fark görülmedi. Operatif durumlarda glisemik kontrol amacıyla serum glukoz seviyesinin klinik pratikte sıklıkla kullanıldığı, buna rağmen serum fruktozamin seviyelerinin ise genellikle değerlendirilmediği görülmüş ve genel sağlık kontrolü taramalarında stres hiperglisemisinin, hiperglisemi ile ayrımının yapılması için fruktozamin ölçümlerinin faydalı olabileceği sunulan çalışma sonuçları çerçevesinde değerlendirilmiştir.

Kedilerde glukoz metabolizmasındaki bozuklukların erken tanısında glukoza kıyasla daha uzun bir kan glukozu seviyesini yansıtmasından dolayı fruktozamin kullanılmaktadır (Perez-Lopez ve ark. 2020). Perioperatif dönemde serum glukoz ve fruktozamin değişiminin takibi ve fruktozaminin perioperatif yanıtının değerlendirilmesi amacıyla yapılan bu çalışma fruktozamin düzeyinin anestezik ajanlardan veya operasyona bağlı gelişebilecek stres durumundan etkilenmediğini ortaya koydu.

SONUÇ

Örnekleme zamanları arası yapılan karşılaştırma verilerine göre çalışma sonuçları; kedilerde kısırlaştırma operasyonunda kullanılan "medetomidin, butorfanol ve ketamin" anestezisinin ve/veya anestezi/operasyon stresinin serum glukoz seviyelerinde meydana gelen değişimle ilişkili olabileceğini ortaya koydu. Serum fruktozamin seviyelerinde ise, istatistiksel açıdan önemli olmamakla beraber, numerik bir fark tespit edildi. Perioperatif glisemik kontrollerde glukoz ölçümü önemli olmakla birlikte, fruktozamin ölçümünün de, daha uzun süreli bir kan glukoz ortalaması sunması ve anlık dalgalanmalardan etkilenmemesi sebebiyle, bu dönemde farklı nedenlerle gelişebilecek olası hipergliseminin dışlanmasında diyagnostik yönden önem taşıyabileceği değerlendirildi. Serum glukoz düzeyleri özellikle kedilerde stres durumlarından ve yine anestezik maddelerden etkilenebilmektedir. Perioperatif dönem serum fruktozamin seviyesinin nispeten stabil oluşu, bu çalışmada kullanılan anestezik ajanlardan etkilenmediğinin bir göstergesi olarak kabul edilebilir.

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

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RESEARCH ARTICLE

Isolation and Molecular Characterization of Colistin-Resistant *Escherichia coli* in Raw Meat Samples

Mukaddes BAREL[1]*

[1]*Department of Public health, Faculty of Veterinary Medicine, Erciyes University, Kayseri, Türkiye*

ABSTRACT

Antimicrobial resistance occurring in a wide variety of Gram negative bacteria is considered an ongoing public health threat. For this reason, unconscious use of antimicrobials reveals new resistance mechanisms, and these resistance mechanisms are spreading globally. Due to these reasons, our current study aimed to isolate colistin-resistant *Escherichia coli* in raw meat samples and molecular characterization of resistance genes. For this purpose, the *trpA* gene was detected in 90 (75%) of the *E. coli* isolates obtained from 300 samples. *mcr-1* gene was found in 8 (8.8%) of the isolates confirmed as *E. coli*. However, as a result of the PCR analysis, O45:H2, O103:H2, O121:H19, O145:H28, O26:H11, O111:H8 *E. coli* serogroups and virulence genes were not found in any isolate. Additionally, isolates 7 (7.7%), 5 (5.5%), 29 (32.2%), 23 (25.5%), 8 (8.8%), 10 (9%), 2 (2.2%) and 6 (6.6%). It was found to be acid resistant to tetracycline, erythromycin, gentamicin, azithromycin, imipenem, ampicillin and nalidixic acid, respectively.

Keywords; Antimicrobial resistance, *Escherichia coli*, kolistin

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Çiğ Et Örneklerinde Kolistine Dirençli Escherichia coli'nin İzolasyonu ve Moleküler Karakterizasyonu

ÖZ

Çeşitli Gram-negatif bakterilerde meydana gelen antimikrobiyal direnç devam eden bir halk sağlığı tehdidi olarak nitelendirilmektedir. Bu nedenle bilinçsiz antimikrobiyal kullanımı yeni direnç mekanizmalarını ortaya çıkarmakta ve söz konusu bu mekanizmalar küresel olarak yayılmaktadır. Bu nedenler ışığında, mevcut çalışmamızda, çiğ et örneklerinde kolistin dirençli *E. coli* izolasyonu ve direnç genlerinin moleküler karakterizasyonu amaçlanmıştır. Bu amaçla, 300 örnekten elde edilen *E. coli* izolatının 90 (%75)'ınında *trpA* geni saptanmıştır. *E. coli* olarak doğrulanan izolatın 8 (%8.8)'inde *mcr-1* geni bulunmuştur. Ancak yapılan PCR analizi sonucunda hiçbir izolatta O45:H2, O103:H2, O121:H19, O145:H28, O26:H11, O111:H8 *E. coli* serogrupları ve virülens genleri bulunamamıştır. Ayrıca, *E. coli* izolatları disk difüzyon testi sonucunda 7 (%7.7), 5 (%5.5), 29 (%32.2), 23 (%25.5), 8 (%8.8), 10 (%9), 2 (%2.2) ve 6 (%6.6) oranlarında sırasıyla tetrasiklin, eritromisin, gentamisin azitromisin, imipenem, ampisilin, nalidiksik aside asit dirençli bulunmuştur.

Anahtar Kelimeler; Antimikrobiyal dirençlilik, *Escherichia coli*, kolistin

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ORCID ID; *MB: 0000-0002-1170-8632*
*Corresponding author e-mail: mukaddesbarel@erciyes.edu.tr

GİRİŞ

Escherichia coli, dünya genelinde insan ve hayvanlarda enfeksiyonlara neden olabilen ve toplumlara önemli ölçüde tıbbi, sosyal ve bakım maliyetleri getiren fırsatçı bir patojendir (Redweik ve ark., 2020). Gıda kaynaklı bir patojen olan *E. coli* insanlarda genellikle sepsise, farklı yaşlardaki çocuklarda ise orta veya şiddetli enfeksiyonlara neden olmaktadır (Manges ve ark., 2019 ; Wasinski, 2019). *E. coli*'nin hayvanlar ve insanlar arasındaki bulaşı, çiğ veya az pişmiş et, çiğ süt ve suyun hayvan dışkısı ile kontaminasyonu aracılığıyla olmaktadır. Kolistine dirençli bakteriler ise son on yılda çevre, gıda, su ve bitkilerde varlığını göstermiştir (Koskeroglu ve ark., 2023). Gram negatif bakterilerin sahip olduğu plazmidler ve integronlarda mevcut gen kasetleri gibi mobil genetik elementler, bu bakteriler arasında direnç genlerinin yayılmasında kilit rol oynamaktadır. Bu nedenle insan ve veteriner hekimliğinde yapılan tedavinin başarısız olmasına ve tedavinin uzamasına neden olmaktadır (Kim ve ark., 2018; Poirel ve ark., 2018; Berglund, 2018; Liu, 2018). Antibiyotikler patojen bakterileri hedef almakla birlikte, aynı zamanda memeli bağırsaklarındaki doğal florayı da etkileyerek vücuttaki doğal dengenin bozulmasına ve bağırsaklarda ciddi ikincil etkilere neden olmaktadır (Enany ve ark., 2019). Son yıllarda bakteriyel hastalıkların tedavisinde ve önlenmesinde antibiyotiklerin yaygın ve uygunsuz kullanımı (hayvanlarda ve insanlarda tarım ve tedavi amaçlı) nedeniyle çoklu ilaca dirençli bakterilerin ortaya çıkışı önemli bir halk sağlığı problemi haline gelmiştir (Hizlisoy ve ark., 2017; McLellan ve ark., 2018; Wolfensberger ve ark., 2019 ; Komijani ve ark., 2018). Söz konusu dirençli bakterilerin ortaya çıkışı kolistin gibi güçlü antibiyotiklerin fazla kullanımına neden olmuştur. Bu sebeple hastalıklara neden olan patojen mikroorganizmaları azaltılabilmesi için etkili ve güvenli terapötik alternatiflere acil ihtiyaç duyulmaktadır (Shahin ve ark., 2020). Söz konusu bu nedenlere bağlı olarak, mevcut çalışmamızda çiğ et örneklerinde kolistin dirençli *E. coli* izolasyonu ve direnç genlerinin moleküler karakterizasyonu amaçlanmıştır.

MATERYAL ve METOT

Materyal

Örneklerin Toplanması

Bu çalışmada, 2020-2022 yılları arasında 12 ay boyunca Kayseri ilinde bulunan satış noktalarından (beş adet) her bir ziyarette 25 adet (beşer adet) örnek olmak üzere toplamda 300 adet çiğ et örneği elde edilmiştir. Toplanan örnekler steril poşetler içerisine alınarak soğuk zincirde aynı gün içerisinde Erciyes Üniversitesi veteriner fakültesi Besin Hijyeni ve Teknolojisi laboratuarına getirilmiş ve analiz edilinceye kadar +4°C'de buzdolabında muhafaza edilmiştir.

Metot

E. coli'nin fenotipik identifikasyonu

Tüm örneklerde *E. coli* izolasyonu ISO 16649-2 kriterlerine göre yapılmıştır. Bu amaçla Eozin Metilen Blue (EMB) Agarda (Merck,103858) spesifik metalik rengi (yeşil) veren tüm şüpheli koloniler *E. coli* olarak kabul edilmiştir. Elde edilen şüpheli koloniler alınarak Blood agara (Merck,103879) ekilmiş ve 37°C'de 18-24 saat inkübasyona bırakılmıstır. İnkubasyon sonucunda elde edilen koloniler %10 oranında gliserol içeren Brucella broth (Merck. B3051)'lu cryovial tüplere alınarak -81°C'de mufaza edilmiştir.

DNA Ekstraksiyonu

Bakteri DNA'sını elde etmek amacıyla InstaGene Matrix kiti (Bio-Rad, ABD) kullanılmış olup, bu işlem kit protokolünde belirtildiği gibi gerçekleştirilmiştir. Bu sürenin sonunda elde edilen süpernatant yeni bir ependorfa alınarak, hedef DNA olarak kullanılmak üzere -20° C'de saklanmıştır.

Plazmit Ekstrasyonu

Kolistine dirençli suşlardan plazmid ekstraksiyonu, üreticinin protokolüne göre QIAprep Spin Miniprep Columns (QIAGEN, 27115) kiti kullanılarak gerçekleştirilmiştir. Elde edilen RNA tüm genom analizi için firmaya (Sugenomik Biotechnology) gönderilmiştir (Şekil 2).

E. coli'nin Moleküler İdentifikasyonu

Fenotipik testlerle *E. coli* olarak tespit edilen izolatların doğrulanması amacıyla PCR yöntemi kullanılmıştır (Clermont et al., 2013). Reaksiyon karışımı; her bir primerden 0.2 µM, 200 µM dntp, 2.5 µl PCR reaksiyon buffer, 3mM $MgCl_2$ U Taq DNA polimeraz (Thermo Scientific; EP0402, ABD) ve 1 µl DNA'dan oluşmuştur. Termal döngü koşulları; 94° C'de 5 dk ilk denatürasyon; 94°C'de 10 sn son denatürayon, 59° C'de 20 sn primer bağlanması (Tablo 1), 72° C'de 10 sn uzama, 72° C'de 5 dk son uzama aşaması olmak üzere 30 döngü ve 25 µL hacimde gerçekleştirilmiştir.

Tablo 1. Çalışmada kullanılan primer çiftleri
Table 1. Primer pairs used in the study

GENES	FORWARD	REVERSE	SIZE (bp)	REFERENCES
O45	TGCAGTAACCTGCACGGGCG	AGCAGGCACAACAGCCACTACT	238	DebRoy et al., 2011
O121	TCCAACAATTGGTCGTGAAA	AGAAAG TGTGAAATGCCCGT	628	DebRoy et al., 2011
O145	TTCATTGTTTTGCTTGCTCG	GGCAAGCTTTGGAAATGAAA	750	DebRoy et al., 2011
O26	CAATGGGCGGAAATTTTAGA	ATAATTTTCTCTGCCGTCGC	155	DebRoy et al., 2011
O111	TGTTTCTTCGATGTTGCGAG	GCAAGGGACATAAGAAGCCA	438	DebRoy et al., 2011
O103	TTGGAGCGTTAACTGGACCT	GCTCCCGAGCACGTATAAAG	321	DebRoy et al., 2011
H2	TGATCCGACATCTCCTGATG	CCGTCATCACCAATCAACGC	228	Banjo et al., 2018
H19	GCTGGCGATACATTTACCGC	CGCCGCTGTCATCAATGTTT	592	Banjo et al., 2018
H28	CTGGCATACAACAGGCACAC	TCAGCTTTGGTGTAAGCGTC	285	Banjo et al., 2018
H11	AACAACAACTGCAGCGGATG	TCGGGCTACCACCTTCTGAT	341	Banjo et al., 2018
H8	CGGCGCGGTTAAGAATGATG	CCGTTTTACCATCTGCGCTG	467	Banjo et al., 2018
trpA	CGGCGATAAAGACATCTTCAC	GCAACGCGGCCTGGCGGAAG	489	Clermont et al., 2012
Mcr-1	ACGCCATCTGCAACACCAA	GCCAACGAGCATACCGACAT	-	-

Disk Difüzyon Testleri

E. coli olarak identifiye edilmiş bakterilerin çeşitli antibiyotiklere karşı direnç durumunu belirlemek amacıyla disk difüzyon testi Klinik ve Laboratuvar Standartları Enstitüsünün (2016) önerdiği prosedüre göre yapılmıştır (Bauer ve ark., 1996). Antibiyotik duyarlılık test diskleri seftriakson (CRO 30 µg), sefalotin (KF 30 µg), sefpim (FEP 30 µg), sefksim (CFM 5 µg), fosfomisin (FOS 50 µg), mecillinam (MEL 25 µg), tetrasiklin (TE 30 µg), sülfametoksazol -trimetoprium (SXT 25 µg), levofoksasin (LEV 5 µg), eritromisin (E 15 µg), azitromisin (AZM 15 µg), imipenem (IPM 10 µg), ampisilin (AMP 10 µg), nalidiksik asit (NA 30) µg), siprofoksasin (CIP 5 µg), gentamisin (CN 10 µg), kloramfenikol (C 30 µg) ve aztreonam (ATM 10 µg) Müller- Hinton agar (Merck, 103876) üzerine yerleştirilmiştir. Petriler, aerobik 37° C'de 18-24 saat inkubasyona bırakılacak, inkubasyon periyodu sonunda oluşan zon çapları ölçülerek değerlendirme yapılmıştır (CLSI, 2016).

Virülens genlerinin PCR ile tespiti

Fenotipik testlerle *E. coli* olarak tespit edilen izolatlara ait virülens gen (*stx1, stx2 eae* ve *hlyA*) tespiti PCR yöntemi ile yapılmıştır. Reaksiyon karışımı; her bir primerden 0.5 µM, 400 µM dntp, 1X PCR buffer, 3mM $MgCl_2$ 1.5 U Taq DNA polimeraz (Thermo Scientific; EP0402, ABD) ve 1 µl kalıp DNA olmak üzere toplamda 50µL hacimde gerçekleştirilmiştir.

Termal döngü koşulları; 94° C'de 2 dk ilk denatürasyon aşamasından sonra 94° C'de 20 sn son denatürayon, 54° C'de 1 dk primer bağlanması, 72° C'de 1 dk uzama ve 72° C'de 10 dk son uzama aşaması olmak üzere 35 döngüden oluşmuştur (Barel ve ark., 2022).

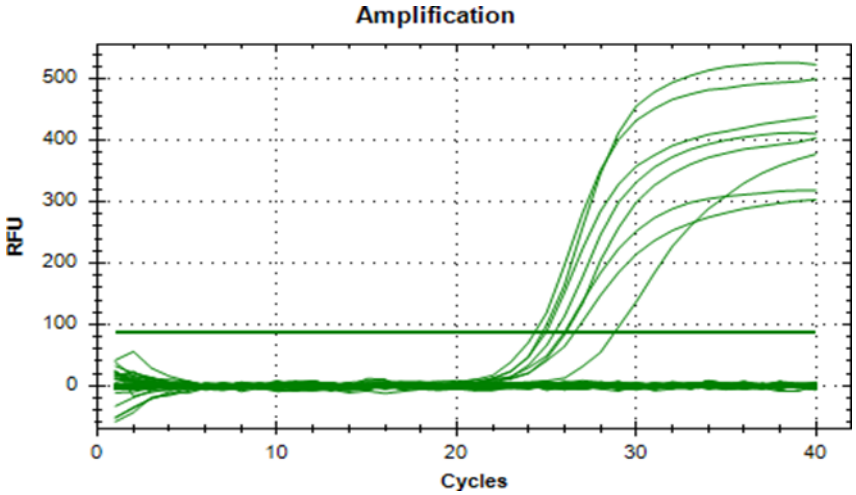
Real Time PCR ile Kolistin Direnç Geninin (mcr-1) Tespiti

E. coli olarak belirlenen izolatların tespiti amacıyla Real Time PCR kullanılmıştır. Reaksiyon toplam 20µL hacminde gerçekleştirilmiş olup, her bir primerden 0,1 µM DNA'dan 2 µl olacak şekilde Syber green supermix (Bio-Rad) kullanılarak yapılmıştır. Termal döngü koşulları; 95° C'de 10 dk ilk denatürasyon aşamasından sonra 95° C'de 15 sn son denatürayon, 63° C'de 2 dk primer bağlanması ve 72° C'de 10 sn uzama olacak şekilde 40 döngüden oluşmuştur (Şekil 1).

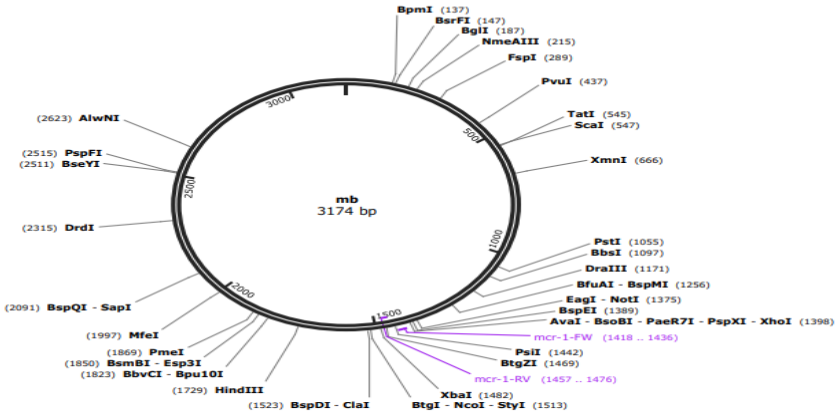
BULGULAR

Çalışmada 300 örnekten konveksiyonel metot ile elde edilen 90 (%75) izolatta *trpA* geni saptanmıştır. Buna

ek olarak yapılan PCR analizi sonucunda hiçbir izolatta *E. coli* serogupları (O45:H2, O103:H2, O121:H19, O145:H28, O26:H11, O111:H8) bulunamamıştır. Ayrıca, analiz sonucuna göre toplamda 8 (%8.8) *mcr-1* geni bulunmuştur (Şekil 1). Bu çalışmada hiçbir izolatta virülens genlerine rastlanılmamıştır. Ayrıca, *E. coli* izolatları 7 (%7.7), 5 (%5.5), 29 (%32.2), 23 (%25.5), 8 (%8.8), 10 (%9), 2 (%2.2) ve 6 (%6.6) oranlarında sırasıyla tetrasiklin, eritromisin, gentamisin azitromisin, imipenem, ampisilin, nalidiksik aside asit dirençli bulunmuştur.



Şekil 1: Real time analizi sonucu *mcr-1* geni pozitif olan *E. coli* izolatlarına ait Cq değerleri
Figure 1: Cq values of E. coli isolates with mcr-1 gene positive as a result of real time analysis



Şekil 2: Tüm genom analizine ait sirküler grafik
Figure 2: Circular graph of whole genome analysis

TARTIŞMA

Çalışmada 300 örnekten konveksiyonel metot ile elde edilen 90 (%75) izolatta *trpA* geni saptanmıştır. Ghafur ve ark. (2019) çiğ gıdalar ile yaptıkları bir çalışmada *E. coli* düzeyini %20 olarak rapor etmiştir. Aklilu ve Raman, (2020) tarafından yapılan başka bir çalışmada ise *E. coli* %54 düzeyinde olduğu bildirilmiştir. Tüm bu çalışmalara ek olarak, Guo ve ark. (2021) yaptıkları çalışmada ise *E. coli* düzeyini %24 olarak rapor etmiştir. Yapılan çalışmaların (Ghafur ve ark., 2019; Aklilu ve Raman, 2020; Guo ve ark., 2021) pozitif *E. coli* oranları bu çalışmadaki orandan düşük olduğu görülmüştür. Bu çalışma sonucundan yüksek olarak Adzitey (2020) çiğ et örnekleriyle yaptıkları bir çalışmada *E. coli* düzeyini %90 olarak bildirmiştir. Sonuçlar arasındaki farklılıklar örnekleme yöntemine, test edilen numunelerin farklılığına ve tespit tekniğine atfedilebilir (Barel ve ark., 2022).

Bu çalışmada yapılan PCR analizi neticesinde hiçbir izolatta O45:H2, O103:H2, O121:H19, O145:H28, O26:H11, O111:H8 *E. coli* serotipleri bulunamamıştır. Ancak, Mokhta ve Karmi (2021) tarafından sucuk kıyma ve tavuk eti ile yapılan bir çalışmada 2 izolatta O26:H11 ve O119:H6 serotipi tespit edildiği bildirilmiştir. Bu sonucun mevcut çalışmamızdaki sonuçtan yüksek olduğu görülmüştür. Bu çalışmanın sonuçlarına paralel olarak Cho ve ark. (2020) tarafından yapılan bir çalışmada hiçbir izolatta *E. coli* serotiplerine rastlanılmadığı belirtilmiştir. Bu çalışma ve diğer çalışmadaki *E. coli* serotiplerine rastlanılmaması, analize tabi tutulan örneklerin azlığı veya az sayıda serogrup içermesinden kaynaklı olabileceği bildirilmiştir (Renter ve ark., 2007).

Analiz sonucuna göre bu çalışmada toplamda 8 (%8.8) izolatta *mcr-1* geni bulunmuştur. Aklilu ve Raman (2020) yaptıkları bir çalışmada pozitif *mcr-1* genini %93 oranında bulduklarını rapor etmiştir. Yapılan başka bir çalışmada *mcr-1* geni %20 oranında bulunmuştur (Adiguzel ve ark., 2021). Zhang ve ark. (2022) tarafından yapılan başka bir çalışmada ise *mcr-1* geni %20 oranında rapor edilmiştir. Ancak, Rega ve ark. (2021) tarafından yapılan başka bir çalışma ise hiçbir izolatta *mcr-1* geni tespit edilemediği bildirilmiştir. Yapılan tüm çalışma sonuçları (Aklilu ve Raman, 2020; Adiguzel ve ark., 2021; Zhang ve ark., 2022) bu çalışma sonucundan oldukça yüksek olduğu görülürken, Rega ve ark. (2021) tarafından yapılan çalışma sonucundan yüksek olduğu görülmüştür.

Bu çalışmada hiçbir izolatta virülens genlerine rastlanılmamıştır. Nehoya ve ark. (2020) yaptıkları bir çalışmada izolatların %8'inde *eae* ve *stx1* birlikte bulunurken, %40'ında ise *eae*, *stx1* ve *stx2* virülans genleri birlikte bulunduğu bildirilmiştir. Buna ek olarak, Babolhavaeji ve ark. (2021) tarafından yapılan bir çalışmada virülens gen oranlarını *stx1* 1 (%12.5), *stx1/hly* 5 (%62.5) ve *stx2/hly* 2 (%25) olarak rapor edilmiştir. Momtaz ve Jamshidi (2013) yaptıkları bir çalışmada *stx1*, *eaeA* ve *hly* virülans genlerinin oranını 5 (%9.80) olarak bulmuştur. Yapılan tüm çalışma sonuçları (Momtaz ve Jamshidi, 2013; Nehoya ve ark., 2020; Babolhavaeji ve ark., 2021) bu çalışma sonucundan yüksek olduğu görülmüştür. *E. coli* izolatlarında *stx* geninin bulunmaması, Karch ve ark. (1992) bildirdiği gibi zenginleştirme sırasında *stx* geninin kendiliğinden kaybıyla ilişkili olabileceği belirtilirken, plazmit üzerinde taşınan *hlyA* geninin bakteri kültürleme sonrasında plazmid ile kaybolabildiği bu durumun tüm izolatlarda *hlyA* geninin belirlenememesinin bir nedeni olabileceği bildirilmiştir (Ferdous, 2017; Wetzel ve LeJeune, 2007). İzolatların 7 (%7.7), 5 (%5.5), 29 (%32.2), 23 (%25.5), 8 (%8.8), 10 (%9), 2 (%2.2) ve 6 (%6.6) oranlarında sırasıyla tetrasiklin, eritromisin, gentamisin azitromisin, imipenem, ampisilin ve nalidiksik asite dirençli olduğu tespit edilmiştir. Bu çalışma sonucuna benzer olarak Kassem ve ark. (2020) ve Mgaya ve ark. (2021) tarafından yapılan bir çalışmada tetrasikline (%91.9) sülfametoksazol-trimetoprim (%80.5), ampisilin (%70.9), siprofloksasin (%40.2) ve %25 sefotaksim, gentamisin (%10.8) ve imipenem (%8.6) olarak bildirilmiştir. Buna karşın, bu çalışma sonucundan yüksek olarak Mashak ve ark., (2018) yaptıkları çalışmada ampisilin, gentamisin, tetrasiklin ve siprofloksasin oranlarını sırasıyla (%100), (%90.47), (%85.71) ve (%71.42) olarak bildirmiştir.

SONUÇ

Çalışmada 300 örnekten konveksiyonel metot ile elde edilen 90 (%75) izolatta *trpA* geni saptanmıştır. Hayvanların kesim işlemi sırasında dışkı kontaminasyona dikkat edilmesi, üretimden tüketime kadar ki her aşamada et ve et ürünlerinin patojen *E. coli* türleri ile kontaminasyonuna sebep olan engellerin ortadan kaldırılması, antibiyotiklere dirençli *E. coli* varlığının azaltılması halk sağlığı açısından büyük önem arz etmektedir. Ayrıca, kesimhanelerde çalışan personele gerekli ve yeterli hijyen eğitimlerinin etlerin dışkı ile kontaminasyonun engellenmesinde oldukça önemli olduğu sonucuna ulaşılmıştır.

Çıkar çatışması: Yazarların rapor edecekleri herhangi bir çıkar çatışması yoktur.

Yazar katkı Oranları: MB, çalışmanın proje fikrine, tasarımına ve yürütülmesine katkıda bulundu, verilerin toplanmasına katkıda bulundu, taslağı hazırladı ve yazdı, makaleyi eleştirel olarak inceledi.

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RESEARCH ARTICLE

Determination of Malondialdehyde, Nitric Oxide, Reduced Glutathione, Sialic Acid and Ceruloplasmin Levels in Sheep Liver Tissue With Hydatic Cyst

Semistan KIZILTEPE[1*], Caglar OKULMUS[2], Oguz MERHAN[3], Neriman MOR[3], Erdogan UZLU[4]

[1]Igdır University Tuzluca Vocational School of Higher Education, Igdır Türkiye
[2]Izmir/Bornova Veterinary Control Institute Department of Biochemistry, Izmir Türkiye
[3]Kafkas University, Faculty of Veterinary Medicine, Kars Türkiye
[4]Balıkesir University, Faculty of Veterinary Medicine, Balıkesir Türkiye

ABSTRACT

Cystic echinococcosis is a prevalent helminth-zoonosis that poses a significant threat to human and animal health worldwide. Although it typically shows an asymptomatic clinical course, it has been reported to cause many damages and biochemical changes in tissues and organism. The aim of this study was to investigate the concentrations of nitric oxide (NO), reduced glutathione (GSH), malondialdehyde (MDA), ceruloplasmin (Cp), and total sialic acid (TSA) in sheep liver tissue affected by cystic echinococcosis. The study was carried out on sheep between 4-5 years of age brought to the slaughterhouse in the Igdir territory. The livers were examined post-mortem for cystic echinococcosis and cystic structures. Sheep liver tissues that tested positive for protoscolex were designated as the 'infected group', while healthy sheep liver tissues without lesions were assigned to the 'control group'. The results showed significantly higher levels of NO, MDA, Cp and TSA in the infected group compared to the control group ($p<0.05$), while GSH levels were significantly lower ($p<0.05$). These findings indicate that cystic echinococcosis in sheep is closely associated with mechanisms of inflammation, oxidative stress, and tissue damage. Moreover, our study provides insights into the oxidative response of cystic echinococcosis in liver tissue and enhances our understanding of the disease's pathogenesis.

Keywords: Cystic Echinococcosis, Inflammation, Oxidative Stress, Sheep

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Hidatik Kistli Koyun Karaciğer Dokusunda Malondialdehit, Nitrik Oksit, İndirgenmiş Glutatyon, Sialik Asit ve Seruloplazmin Düzeylerinin Belirlenmesi

ÖZ

Kistik ekinokokkoz, dünya çapında insan ve hayvan sağlığına ciddi bir tehdit oluşturan yaygın bir helmint-zoonozdur. Genellikle semptomsuz bir klinik seyir göstermesine rağmen, birçok doku ve organizmada hasara ve biyokimyasal değişikliklere neden olduğu bildirilmiştir. Bu çalışmanın amacı, kistik ekinokokkoz etkisi altındaki koyun karaciğer dokusunda nitrik oksit (NO), indirgenmiş glutatyon (GSH), malondialdehit (MDA), seruloplazmin (Cp) ve toplam sialik asit (TSA) konsantrasyonlarını araştırmaktı. Çalışma, Iğdır bölgesinde kesimhaneye getirilen 4-5 yaş arası koyunlar üzerinde gerçekleştirildi. Karaciğerler, ölümden sonra kistik ekinokokkoz ve kistik yapılar için incelendi. Protoskol eksisi pozitif çıkan koyun karaciğer dokuları "enfekte grubu" olarak adlandırıldı, lezyon olmayan sağlıklı koyun karaciğer dokuları ise "kontrol grubu" olarak atanmıştır. Sonuçlar, enfekte grubun NO, MDA, Cp ve TSA seviyelerinin kontrol grubuna göre önemli ölçüde yüksek olduğunu gösterdi ($p<0.05$), buna karşın GSH seviyelerinin önemli ölçüde düşük olduğunu ortaya koydu ($p<0.05$). Bu bulgular, kistik ekinokokkozun koyunlarda iltihaplanma, oksidatif stres ve doku hasarı mekanizmalarıyla yakından ilişkili olduğunu göstermektedir. Ayrıca, çalışmamız kistik ekinokokkozun karaciğer dokusundaki oksidatif yanıtını anlamamıza ve hastalığın patogenezi hakkındaki anlayışımızı artırmamıza yardımcı olmaktadır.

Anahtar kelimeler: İnflamasyon, Kistik Ekinokokkozis, Oksidatif Stres, Koyun

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ORCID ID; *SK: 0000-0003-2064-0657, CO: 0000-0003-3563-3716, OM: 0000-0002-3399-0667, NM: 0000-0002-3674-8120, EU: 0000-0002-3064-6633*
*Corresponding author e-mail: semistan.kiziltepe@igdir.edu.tr

INTRODUCTION

Echinococcus granulosus (E. granulosus) is a cestode parasite that primarily resides in the intestines of wolves, jackals, foxes, dogs, and cats. The parasite's eggs are excreted into the environment through the feces of infected carnivores (Craig et al. 2007). The main mode of transmission is the consumption of food contaminated with feces containing parasite eggs. Additionally, uncontrolled contact with contaminated items or intermediate hosts can also contribute to transmission. Upon oral ingestion, the eggs form oncospheres due to the action of stomach acid. These oncospheres then travel from the small intestine to the liver through the portal vein, where they invade and develop. Oncospheres that enter the circulation can also settle in various organs such as the brain, eyes, lungs, heart, kidneys, spleen, pleura, and bones. The clinical manifestation that occurs in the intermediate host organism, resulting from the growth of larvae derived from settled oncospheres in the intermediate host organs, is known as cystic echinococcosis (Mc. Manus et al. 2003; Oku et al. 2004; Craig et al. 2007; Ersayit et al. 2009).

Diagnosis of cystic echinococcosis typically involves the use of radiology, ultrasonography (USG), and serological methods. However, it can often be misdiagnosed as a tumor, abscess, or other types of cysts (Nart 2004; Şener et al. 2004; Özcel et al. 2007). In animals, the definitive diagnosis of cystic echinococcosis is often not cost-effective or practical. Generally, cystic echinococcosis is an asymptomatic disease in animals and is frequently detected during postmortem examinations in slaughterhouses. Despite being asymptomatic, cystic echinococcosis poses significant economic losses and health risks, making it a highly important parasitic disease (Gıcık et al. 2004; Latif et al. 2010; Demir and Mor 2011; Saadi et al. 2020).

Cystic echinococcosis is a prevalent helminth-zoonosis that poses a significant threat to human and animal health worldwide. In Turkey, factors such as extensive sheep farming, inadequate wildlife control, and insufficient implementation of control measures (such as parasitic spraying and breeder education) contribute to the high prevalence of cystic echinococcosis (Yıldız and Gürcan 2003; Gıcık et al. 2004).

In parasitic infestations, the disruption of the antioxidant defense balance leads to an increased production of reactive oxygen species, which can cause damage to host cells. Various studies investigating different parasitic agents have reported changes in antioxidant levels and the occurrence of oxidative stress during infestations. Lipid peroxidation (MDA) and other oxidative stress mechanisms (Cp, NO, TSA, and GSH) have been implicated in the pathogenesis of several parasitic diseases affecting animals (Sanchez-Campos et al. 1999; Kilic et al. 2003; Derda et al. 2004; Kolodziejczyk et al. 2006; Şimşek et al. 2006; Kaya et al. 2007; Gabrashanska et al. 2008; Saleh 2008; Saleh et al. 2009; Dimri et al. 2010; Heidarpour et al. 2012).

Numerous studies have documented the occurrence of oxidative stress in parasitic diseases (Boczon et al. 1996; Shousha et al. 1999; Sanchez-Campos et al. 1999; Derda et al. 2004; Şimşek et al. 2006; Saleh, 2008; Saleh et al. 2009; Dimri et al. 2010). Moreover, oxidative stress plays a significant role in the general pathogenesis of various liver diseases. Assessing oxidative damage in live animal liver tissue is challenging, which is why oxidative stress markers are often measured in blood and blood products in such diseases.

Several studies have been conducted to determine the antioxidant and oxidative stress markers in the blood of humans, cattle, sheep, and camels with cystic echinococcosis (Kilic et al. 2010; Heidarpour et al. 2012, Heidarpour et al. 2013 a, b; Mahmood et al. 2020). In contrast, some studies suggest that direct detection methods are more reliable for evaluating antioxidant status and oxidative stress (Fang et al. 2002; Değer et al. 2008; Aslam et al. 2023). However, there is a lack of reports on direct tissue analysis of oxidative stress and antioxidant defense systems specifically related to cystic echinococcosis in sheep.

In recent years, the veterinary field has seen increased investigation into oxidative stress parameters such as MDA, NO, and GSH, as well as acute phase proteins like TSA and Cp. However, there is still limited research on how these parameters change in parasitic conditions such as cystic echinococcosis, which continues to pose a risk in industrial livestock farming and has zoonotic implications. Therefore, the current study aims to investigate the levels of liver MDA, Cp, NO, TSA, and GSH in infected sheep, with the goal of assessing the oxidative response and providing insights into the pathogenesis of the disease.

MATERIALS AND METHODS

The study utilized liver samples obtained from between sheep 4-5 years of age brought to the slaughterhouse from sheep farms in the Iğdır territory. Prior to inclusion in the study, a comprehensive health examination was conducted on all sheep brought for slaughter. After slaughtering the animals, all organs were examined to identify hydatid cysts, and only sheep with liver cysts were included in the study. During sample collection, particular attention was given to ensuring that each liver had at least three cyst foci. The cyst contents of these samples were examined under a microscope for parasitological analysis, and a total of 40 sheep livers with positive protoscoleces (fertile cysts) were assigned to the experimental group. The control group consisted of 10 sheep livers that did not show any pathological lesions during general organ examinations and appeared healthy in the physical examination.

Tissue samples from the study group were taken from the cyst site and cyst membrane, measuring approximately 1 cm^3 in size. Tissue samples from the control group were obtained from the middle lobe of the liver, also measuring approximately 1 cm^3. All samples from both groups were stored at -20 °C for further analysis.

Homogenization of Liver Tissue

Tissue sample taken from liver were immediately fixed with PBS (7.4 pH) at +4 °C and homogenized at 290 g for 3 minutes with the help of a cooling homogenizer (Wiggen-Hauser D-500, Germany). During homogenization, the samples were kept in ice for 15-20 seconds once a minute to prevent heating. The homogenates were centrifuged for 15 minutes at 4 °C at 2400 rpm (Hermle Z 326 K, Germany), and the supernatants obtained were stored at -80 °C until analyzed.

Biochemical Analyses

The concentrations of nitric oxide (NO), reduced glutathione (GSH), malondialdehyde (MDA), ceruloplasmin (Cp) and total sialic acid (TSA), respectively, Miranda et al. (2001), Beutler et al. (1963), Yoshioka et al. (1979), Colombo and Richterich (1964) and Sydow et al. (1988) spectrophotometrically measured (Epoch®, Biotek, USA) according to the method reported.

Statistical Analysis

The data of the study were statistically evaluated using the SPSS 20.0 (SPSS Inch. Chicago, IL, USA) package program. Kolmogorov-Smirnov test was performed and it was determined that the groups showed normal distribution. Student's t test was used to compare groups.

RESULTS

Table 1 displays the levels of MDA, NO, GSH, Cp, and TSA obtained from sheep livers with hydatid cysts (Figure 1) and healthy sheep livers. In comparison to the control group, the infected group exhibited significantly higher levels of MDA, TSA, Cp, and NO, along with lower levels of GSH ($p<0.05$). The disparity between the measurements was particularly significant in the MDA and TSA parameters ($p<0.001$).

Table 1. Malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide (NO), ceruloplasmin (Cp) and total sialic acid (TSA) levels in liver tissue.

Parameters	Control group	Infected group	P value
MDA (µmol.g−1 wet tissue)	0.61±0.14 ^{x}	2.28±0.24^{y}	p<0.001
TSA (mg.g^{-}−1 wet tissue)	0.83±0.12 ^{x}	1.34±0.08 ^{y}	p<0.001
Cp (mg.g−1 wet tissue)	0.27±0.11 ^{a}	0.73±0.09 ^{b}	p<0.05
NO (µmol.g^{-}−1 wet tissue)	0.83±0.05 ^{a}	1.29±0.09 ^{b}	p<0.05
Reduced GSH (µmol.g−1 wet tissue)	5.84±0.36 ^{a}	4.66±0.19 ^{b}	p<0.05

***** The difference between groups with different (a, b) signs in the same lines is significant ($p<0.05$).
**The difference between groups with different (x, y) signs in the same lines is significant ($p<0.001$).

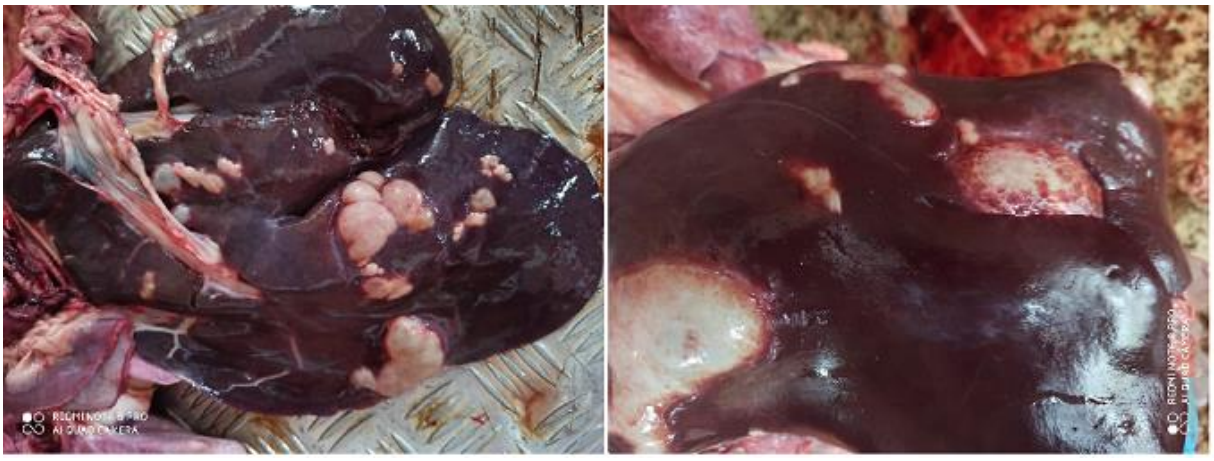

Figure 1: Sheep liver samples with hydatid cyst after slaughter at the abattoir.

DISCUSSION

Cystic echinococcosis is a parasitic and zoonotic disease that exhibits a higher incidence in regions where traditional sheep breeding is common and rural populations are concentrated. Typically, cystic echinococcosis has an asymptomatic clinical course. Various studies have demonstrated that parasites can induce biochemical changes by damaging the tissues and organs they inhabit (Mert et al. 2003; Ayaz et al. 2006; Şahin and Akgül 2006).

One of the reactions observed in cellular damage caused by free radicals is lipid peroxidation in cell membranes. Elevated levels of MDA, an end product of lipid peroxidation, are considered an indicator of oxidative damage in multiple tissues and organs. Aslam et al. (2023) found significantly higher concentrations of MDA ($p<0.05$) in cystic echinococcosis cysts and infected buffalo liver tissues compared to uninfected liver tissues in their study. Similarly, in the present study, it is hypothesized that cystic echinococcosis cysts cause significant cellular damage in liver tissue due to their space-occupying effect and mechanical pressure. This damage is believed to be the underlying cause of the elevated MDA levels, which serve as a strong indicator of oxidative stress due to increased lipid peroxidation. In our study, infected sheep exhibited higher MDA levels compared to the control group. Moreover, it is worth noting that several studies conducted with humans, camels, cattle, and sheep infected with Echinococcus granulosus have also reported significantly higher MDA levels compared to healthy individuals, which supports the findings of our study (Heidarpour et al. 2012, 2013a; Merhan et al. 2017; Aslam et al. 2023).

Acute-phase proteins serve as clinically useful and convenient biochemical markers for disease diagnosis, treatment, and prognosis monitoring (Nakajima 1993; Mc Pearson 1996; Gruys et al. 1994; Floris et al. 2000; Bozukluhan et al. 2020). Additionally, sialic acid undergoes significant changes in diseases associated with tissue damage or inflammation, where the acute-phase response is stimulated. The level of sialic acid is considered an indicator of the acute-phase reaction due to its structural characteristics (Erdogan et al. 2008; Yarım et al. 2010). Studies have shown that serum TSA levels increase in buffalo and cattle naturally infected with cystic echinococcosis (Yarım et al. 2010; Mohammadpour et al. 2021). In our current study, we observed statistically significant elevation ($p<0.001$) in TSA concentrations in the cystic echinococcosis-infected group compared to the control group. We attribute this increase to the induced acute-phase reaction triggered by parasitic infection.

Peptide-structured glutathione (GSH), which can be synthesized in the liver, plays a crucial role in the antioxidant defense system that combats oxidative damage caused by free radicals and peroxidases (Karaman et al. 2008; Koltas et al. 2008). During parasitic infections, the glutathione system demonstrates its protective effect against the detrimental effects of lipid peroxidation by interacting with free radicals and peroxides (Dede et al. 2000; Değer et al. 2008). Previous studies conducted on cattle infested with Dictyocaulus viviparus (Değer et al. 2008), goats naturally infected with Haemonchus contortus (Rashid and Irshadullah 2019), and buffaloes infested with Fasciola gigantica (Rehman et al. 2021), as well as cystic echinococcosis-infected buffaloes (Kolodziejczyk et al. 2006), have reported a decrease in GSH concentration in serum or liver tissue examinations, highlighting the history of liver tissue damage in the pathogenesis. In our study, we also found lower liver tissue GSH concentrations ($p<0.05$) in the infected group compared to the control group, aligning with the findings of the aforementioned research. We believe that this observation is a result of the reaction occurring with the protective effect of GSH as an antioxidant in response to the oxidative stress induced by the damage to cells, tissues, and organs infested with cystic echinococcosis.

Ceruloplasmin (Cp) is another important acute-phase protein for sheep (Bozukluhan et al. 2018; Ulutaş et al. 2008). In certain infections, it has been reported to increase up to half of the normal value (Nispet et al. 2008). In addition to its acute-phase protein properties, Cp is involved in the transport of antioxidants and copper in the bloodstream (Ulutaş et al. 2008; Gökce and Bozukluhan 2009; Tuna and Ulutaş 2015). This characteristic makes it a potential diagnostic marker for diseases (Erkılıç et al. 2019; Kırmızıgül et al. 2020). In a study conducted by Nisbet et al. (2008) in cattle with cystic echinococcosis, they found that serum Cp levels were higher in the patient group compared to the group of healthy animals. Similarly, in a study by Eser et al. (2013) in individuals with pulmonary echinococcus (PE), they reported higher Cp values in the PE group compared to the healthy group. They also observed a significant decrease in Cp values in the PE group after surgical removal of the cysts, compared to the initial values obtained from the patients. In our study, the concentration of Cp in the cystic echinococcosis-infected group was found to be higher than in the control group ($p<0.05$). This elevation is likely attributed to the inflammatory changes associated with cystic echinococcosis.

CONCLUSION

In conclusion, the significant differences observed in MDA, NO, GSH, TSA and Cp levels in cystic echinococcosis-infected sheep liver tissues compared

to healthy sheep indicate the presence of significant oxidative stress in the livers of infected sheep. In addition, it is thought that direct measurement of these parameters from liver tissue may be a more reliable diagnostic method.

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

Authors' Contributions: SK and CO performed the research, OM, NM and CO analysed the data, SK, EU and CO designed the research study and wrote the paper.

Ethical Approval: This research was approved by The Ethics Committee of the Faculty of Veterinary Medicine, Kafkas University (KAU-HADYEK, Ref No: 2021/81, Tarih: 4/2021)

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RESEARCH ARTICLE

Investigation of the Protective Effects of Black Garlic Extract an Experimental Gastric Ulcer Model in Rats

Serdar AKTAŞ[1*]

[1]Aydin Adnan Menderes University, Faculty of Veterinary Medicine, Aydın, Türkiye

ABSTRACT

This study aimed to evaluate the effects of black garlic extract in preventing gastric ulcers. For this purpose, twenty-eight 2-3 month-old *Spraque dawley* rats were randomly divided into four groups: the Control group (CG), Ulcer group (UG), Ulcer + pantoprazole treatment group (PTG), and Ulcer + Black garlic group (BGG). Stomach ulcers were induced by administering indomethacin at a dose of 25 mg.kg^{-1} to all groups except the control group. Then, pantoprazole (20 mg.kg^{-1}) and black garlic extract (275 mg. rat) were administered to the treatment groups. After the study, stomach samples were collected and macroscopic (ulcer scoring and ulcerative area mm^2) and histopathology examination (HE) were performed. In biochemical analysis, MDA (pg.ml^{-1}), SOD (ng.ml^{-1}) and CAT (ng.ml^{-1}) levels were determined in the tissue. According to the macroscopic results obtained; Statistically significant changes were found between BGG and UG ($p<0.0001$). PTG showed better effects than all groups. In histopathology; no lesion was found on CG. There was a similarity between PTG and CG. While there were intense bleeding and ulcer foci in UG, only limited focal ulcers were found in BGG. MDA, the highest values were seen in UG. There was no statistical significance between PTG and BCG. The lowest SOD activity was in UG, and there was a similarity in PTG and BGG. The highest CAT activity was determined in CG and the lowest in UG. In conclusion; black garlic extract applied in a single and high dose (5% w.v-1, 275 mg. rat) showed partial protective activity against stomach ulcers.

Keywords: Black garlic extract, pantoprazole, stomach ulcer, ulcer model.

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Siyah Sarımsak Ekstraktının Ratlarda Deneysel Mide Ülseri Modelinde Koruyucu Etkilerinin Araştırılması

ÖZ

Bu çalışmada gastrik ülserin önlenmesinde siyah sarımsak ekstraktının etkilerinin değerlendirilmesi amaçlandı. Bu amaçla 2-3 aylık 28 adet *Spraque dawley* ırkı rat rastgele olarak 4 gruba ayrıldı. Kontrol grubu (CG), Ülser grubu (UG), Ülser + pantoprazole tedavi grubu (PTG) ve Ülser + Siyah sarımsak grubu (BGG). Kontrol grubu hariç diğer gruplara 25 mg.kg^{-1} dozda indomethazin verilerek mide ülseri oluşturuldu. Daha sonra tedavi gruplarına Pantoprazol (20 mg.kg^{-1}) ve siyah sarımsak eksraktı (275 mg.rat) uygulandı. Çalışma sonrasında mide örnekleri alınarak makroskobik (ülser skorlaması ve ülseratif alan mm^2) ve histopatoloji incelemesi (HE boyaması) yapıldı. Biyokimyasal analizlerde oksidan/antioksidan parametre analizleri kapsamında malondialdehid (MDA, pg. ml^{-1}) seviyesi ile süperoksid dismutaz (SOD, ng. ml^{-1}) ve katalaz (CAT, ng. ml^{-1}) aktiviteleri değerlendirildi. Elde edilen makroskobik sonuçlara göre; BGG ve UG arasında istatisliksel anlamlı değişimler bulundu ($p<0.0001$). PTG tüm gruplara göre daha iyi etki gösterdi. Histopatolojide; CG'de herhangi bir lezyona rastlanmadı. PTG ile CG arasında benzerlik vardı. UG'de yoğun kanama ve ülser odakları mevcutken, BGG'de sadece sınırlı fokal ülser bulundu. MDA'da değerler en yüksel UG'de görüldü. PTG ve BCG arasında istatisliksel anlam yoktu. SOD aktivitesi en düşük UG olup, yine PTG ve BGG'de benzerlik vardı. CAT aktivitesi en yüksek CG'de en düşük ise UG'de belirlendi. Sonuç olarak; tek ve yüksek dozda (%5 w.v, 275 mg.rat) uygulanan Siyah sarımsak ekstraktı mide ülserine karşı kısmi koruyucu etkinlik göstermiştir.

Anahtar kelimeler: Mide ülseri, pantoprazole, siyah sarımsak ekstraktı, ülser modeli

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ORCID ID; *SA: 0000-0003-3243-8093.*
*Corresponding author e-mail: saktas@adu.edu.tr

INTRODUCTION

The gastric ulcer has an important place among gastrointestinal problems and if left untreated, it can cause complications, stomach and duodenal cancers and even death. Many etiological factors can cause this problem. Nonsteroidal anti-inflammatory drugs, alcohol, stress, and Helibacterium pylori infections are the main ones (Sabiu et al. 2015). The size and number of ulcers formed in the stomach tissue may vary. Ranging between 1-5 mm in diameter, these ulcers may extend deeply from the mucosa to the muscular layer, resulting in a slow or incomplete healing process influenced by digestive fluids. Antacid drugs, H_2 receptor blockers and proton pump inhibitors are currently used in ulcer treatment. However, these drugs have side effects and long-term problems, such as absorption problems and the elimination of the initial barrier effect of the stomach.

In stomach ulcer research, the ulcer tissue must heal quickly by increasing the durability of the stomach tissue. For this purpose, many experimental models (non-steroids, alcohol and stress) and studies are available. Herbal extracts with antioxidant and antimicrobial effects against *Helibacterium pylori* are important in experimental ulcer studies. *Pistacia atlantica* essential oil has been reported to have a protective effect against gastric ulcers caused by ethanol and antibacterial activity on *Helicobacter pylori* (Memariani et al. 2017). The aqueous extract of *Carica papaya* seed exhibited anti-ulcerogenic and antioxidant effects (Oloyede et al. 2015), while *Xylocarpus granatum* fruit was found to be mucosal protective in rats (Lakshmi et al. 2010).

In recent years, many operations have been carried out to remove the unpleasant odour of garlic and increase its taste. Black garlic; It is a form of normal garlic (*Allium sativum*) with high antioxidant levels, formed as a result of heat treatments and fermentation steps. It has been stated that this form may be useful in preventing and reducing the effects of various diseases. Since the sulfur compounds and allicin it contains decrease during fermentation, its disturbing effects are no longer a problem. Black garlic extract; has been determined in previous studies to it could exhibit anti-inflammatory, anti-obesity, hepatoprotection, hypolipidemia, anti-cancer, anti-allergy, immunomodulation, nephroprotection, cardiovascular protection and neuroprotective effects (Tak et al. 2014; Jeong et al. 2016; Kim et al. 2017).

There are very few studies on the gastrointestinal effects of black garlic. This study aimed to determine the potential protective effects of single and high doses of black garlic extract on induced gastric ulcers in rats.

MATERIALS and METHODS

This study Animal Experiments Local Ethics Committee approval was taken. Twenty-eight 3-month-old *Spraque dawley* rats were randomly divided into four groups (n=7 rats per group). Group 1; Control group (CG), Group 2; Ulcer group (UG), Group 3; Ulcer +Standard treatment group (PTG), Group 4; Ulcer + Black garlic group (BGG). Indomethacin (Endol capsule/Deva Ilac/İstanbul/Türkiye) was administered orally by gavage at a dose of 25 mg.kg-1 to all rats except the control group, after a 12-hour fasting period. 10 minutes before indomethacin was administered to the treatment groups (Group 3); Pantoprazole (20 mg.kg^{-1}, Pulcet capsule, Nobel Ilac İstanbul/Türkiye) and black garlic (MDC Black Garlic, İstanbul-Türkiye) was administered to Group 4 by preparing its aqueous extract in powder form (5% w.v^{-1}, 275 mg. rat) was administered orally. 6 hours after drug administration, the rats were euthanized by cervical dislocation under ketamine (Keta control, Doga İlaç/İstanbul/Türkiye) and xylazine (Xylazınbio 2%, Bioveta) anaesthesia. After the stomach tissues were removed, they were washed in 0.9% NaCl solution.

Macroscopic evaluation

Stomach tissues were placed on a clean surface and ulcer areas were visualized. Ulcer areas (mm^2) on the stomach surface were determined using millimetric paper. In ulcer scoring, the ulcer level (0-5 scale) was determined and recorded separately for each animal, as stated in previous studies (Cantarella et al. 2005).

Histopathological evaluation

After the experimental study, the stomach tissues removed after euthanasia were divided into 2 equal parts. Gastric tissue samples taken for histopathology were stored in 10% formaldehyde solution. 4 μm sections were taken from the tissue samples taken into paraffin blocks and examined by Hematoxylin-Eosin (HE) tissue staining. The severity of the ulcer and the level of healing were determined by 3 experts in pathology.

Oxidant-antioxidant balance parameters

Other stomach tissue samples taken from each rat were homogenized in an ice cube in PBS (Phosphate buffer saline) for 1 minute in a tube with 2000 rotations per minute. The resulting homogenates were centrifuged at 7000 rpm for 10 min at 4 °C in a refrigerated centrifuge, and the supernatants were stored at −80°C until subsequent analysis. Total protein (TP, Abcam Company ab113463) levels and malondialdehyde (MDA Assay Kit, ab238537), glutathione (GSH Colorimetric ab102530), superoxide dismutase (SOD Abcam Company, ab285309), and catalase (CAT, BT Lab E0869Ra) activities in the

stomach tissue were measured from the prepared samples.

Statistical evaluation

All numerical values obtained will be evaluated by one-way analysis of variance (ANOVA) if appropriate, or by Kruskal Wallis H-Test if not appropriate. SPSS 22 and GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) were used in the study. In the study, $p<0.5$ was considered significant.

RESULTS

Stomach ulcer areas are considered in macroscopic evaluation; No lesion was found in the control group (CG). While the highest ulcer area (mm^2) was seen in UG, the lowest amount was seen in the PTG group. Statistical significance was found between PTG and UG. (Figures 1 and 2, $p<0.0001$).

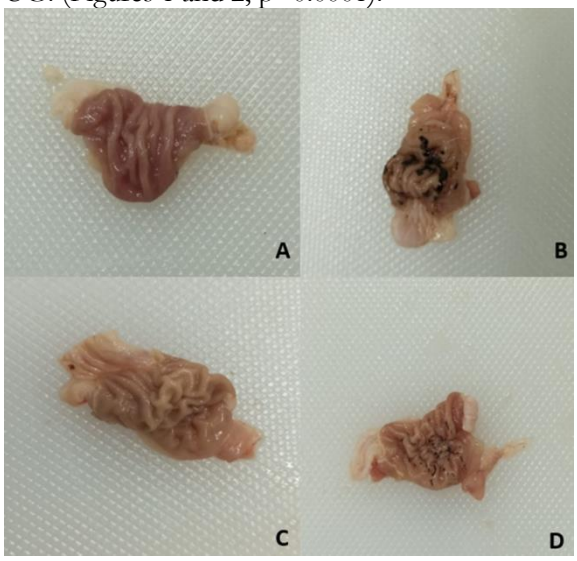

Figure 1: Macroscopic appearance of the stomach tissues; A: Control group (CG), B; Ulcer group to which indomethacin was applied (UG), C; Pantoprazole treatment group (PTG), D; Black garlic treatment group(BGG).

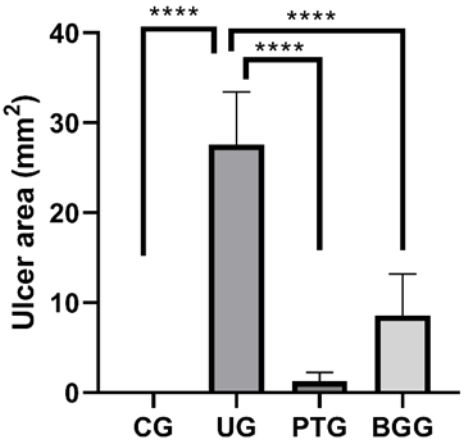

Figure 2: Ulcer areas (mm^2) formed after indomethacin application in gastric tissue samples. Control group (CG), Ulcer group (UG), Ulcer + Pantoprazole treatment group (PTG) and Black garlic group (BGG). Statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

A statistical difference was found between BGG, UG and CG in ulcer index (0-5 scale) values. The highest ulcer index was seen in UG, while the lowest level was seen in PTG. There was statistical significance between PTG and BGG (Figure 3, $p<0.01$).

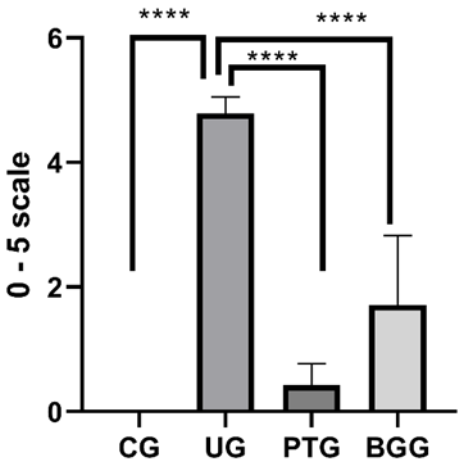

Figure 3: Ulcer scale (0-5) all groups. Control group (CG), Ulcer group (UG), Ulcer + Pantoprazole treatment group (PTG) and Black garlic group (BGG). Statistically significant (*$p< 0.05$, **$p< 0.01$, ***$p< 0.001$, ****$p < 0.0001$).

In histopathological evaluation; In the material examined with serial sections on CG, mucous columnar epithelium in the stomach and gastric glands in the submucosa were observed as normal. Multiple bleeding ulcerative foci and erosions were observed in the UG. In the PTG group, there was an appearance close to normal tissue. Mucosa and glands were normal. No ulcer was detected. In BGG, there were a few focal mucosal damages and ulcers. An increase in mucus cells and mucus was observed. The multilayered cutaneous stomach section was normal in all groups. The fact that fewer ulcer areas were observed in BGG than in UG showed that black garlic has incomplete partial protective effects.

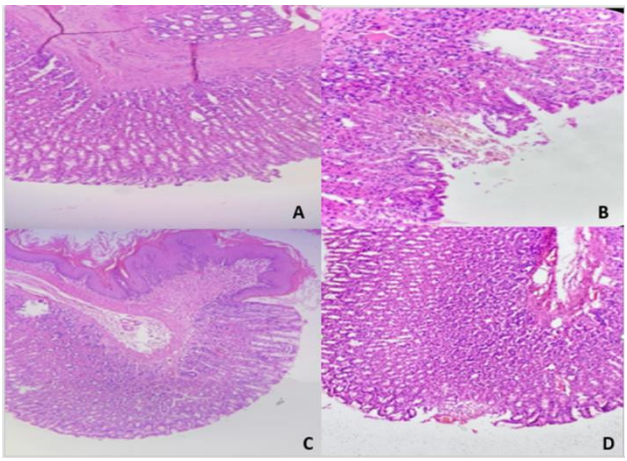

Figure 4: Evaluation of gastric tissue samples by Hematoxylin and Eosin staining (HEx20 Magnification). **(A):** Control group; normal stomach tissue. **(B):** Ulcer group; mucosal damage, cell infiltrates, multiple ulcers and haemorrhage. **(C):** Ulcer + Pantoprazole treatment group; Near normal mucosa, **(D):** Black garlic group; Very low rate of focal mucosal damage.

When examined in terms of oxidative stress parameters, MDA levels (pg. ml-1) were highest in UG and lowest in CG. A statistically significant decrease was observed in BGG compared to UG ($p<0.01$). No statistical significance was found between PTG and BGG (Figure 5).

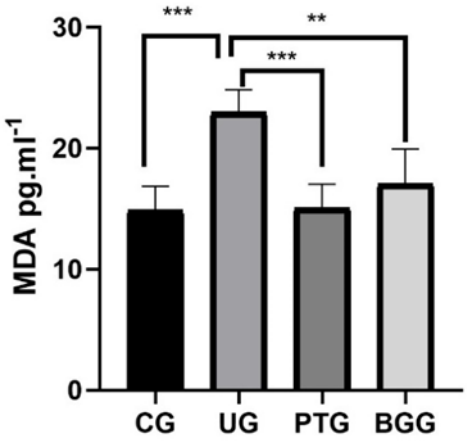

Figure 5: MDA (pg.ml-1) levels in samples obtained from gastric tissue homogenates. Control group (CG), Ulcer group (UG), Ulcer + Pantoprazole treatment group (PTG) and Black garlic group (BGG). Statistically significant (*p<0.05, **p<0.01, ***p<0.001).

The highest SOD activity (ng. ml-1) was seen in CG and the lowest level was seen in UG. There was a statistical difference between BGG and UG ($p<0.5$). There was no statistical difference between BGG and PTG (Figure 6).

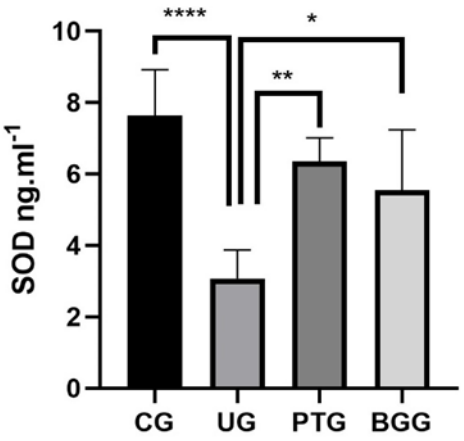

Figure 6: SOD (ng.ml-1) activities in samples. Control group (CG), Ulcer group (UG), Ulcer + Pantoprazole treatment group (PTG) and Black garlic group (BGG). Statistically significant (*p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

While there was a statistical difference between PTG and UG in CAT activity (ng. ml-1, $p<0.5$), no significance was found between UG and BGG (Figure 7).

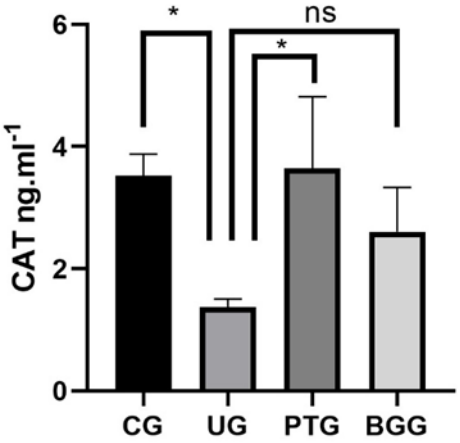

Figure 7: CAT (ng.ml-1) activities in samples. Control group (CG), Ulcer group (UG), Ulcer + Pantoprazole treatment group (PTG) and Black garlic group (BGG). Statistically significant (*p < 0.05, **p <0.01). Ns: Not significant. No statistical significance was found between UG and BGG values.

DISCUSSION

Stomach ulcers are one of the gastrointestinal problems that significantly reduce the quality of life, which may have different etiological reasons. Non-steroids are one of the factors playing a role in aetiology. Indomethacin is a non-steroidal drug and has many side effects. They have side effects on the stomach (especially ulcers) when used in normal doses and sometimes unconsciously in high amounts (Suleyman et al. 2010). There is a search for new

treatments for ulcer treatments due to the negative effects of currently used drugs, such as long-term absorption disorders and eliminating the barrier effect of the stomach. Black garlic extract is one of them. The amount of total phenolic substances and flavonoids is much higher than normal garlic (Ahmed and Wang 2021). Antioxidant compounds such as lycopene show protection by showing antioxidant effects in gastric ulcer models (Boyacioglu et al. 2016). In another study, it was stated that there was a higher level of protection at the 200 $mg.kg^{-1}$ dose in the stomach ulcer model created by applying 100 and 200 $mg.kg^{-1}$ doses for 30 days (Badr et al. 2014).

Possible protective efficacy can be easily observed and measured macroscopically in gastric ulcer models created with indomethacin. Calculating ulcer areas and ulcer scores is one of them. In a previous study, it was reported that there was a statistical decrease in ulcer areas in the stomach tissue as a result of the application of silymarin at different doses (25, 50 and 100 $mg.kg^{-1}$) in the stomach ulcer model created in rats (Boyacioglu 2019). In this study, there was a statistically significant decrease in ulcer scores and areas (mm^2) in the BGG group compared to the UG group ($p<0.0001$). However, the use of higher doses of black garlic extract compared to previous studies, did not achieve higher levels of protection.

In histopathological evaluation, while no ulcers and mucosal damage were found in CG and PTG, a high rate of haemorrhage and ulcer foci were observed in UG. In BGG, fewer focal ulcer foci were detected (Figure 4). This showed that the protective effectiveness of black garlic remained at a partial level. In a previous study, it was determined that black garlic applied to rats at a dose of 200 $mg.kg^{-1}$ for 10 days had protective effects on stomach ulcers but was lower than omeprazole application (El-Ashmawy et al. 2016). In the same study, it was stated that omeprazole showed 94.5 % anti-ulcerative effects, while black garlic extract showed a protective effect of 83.4 %. In this study, black garlic extract used in higher doses compared to previous studies did not show protection reaching higher levels. This showed that increasing doses were not effective in protection.

Some researchers have stated that black garlic increases the inflammatory response due to its high sugar content compared to fresh garlic and that it is only more effective in ROS-related issues (Kim et al. 2017). They found that the anti-inflammatory activity of black garlic fermented with *Lactobacillus rhamnosus* was dependent on the activation of NF-ϰB, inhibition of cytokine production, and expression of iNOS and COX-2 (Tak et al. 2014). In previous studies, black garlic has been shown to exert protective effects by reducing oxidative stress parameters in rats subjected to nephrotoxicity (Maldonado et al. 2003). In another study, it was reported that it similarly reduced 8-hydroxydeoxyguanosine and MDA levels and regulated TGF-β1, SOD, CAT, and GSH levels in a colistin-induced renal failure model (Lee et al. 2019). In this study, black garlic application statistically significantly reduced MDA levels in BGG compared to UG ($p<0.01$). There is no significance between BGG and PTG for the same values. These values showed that black garlic has significant effects on reducing MDA levels. Considering the SOD activity, it is seen that there is statistical significance between BGG and UG ($p<0.05$), but there is no significance between BGG and PTG. It has been observed that black garlic has beneficial effects in maintaining antioxidant enzyme levels. Considering the activities of another antioxidant, CAT, it was seen that the levels were higher in the PTG group. It has been determined that black garlic has limited effectiveness in protecting CAT activities. Additionally, due to the oxidation of γ-glutamyl cysteine, black garlic does not produce irritating and soluble sulfur-containing compounds, thus it does not irritate the digestive system. It has also been determined that it can help reduce constipation because it causes increased motility in the digestive system. Aged black garlic extract induces gastric cancer cell growth inhibition in vitro and in vivo (Wang et al. 2012). It also induces apoptosis in the HT29 colon cancer cell line (Dong et al. 2014).

CONCLUSION

It was found that the application of black garlic extract in a single and high dose (275 mg. rat) showed partial gastroprotective effects in the gastric ulcer model induced by a nonsteroidal anti-inflammatory drug (indomethacin). Although its protective effects are not as strong as pantoprazole, it is thought to have the potential for use in prophylaxis and treatment. Since black garlic has compounds that dissolve only in oil, it is thought that it may be beneficial to conduct this study in oil and different solvents (such as carboxy methyl cellulose).

Ethical Approval: Aydin Adnan Menderes University Animal Experiments Local Ethics Committee (Approval No: 64583101/2023/156)

Funding: Self-funded.

Conflict of Interest: There is no conflict of interest with any person or institution in this study.

Limitations of the Study: In this study, determination levels of TNF-α, IL-2, PGE1 and PGE2 could not be performed due to budget constraints.

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RESEARCH ARTICLE

Effect of Postpartum Drenching of Fluid-Electrolyte and Energy Supplement on Fertility Parameters in Holstein Dairy Cows

Cangir UYARLAR[1*], Oktay YILMAZ[2]

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

ABSTRACT

In this study, the effect of orally given fluid-electrolyte and energy supplementation immediately after the delivery on some metabolic and fertility parameters was investigated. Holstein dairy cows which were housed in a private dairy company under identical management and feeding programs were divided into two groups as experiment (n=70) and control (n=75). Cows in the experiment group were treated by orally given 5 kg fluid-electrolyte and energy supplementation dissolved in 20 litres lukewarm water for three consecutive days, whereas the control group received only the 20 litres of lukewarm water. The ear tag numbers of all cows used in the study were recorded and monitored until next delivery. Blood samples were collected from the coccygeal vein of the cows on the postpartum 4th day. It was determined that the concentrations of βHBA in the experiment group were lower than in the control group ($p<0.01$). However, the concentrations of NEFA and glucose did not differ between the experiment and the control groups ($p>0.05$). It was seen that the synchronization rate was lower in the experiment group than that detected in the control group ($p<0.05$). The pregnancy rate did not differ between groups ($p>0.05$). Nevertheless, the service number per pregnancy displayed a higher incidence in the control group as compared to the experiment group ($p<0.001$). Similarly, calving to pregnancy interval and the calving interval was higher in the control group ($p<0.05$). Total lactation milk yield in the experiment group was higher as compared to the control group ($p<0.05$). In conclusion, it was observed that orally given fluid-electrolyte and energy supplementation immediately after delivery improved the fertility parameters in Holstein dairy cows.

Keywords: Fluid-Electrolyte, βHBA, Dairy cow, Fertility

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Holştayn Süt İneklerinde Doğum Sonrası Sıvı-Elektrolit Ve Enerji Katkısının Fertilite Parametreleri Üzerine Etkisi

ÖZ

Bu çalışmada süt ineklerinin doğumlarından hemen sonra üç gün boyunca içirilen sıvı-elektrolit ve enerji desteğinin bazı metabolik ve fertilite parametreleri üzerine etkisi araştırıldı. Ticari bir süt işletmesinde aynı çiftlik yönetimi ve besleme programı altında barındırılan Holştayn süt inekleri deneme (n=70) ve kontrol (n=75) olmak üzere iki gruba ayrıldı. Deneme grubundaki ineklere üç gün boyunca ağızdan 5 kg/gün sıvı-elektrolit ve 20 litre ılık suda eritilmiş enerji takviyesi verilirken, kontrol grubundaki ineklere sadece 20 litre ılık su verildi. Çalışmada kullanılan tüm ineklerin kulak küpesi numaraları kayıt altına alındı ve bir sonraki doğuma kadar takip edildi. Bütün hayvanların kuyruk venalarından postpartum 4. günde kan örnekleri alındı. Deneme grubundaki kan βHBA konsantrasyonunun kontrol grubuna göre daha düşük olduğu belirlendi ($p<0.01$). Ancak kan NEFA ve glikoz konsantrasyonları deneme ve kontrol grupları arasında farklılık göstermedi ($p>0.05$). Senkronizasyon oranının deneme grubunda kontrol grubuna göre daha düşük olduğu görüldü ($p<0.05$). Gebelik oranı gruplar arasında farklılık göstermedi ($p>0.05$). Kontrol grubunda ise gebelik başına tohumlama sayısı deneme grubuna göre daha yüksek görüldü ($p<0.001$). Benzer şekilde doğum-gebe kalma aralığı ve buzağılama aralığı da kontrol grubunda daha yüksek olduğu izlendi ($p<0.05$). Deneme grubunda laktasyon süt veriminin kontrol grubuna göre daha yüksek olduğu belirlendi ($p<0.05$). Sonuç olarak Holştayn süt ineklerinde doğumdan hemen sonra oral olarak verilen sıvı-elektrolit ve enerji takviyesinin fertilite parametrelerini iyileştirdiği kanısına varıldı.

Anahtar Sözcükler: Sıvı-Elektrolit, βHBA, Süt ineği, Fertilite.

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ORCID ID; *CU 0000-0002-7803-2454 OY: 0000-0002-9722-5155*

*Corresponding author e-mail: cangiruyarlar@hotmail.com

INTRODUCTION

Dairy cows face a metabolically stressful period after parturition due to the enormous loss of nutrients such as water, energy and electrolytes (Grummer 1995; Enemark et al. 2009). Meanwhile, due to suppressed dry matter intake in the late pregnancy period, animals are not able to meet their nutrient requirements amelioration from the diet. Especially, energy deficiency is quite remarkable and it leads to a severe negative energy balance shortly after parturition (Drackley et al. 1991). Insufficient energy intake causes dramatic metabolic changes such as a decrease in blood glucose whereas there is an increase in non-esterified fatty acids (NEFA) and beta hydroxybutyric acid (βHBA) (Oetzel 2007). Moreover, subclinical ketosis occurs in dairy cows while serum βHBA levels are higher than 1.2 mmol/l. Subclinical ketosis is one of the most dangerous metabolic disorders induced by reduced milk yield, impaired reproductive performance (Collard et al. 2000) and increased incidence of culling rate (Uyarlar et al. 2018). Numerous studies demonstrated the relationship between hyperketonemia and reduced productivity in dairy cows (Dohoo and Martin 1984; Drackley 1999; Herdt 2000; Ospina et al. 2010b; McArt et al. 2012). Moreover, subclinical ketosis is related with increased incident in puerperal metabolic disorders (Ospina et al. 2010a).

The reproductive performance of dairy cows is closely related to the energy status in dairy cows (Grummer 2007). Oftentimes, delayed onset of luteal activity, decreased conception rate in the first insemination and increased number of the days from calving to conception in dairy cows are occurred in severe negative energy balance (Staples et al. 1990; Reist et al. 2003). Moreover, Rutherford et al. (2016) demonstrated that subclinical ketosis caused impaired reproductive performance by prolonging calving to first standing heat, calving to first insemination and calving to pregnancy intervals. In addition, drenching dextrose, electrolytes and vitamins is a prophylactic administration, when severe dehydration occurs (Enemark et al. 2009). However, nutritionists traditionally suggest oral administration of glucose precursors such as propylene glycol, glycerol, and calcium propionate to dairy cows during the transition period (Overton and Waldron 1996). This treatment aids in decreasing the energy gap, whereas it does not help to alleviate the loss of water, electrolytes and vitamins due to the parturition (Stokes and Goff 2001; Enemark et al. 2009). From this point of view, this study was aimed to determine the effect of orally given fluid-electrolyte and energy supplementation for three days after parturition on fertility parameters in Holstein dairy cows.

MATERIALS and METHODS

Animals and Housing

The study was conducted in a private Holstein Friesian dairy cow farm which were located in Türkiye. The cows had same management and feeding programs and were milked thrice daily. The dry period was separated to the far-off (50-60 days before the expected delivery) and close-up (21 days before the expected days) periods. After parturition, the cows were housed in fresh group (3-30 days postpartum). The individual milk yield of the cows which were taken from an automatic milking system was daily recorded (DairyPlan, GEA, Germany) until next delivery and the lactation milk yield were calculated at the end of study. The cows were grouped according to the individual milk yield following the fresh period.

Experimental Design

A total of 145 Holstein dairy cows immediately after delivery were divided into two groups. Cows in the experiment group (n = 70) were treated by orally given 5 kg fluid-electrolyte and energy supplementation (Polihidra, Polimed, Türkiye) dissolved in 20 litres lukewarm water for three consecutive days, whereas control group (n = 70) received only 20 litres of lukewarm water. The ear tag numbers of all cows used in the study were recorded and the cows were monitored for the reproductive parameters (synchronization rate, pregnancy rate, service number per pregnancy, calving to pregnancy interval and calving interval) until next delivery.

Diets

The animals were fed by same diet which were prepared and served as total mix ration (TMR). Nutrient composition and feedstuff formulation of the diet are presented in Table 1.

Measurement of metabolic profile

Blood samples were collected from coccygeal vein of the cows on the postpartum 4th day. Sera were harvested from blood samples following the centrifugation at 5.000 rpm for 10 min. The serum concentrations of glucose were analyzed by the commercially available reagents of Cobas C111 analyzer (Roche Diagnostic, Mannheim, Germany), whereas betahydroxybutyric acid (βHBA, #RB1008, Randox Laboratories Ltd., Crumlin, UK) and non-esterified fatty acid (NEFA, #FA115, Randox Laboratories Ltd., Crumlin, UK) were measured by ELISA (ChemWell, 2910, Awareness Technology, Inc., Florida, USA).

Monitoring of Reproductive Parameters

The voluntary waiting period was 55-65 days on average. The first postpartum (pp) artificial insemination (AI) was performed by G6G synchronization protocol (Yilmaz et al. 2011) or

standing heat observation. When the cows showed standing heat 18-24 days after AI detected by observation and/or pedometer activity, the estrous was confirmed by transrectal ultrasonography (6 MHz, DP 10, Mindray, China) and the cows were re-inseminated. If the cows did not display standing heat, the pregnancy status (pregnant or non-pregnant) was diagnosed by transrectal ultrasonography 33-35 days after AI. Single dose or double dose of prostaglandin $F_{2\alpha}$ 14 days apart or Ovsynch protocol were used for the insemination of non-pregnant cows. All data were daily recorded to the farm computer system. When the cows reached to the next delivery, the data were evaluated for the calculation of the synchronization rate (%), pregnancy rate (%), service number per pregnancy, calving to pregnancy interval (day), and calving interval (day).

Statistics

The distribution of normality of data were analysed by Shapiro-Wilk normality test. The data regarding the concentrations of blood betahydroxybutyric acid, non-esterified fatty acid, glucose, service number per pregnancy, calving to pregnancy interval, and calving interval were evaluated by t-test, whereas synchronization rate, pregnancy rate and embryonic death rate were evaluated by chi-square test (SPSS 26.0, IBM, USA). Statistical significance was set at $p<0.05$.

RESULTS

The blood concentrations of βHBA, NEFA and glucose in the experiment and control groups are given at Table 2. The concentrations of βHBA in the experiment group (0.74±0.34 mmol/l) was lower than those measured in the control group (0.91±0.49 mmol/l) and the difference was statistically significant ($p<0.01$). However, the concentrations of NEFA in the experiment group (0.73±0.39 mmol/l) was slightly lower than those measured in the control group (0.87±0.50 mmol/l) but the difference was not statistically significant ($p>0.05$). The glucose concentrations did not differ between the experiment (53.73±3.71 mg/dl) and control (53.68±3.77 mg/dl) groups ($p>0.05$).

All related reproductive parameters and lactation milk yield in the experiment and control groups are given at Table 3. Accordingly, it was seen that the synchronization rate was lower in the experiment group (50.90 %) than those detected in the control group (70.90 %) and the difference was statistically significant ($p<0.05$). The pregnancy rate was slightly higher in the experiment group (75.70 %) than those measured in the control group (73.30 %) throughout the study but the difference was not significant statistically ($p>0.05$). On the other hand, the service number per pregnancy was displayed a higher incidence in the control group (2.21±0.85) as compared to the experiment (1.68±0.69) group ($p<0.001$). Similarly, calving to pregnancy interval was higher ($p<0.05$) in the control group (137.27±33.25) than those measured in the experiment group (124.22±31.39), whereas calving interval was also high ($P<0.05$) in the control group (415.27±33.25) as compared to the experiment group (402.22±31.39). Finally, the lactation milk yield in the experiment group was 32.90±2.99 litres, whereas the control group had 31.37±3.01 litres lactation milk yield and the difference was statistically significant ($p<0.05$).

DISCUSSION

The data obtained in this study provide evidence that electrolyte and energy supplementation with vitamin support can be beneficial for the improvement of metabolic and fertility parameters. In the present study, it was demonstrated that serum βHBA concentrations on four days in milk (DIM) were the lowest in the experiment group which were treated by orally given 5 kg fluid-electrolyte and energy supplementation dissolved in 20 litres lukewarm water at postpartum first 3 days. Moreover, a non-significant drop was also noticed in NEFA concentrations in the experiment group, whereas the serum glucose level did not differ on four DIM. Blood βHBA level is one of the most reliable parameters for monitoring energy balance and the incidence of ketosis which is characterized by loss of appetite, reduced dry matter intake and body condition loss in transition dairy cows (Grummer 2007; Overton et al. 2017; Lei and Simones 2021). Animals with serum βHBA level higher than 1.2 mmol/l are considered as hyperketonemic and sub-clinically ketotic (Iwersen et al. 2009; Konkol et al. 2009). Subclinical ketosis can occur any day in the first 100 days of lactation (Duffield 2000). However, the first week of lactation is the most critical period for the occurrence of hyperketonemia and the development of subclinical ketosis (McArt et al. 2012). This fact was the reason why the fourth day of lactation was chosen for blood sampling in the present study. It has been well documented that during transition period, all dairy cows experience dramatic changes including in the metabolic, physiological and/or nutritional status which can be resulted in a period of negative energy balance, when the diet do not meet the energy demand (Grummer 2007). Negative energy balance in dairy cattle resulting from insufficient energy intake immediately after parturition is considered the most important problem for dairy cattle nutritionists (Churakov et al. 2021). The increasing βHBA and NEFA or decreasing concentrations of glucose are the expected results during negative energy balance period in dairy cows (Grummer 1993; Drackley 1999; McArt et al. 2012). The present study revealed that the experiment group displayed better energy status with lower βHBA and NEFA concentrations than the control group.

Table 1. Diet Composition

Feedstuffs (DM%)	Far off (Dry Period)	Close Up (Dry Period)	Fresh Period (Lactation)
Corn Silage	26.85	35.43	31.52
Wheat Straw	40.58	15.52	0.00
Alfalfa Hay	11.84	5.58	16.99
Soybean Meal	0.00	5.61	19.39
Sunflower Meal	8.39	7.68	4.11
Corn Steam Flakes	6.72	14.79	18.42
Barley Grain Ground	3.40	7.47	2.66
Rumen Protected Fat[1]	0.00	2.92	2.97
Premix[2]	0.69	1.47	1.79
Limestone	1.15	0.00	1.19
Salt	0.38	0.34	0.72
Rumen Protected Lysine[3]	0.00	0.00	0.09
Rumen Protected Methionine[4]	0.00	0.00	0.15
Magnesium Sulfate	0.00	1.68	0.00
Calcium Chloride	0.00	1.51	0.00
Nutrients			
CDMI[5]	13.53	11.85	16.65
Crude Protein (DM %)	10.15	12.46	18.28
MP[6] (g/day)	1032.3	1101.5	1895.2
NEl (Mcal/day)	14.78	18.89	28.63
NEl (Mcal/kg)	1.13	1.59	1.72
NFC (DM %)	26.12	37.05	39.36
NDF (DM %)	53.55	35.64	27.53
ADF (DM %)	37.15	23.19	17.68
Ether Extract (DM %)	2.23	5.33	5.49
Ca (DM %)	0.76	0.82	0.62
P (DM %)	0.38	0.37	0.38
Lysine (MP %)	2.45	2.42	6.82
Methionine (MP %)	6.75	6.89	2.51
(NA+K)-(Cl-S)	**17.72**	**-41.3**	**17.15**

[1] Fractionated rumen protected fat (Polimed, Turkiye), Fat 98% (Minimum), Palmitic Acid 85% (Minimum), Stearic Acid 3,5% (Maximum)
[2] Premix composition in 1 kg; 3000000 IU of Vitamin A, 500000 IU of Vitamin D, 9000 mg of Vitamin E,12000 mg of Mn, 5000 mg of Fe, 50000 mg of Zn, 9000 mg of Cu, 400 mg of I, 75 mg of Co, 250 mg of S
[3] Lysipearl (Kemin, Belgium)
[4] Metipearl (Kemin, Belgium)
[5] Calculated Dry Matter Intake (NRC, 2001)
[6] Metabolizable Protein (NRC, 2001).

Table 2. The concentrations of blood betahydroxybutyric acid (βHBA), non-esterified fatty acid (NEFA) and glucose in cows in the experiment (n = 70) and the control (n = 75) groups.

Parameters	Experiment (n =70)	Control (n =75)	P
βHBA (mmol/l)	0.74±0.34	0.91±0.49	0.003
NEFA (mmol/l)	0.73±0.39	0.87±0.50	0.057
Glucose (mg/dl)	53.73±3.71	53.68±3.77	0.937

Experiment group (n = 70) was treated by orally given 5 kg fluid-electrolyte and energy supplementation dissolved in 20 litres lukewarm water, whereas control group (n = 75) received only 20 litres of lukewarm water.

Table 3. Lactation milk yield (l) and reproductive parameters [synchronization rate (%), pregnancy rate (%), service number per pregnancy, calving to pregnancy interval (day), and calving interval (day)] detected in cows in the experiment (n = 70) and the control (n = 75) groups.

Parameters	Experiment (n =70)	Control (n =75)	P
Synchronization rate (%)	50.90 (27/53)	70.90 (39/55)	0.048
Pregnancy rate (%)	75.70 (53/70)	73.30 (55/75)	0.849
Service number per pregnancy	1.68±0.69	2.21±0.85	0.000
Calving to pregnancy interval	124.22±31.39	137.27±33.25	0.038
Calving interval (day)	402.22±31.39	415.27±33.25	0.038
Lactation milk yield (L)	32.90±2.99	31.37±3.01	0.003

Experiment group (n = 70) was treated by orally given 5 kg fluid-electrolyte and energy supplementation dissolved in 20 litres lukewarm water, whereas control group (n = 75) received only 20 litres of lukewarm water.

These findings are in accordance with another study (Enemark et al. 2009) which reported that oral administration of electrolytes improved the energy balance of dairy cows by reducing βHBA and NEFA concentrations in the first days of lactation. It is suggested that the replacement of electrolyte status with the support of orally given glucose throughout three consecutive days may improve the dry matter intake and/or controlled the incidence of metabolic diseases after parturition. It is needed to clarify the hypothesis that consume the relationship between the postpartum prophylactic treatment and the metabolic or reproductive health status. It has been known that various prophylactic treatments are available in the veterinary field to minimize NEB and related postpartum disorders (Studer et al. 1993; Goff and Horst 1994), consequently maintaining the high productive performance (Miyoshi et al. 2001). It was documented that oral administration of electrolytes with a high amount of water as a prophylactic treatment in fresh cows improves the metabolic haemostasis (Stokes and Goff 2001). Accordingly, MgSO4 helps to increase magnesium concentrations in blood and to maintain the calcium homeostasis at calving due to parathormone induced absorption of dietary calcium (Enemark et al. 2009). On the other hand, KCL provides daily potassium supply from the diet is very important in helping to maintain acid-base balance, water retention, and Mg2+ absorption (Ammerman and Goodrich 1983). It is known that a cow approximately lost 60 litres of uterine fluid (Doreau et al. 1981) and simultaneously plasma protein due to the colostrum secretion (McLennan and Willoughby 1973) at calving which may result electrolyte imbalances in the dam. Moreover, the filling of the rumen with water may help to avert the displacement of the abomasum by restricting rumen movements (Enemark et al. 2009). Therefore, it is speculated that the administration of postpartum large amounts of fluid may serve the replacing of electrolytes which are lost at calving. In accordance with Enemark et al. (2009), it is suggested that drenching with electrolyte-supplemented water helped to improve energy balance in dairy cows and ameliorated the metabolic status of the cows.

Lucy et al. (1992) states that a relationship exists between positive energy status and the diameter of largest follicle on day 10 postpartum, since the gonadotropic hormones tend to be increased by the improving of energy status. In the present study, the artificial inseminations were more likely to be performed following by the observation of standing heat in the experiment group. In another words, the experiment group needed significantly less synchronization protocols as compared to the control group. In early lactation stage, the delayed resumption of postpartum cyclicity is associated with severe NEB which negatively affects the postpartum reproductive efficiency (Jeong et al. 2015). It has been reported that adequate levels of blood glucose 60 mg/dl on average is appropriate for the cow to get pregnant at first insemination (Garverick et al. 2013). Furthermore, it was noted that the animals with more amount of blood glucose came to heat within 2 months compared to those with lesser blood glucose (Veena 2015). In the present study, the concentrations of blood glucose were 53.73±3.71 and 53.68±3.77 in the experiment and the control groups, respectively. It seems that the concentrations of glucose are in the reference range (Mair et al. 2016). Therefore, it is suggested that the lesser proportions of the synchronized cows might be related not only the glucose concentrations but also the electrolyte and vitamin supplementation. It was reported that there have been differences in the blood concentrations of certain vitamins in dairy cows at the different stages of the oestrus cycle (Ataman et al. 2010). Generally, injections of vitamin A, D, E and C in combinations had no effect on the rate of conception or pregnancy in cows (Likittrakulwong et al. 2022). However, it was stated that the injectable vitamin and trace element combination may improve some metabolic and fertility parameters such as blood NEFA and aspartate aminotransferase (ALT) concentrations and pregnancy rate in dairy cows (Yazlik et al. 2021). Moreover, it has been reported

that the mineral supplementation can be used to improve productivity and reproductive well-being (Molefe and Mwanza 2020). In addition, it was reported that under the adequate metabolic conditions the cows showed shorter calving to conception interval and lower insemination index (Çolakoğlu et al. 2020). Although the pregnancy rate did not differ between groups in the present study, the service number, the calving to pregnancy interval and the calving interval were significantly lesser in the experiment group. Therefore, it is suggested that both energy and electrolyte supplementation might be synergistically beneficial to improve the fertility parameters.

The present study showed that the lactation milk yield in the experiment group was significantly higher as compared to the control group. This finding was not accordance with other reports (Enemark et al. 2009) which dextrose supplementation did not exist in the drenching. However, it was reported that the oral supplementation of glucose precursors caused a numeric increase in milk yield (Akhtar et al. 2023), whereas a significant increase in milk yield was observed when the cows had clinical or subclinical ketosis (McArt et al. 2011, Gordon et al. 2013). The discrepancies above mentioned studies might be due to the absence of electrolytes and vitamin supplementation for the prophylactic treatment of the cows.

In conclusion, the present study revealed that orally given electrolyte and energy supplementation dissolved in water at the postpartum three consecutive days induced the decrement in the blood βHBA and NEFA concentrations. Consequently, the decrement in the service number, calving to pregnancy interval and calving interval might be observed in the dairy cows. Therefore, it is suggested that the postpartum short administration of fluid-electrolyte and energy supplementation can be of importance for the improvement fertility and lactation milk yield in the Holstein dairy cows.

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

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

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LETTER TO EDİTOR

Experimental Animal Research in Vaccine Studies during the Pandemic Process

Serdar AKTAŞ[1*]

[1]*Research and Application Hospital, Faculty of Veterinary Medicine, Aydın Adnan Menderes University, Aydın, Türkiye*

The pandemic that emerged in Wuhan, China, in 2019, once again revealed the role of basic sciences and experimental animal research in the world. This situation, also called COVID-19, has spread rapidly all over the world in a short period of 3 months. It has been revealed that vaccination is more important than treatment in reducing mass deaths during the fight against the pandemic. The fact that RNA viruses constantly change their genetic properties was also a major challenge in finding an effective vaccine. However, increasing progress in basic sciences (especially mRNA technology) and the use of humanized mice have contributed greatly to reducing mass deaths. The fact that the Sars-Cov-2 virus does not cause infection in conventional mice and that it can create an infection model in mutant mice to which human lung epithelial genes are transferred has given great impetus to vaccine and new drug treatment studies. The first vaccine model was developed using these mice in vaccine studies. Nearly 176 vaccine types have been developed in the Covid-19 pandemic. These vaccines; viron vaccines are nucleic acid-based (mRNA), viral vector and protein-based vaccines. The mutant mice used in the first step of these vaccines created the disease model almost perfectly and ensured that the results in the target species (Human) progressed with fewer errors. Since the disease occurs in mice carrying human lung epithelium very close to the target species, they have played a major role in testing the protective effects of vaccines. The vaccines prepared towards the end of 2020 were made ready for use. Vaccine production has been carried out at an almost record speed in pandemics in world history. Although vaccines may have different effects and protection levels, the role of vaccines in the pandemic is undeniable. More than 2.5 billion doses of COVID-19 vaccines have been administered worldwide, and approximately 35 million doses continue to be administered every day. Experimental animal research, genetic research and gene transfer that started in the 1970s were used to obtain vaccines that were prepared in a short time and made available to humanity. technologies and mutant mouse technology play a huge role. Although the pandemic process is still not over, it is expected to ease and continue to have effects similar to seasonal flu. In this process, humanity has gained important experience in acting together and rapid vaccine development. The use of experimental animals is of great importance in these processes.

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ORCID ID; *SA: 0000-0003-3243-8093*
*Corresponding author e-mail: saktas@adu.edu.tr

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CASE REPORT

First Report of *Glochidia* (Mollusca: Lamellibranchiata) Infestation in Aquarium Fish (Flowerhorn) from Iran

Salar ZARRABI AHRABI[1*], Shahrokh SHIRAZI[2]

[1]*Department of Basic Health Science, Faculty of Health Sciences, Marmara University, İstanbul, Turkey*
[2] *Department of Veterinary Parasitology, Science and Research Branch, Islamic Azad University, Tehran, Iran*

ABSTRACT

Glochidia are the larva stage of bivalve mollusks (Lamellibranchiata). These temporary obligate parasites are apparent on the fins and gills and rarely on the surface of fish in spring, summer, and autumn. In spring 2018, a flowerhorn (cichlid) with spots appearing body and gills and white small mobile organisms on the floor of an aquarium were referred to the parasitology laboratory in the School of Veterinary Medicine, Science and Research Unit, Tehran Islamic Azad University. The organisms were sampled and clear in Potas 10% fixed by Glycerin gelatin and examined with a light microscope; the samples were diagnosed as *Glochidia*.

Keywords: Bivalve, Flowerhorn, *Glochidia*

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İran'dan Akvaryum Balıklarında (Flowerhorn) *Glochidia* (Mollusca: Lamellibranchiata) İstilasının İlk Raporu

ÖZ

Glochidia, Lamellibranchiata sınıfına ait çift kabuklu yumuşakçaların larva evresini temsil eder. Bu geçici zorunlu parazitler, ilkbahar, yaz ve sonbaharda balıkların yüzgeçlerinde ve solungaçlarında ve nadiren de yüzeyinde görülür. 2018 yılı ilkbaharında, bir çiçek boynuzlu (ciklet) akvaryumun içinde vücut ve solungaçlarda lekelerle birlikte akvaryum tabanında beyaz, küçük ve hareketli organizmaların görünmesi üzerine, bu durum Veteriner Fakültesi, Bilim ve Araştırma Birimi, Tahran İslam Azad Üniversitesi parazitoloji laboratuvarına yönlendirildi. Organizmalardan örnek alındı ve Potas 10%'de sabitlendi, Gliserin jel ile temizlendi ve ışık mikroskobu ile incelendi; örnekler *Glochidia* olarak tanımlandı.

Anahtar kelimeler: Çift kabuklu, Flowerhorn, Glochidia

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ORCID ID; *SZA: 0000-0003-3543-061X, SS: 0000-0001-6819-5391*
*Corresponding author e-mail: salar.zarrabi@marmara.edu.tr

INTRODUCTION

The flowerhorn cichlids are ornamental aquarium fish noted for their transparent colors, and glochidium (plural *Glochidia*) is a larval stage of some freshwater mussels, which are released from female mussels (Neves et al., 1985). These aquatic bivalve mollusks belong to the Uniondale family (Brodniewicz, 1968) and have calcareous bivalve shells, often with little hooks on their inner edge (Paperna, 1996). Freshwater bivalve mollusks exhibit diverse life cycle adaptations for parasitizing obligate hosts. The parasitic larval stage, referred to as *Glochidia*, is a crucial aspect of the life cycle of main freshwater mussels. *Glochidia* have the ability to temporarily attach to the outer surface of suitable hosts, commonly fish, and certain amphibians. This unique strategy serves a dual purpose, providing both nutrition and a means of dispersal for the parasite larvae. (Nikishchenko et al., 2022).

These larvae attach to the fish, utilizing structures like hooks, often targeting the gills or fins of the host fish. This attachment initiates a proliferative reaction in the surrounding area where they are attached (Şereflişan, 2021; Şereflişan, 2018; Arey, 1921; Paperna 1996). *Glochidia* encyst in the gill epithelium and undergo growth before eventually dropping off within a span of 10-30 days. The infestation caused by *Glochidia* is termed Glochidiosis (Gustafson and Iwamoto, 2005). *Glochidia* can bear a resemblance to trematode metacercarial cysts, and in instances of heavy infestation, they can rarely lead to mortality (Nedeau et al., 2005).

There is a need for more consensus regarding the pathogenic impact of *Glochidia* on fish. While some researchers argue that the parasitic larval phase does not hinder recruitment and, therefore, does not substantially affect maintaining the mussel population, high concentrations of *Glochidia* are often associated with decreased swimming ability and higher mortality rates in hosts. Additionally, the relationship is classified as parasitic due to the nutrient transfer from the fish to the mussel (Ieshko et al., 2016).

CASE HISTORY

The case belongs to an aquarium fish enthusiast and owner of a pet shop who referred a case (flowerhorn) to the parasitology laboratory at the Faculty of Veterinary Medicine, Science, and Research Unit, Tehran Islamic Azad University, in spring 2018, upon observing anomalies on the fish such as spots appearing on body and gills and also white small mobile organisms on the floor of an aquarium. As the fish were alive and economically valuable, a necropsy was not feasible. Therefore, a sample was taken from the aquarium base layer for clinical diagnosis. Sample clear in Potas 10% fixed by Glycerin gelatin and examined with a light microscope, revealed the presence of *Glochidia* larvae (Klunzinger et al., 2013), and based on clinical observations, Glochidia infestation was diagnosed on the fish. No specific drug treatment was administered to the case (Smith, 2019). The recommendation included changing the aquarium substrate and water. After one month of follow-up, clinical signs of *Glochidia* infestation were no longer observed, and the fish was successfully sold by the pet shop.

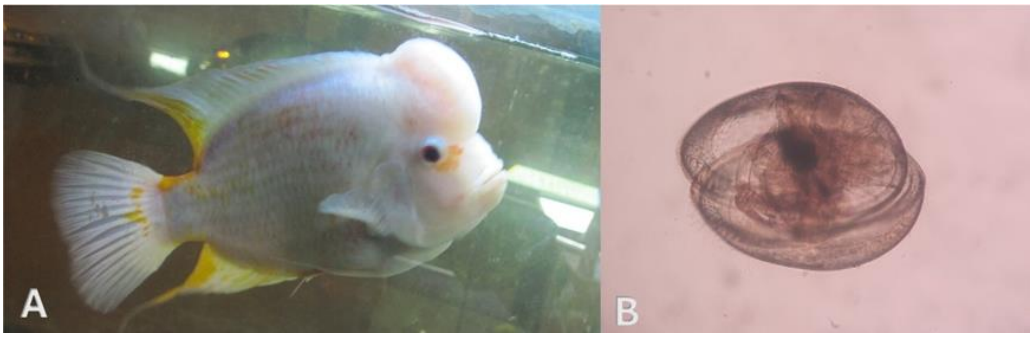

Figure 1: A- Flowerhorn aquarium fish with spots on the body, infested by *Glochidia*. B-*Glochidia* larvae were sampled from the base layer of the aquarium.

DISCUSSION

The flowerhorn fish, belonging to the Cichlid family, are man-made hybrids that do not exist in nature. They are popular ornamental fish, particularly in Southeast Asia and Iran, and are commonly kept in aquariums (Rahmati-Holasoo and Shokrpoor, 2014).

Glochidia infestation is a common occurrence in freshwater fish worldwide and can be found in various aquatic environments such as rivers, lakes, mud-bottomed pools, and ponds (Lee and Mora, 2005; Parasites Paperna, 1996).

Cases of Glochidiosis have been reported in various regions, including Poland and North America (Zieritz et al., 2012). They have developed an identification key for North and Central European Unionid mussels, contributing to understanding and recognizing these parasites in different geographic areas. (Brodniewicz, 1968; Gustafson and Iwamoto, 2005; Zieritz et al., 2012). Due to the ongoing debate and lack of consensus regarding the pathogenic impact of *Glochidial* infection on fish, it is noteworthy that some researchers have reported mortalities among certain fish strains that were experimentally infested with *Glochidia* (Ieshko et al., 2016). Research indicates that elevated rates of *Glochidia* infection can enhance swimming performance and mortality in brown trout, potentially resulting in reduced performance in heavily infested fish in their natural environment. Conversely, some studies reported no mortalities or growth retardation in *Glochidia* infected trout. Comparable outcomes were observed in the experimental infestation of Atlantic salmon parr. These divergent findings underscore the intricate nature of interactions between *Glochidia* and different fish species. The observed discrepancies in the impact of *Glochidia* infection on fish may be attributed to the existence of diverse host fish strains or variations in environmental conditions, such as temperature (Ieshko et al., 2016; Taeubert and Geist, 2013). *Glochidiosis* has been documented in both wild and farmed salmonids in locations such as the Scotland River and Virginia (Hastie and Young, 2001; Neves and Widlak, 1988). According to available references, there are no reports of Glochidiosis in ornamental fish. This could be attributed to the fact that these organisms, in their adult phase, resemble stones and do not move extensively (Nedeau et al., 2005). So, it can probably be transmitted by stones that have been brought from these sources for aquariums. This is the first report of Glochidiosis of flowerhorn in Iran.

CONCLUSION

In conclusion, most reports on Glochidiosis are related to freeliving fishes, particularly in the Salmonidae family. The probability of infestation in aquarium fishes is very low. However, it is crucial to emphasize the importance of maintaining aquarium hygiene and being cautious about transferring objects from the natural environment to the aquarium.

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Author Contribution Rates: Both authors contributed equally to all aspects of the research.

Ethics Committee Information: The current study does not require ethics approval.

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